Role of CFTR and chloride/bicarbonate exchangers in airway epithelial bicarbonate secretion

Salam Haji Ibrahim

Thesis submitted for the degree of Doctor of Philosophy

Newcastle University

Faculty of Medical Sciences

Institute for Cell and Molecular Biosciences

Date of Submission: September, 2015

Abstract

HCO_3^- secretion plays a vital role in regulating the pH and mucus viscosity of airway surface liquid to facilitate airway mucociliary clearance of inhaled pathogen. In cystic fibrosis (CF), reduced HCO_3^- secretion contributes to defective mucociliary clearance which predisposes the lungs to bacterial infection. Calu-3 cells are used as a model of human submucosal gland serous cells which are involved in CFTR (cystic fibrosis transmembrane conductance regulator)-dependent HCO_3^- secretion, a process that appears to involve functional interactions with both apical and basolateral Cl^-/HCO_3^- anion exchangers (AE), but through regulatory pathways that are not well understood. The aim of this thesis was to investigate the signalling mechanisms that regulate CFTR-dependent AE activity in Calu-3 cells.

Under resting conditions, Calu-3 cells showed a DIDS-sensitive Cl^- and HCO_3^--dependent basolateral anion exchange activity consistent with AE2 (SLC4A2) expression. However, apical AE activity was not detected. Increasing cytosolic Ca^{2+}, or removal of extracellular Ca^{2+}, had no effect on basolateral AE activity. In contrast, lowering cytosolic Ca^{2+} with BAPTA-AM, or inhibiting calmodulin (CaM), reduced basolateral AE activity. Furthermore, an intact actin cytoskeleton, as well as active dynamin, were essential for maintaining basolateral AE activity, possibly via supply of new proteins to the basolateral membrane. Inhibiting CK2 or protein phosphatase 1 (PP1) abolished basolateral AE activity, and CK2 inhibition was linked to CaM. This suggests that AE activity was maintained through a novel CaM-dependent mechanism involving phosphorylation/dephosphorylation by CK2/PP1. In support of this, transient transfection of HEK293 cells with mouse AE2, with and without CK2 co-transfection, clearly demonstrated CK2-dependent AE2 activity.

Stimulation of Calu-3 cells with cAMP agonists both activated an apical anion exchanger via a PKA and Epac-dependent mechanism, and inhibited the basolateral anion exchanger, but through a PKA and Epac-independent mechanism. Blocking CFTR with GlyH-101 caused an apparent inhibition of apical AE activity, but addition of basolateral DIDS restored apical activity, suggesting that a basolateral HCO_3^- transporter was activated when CFTR was inhibited. Removal of extracellular Ca^{2+} partially reduced the cAMP-induced inhibition of the basolateral AE activity, but had no effect on cAMP-stimulated apical AE activity. Moreover, increasing cytosolic Ca^{2+}, or lowering cytosolic Ca^{2+} with BAPTA-AM, markedly reduced cAMP-stimulated apical AE activity, but it had no effect on cAMP-induced inhibition of the basolateral anion exchanger. Actin-cytoskeleton disruption had no effect on apical AE activity but dynamin inhibition caused a significant decrease. A similar decrease in apical AE activity was observed when CK2 was inhibited, but in contrast to the basolateral anion exchanger, this appeared to be via a CaM-independent mechanism. Inhibiting CK2, however, had no effect on the cAMP-induced inhibition of the basolateral AE activity, suggesting that CK2 regulation of Calu-3 anion exchangers is through cAMP-independent mechanisms.

These findings provide new insights into the signalling pathways that regulate both the apical and basolateral anion exchangers in Calu-3 cells and help define their respective roles in airway HCO_3^- secretion. The results could potentially open up new avenues for modulating AE activity which could be beneficial in HCO_3^- secretory diseases such as CF.

Acknowledgements

First, and in particular, I would like to sincerely express my gratitude to my supervisor, Dr. Mike Gray, for his constant support, guidance, and for everything he has done for me in order to have for me to complete my PhD. I would like to thank Mike not only for his fantastic guidance and patience with me, but also for his great source of knowledge and for showing me what it requires to be a good scientist. I am also very grateful for his kind help and support with any personal issues that arose during my PhD.

I am also very thankful to my fellow laboratory members, Dr. Mark Turner, Waseema Patel and Dr. Bernard Verdon who have all helped me to be able to complete all the experiments I set out to do.

In addition, I would also like to thank the rest of my fellow members of the Epithelial Research Group at Newcastle University for their friendship, support and technical knowledge. I would like to express my deep sense of gratitude and regard to Maxine Geggie, for her valuable assistance in tissue culture and to Dr. Alison Howard for her help and patience when teaching me certain molecular biology techniques. I should also thank members of Prof Jeff Pearson's lab who gave access to the laboratory and research facilities, and borrowing of their chemicals and other equipment.

I would like to express great thanks to my friends who all have helped me on this journey in some way.

Finally, I would like to thank my family: Mum and Dad and to my brothers and sister who have been a constant source of emotional support. It is my privilege to express a huge thanks to my wife Shaema, for her continuous encouragement, patience and precious support throughout my PhD and my life in general. I am very grateful for everything you have done for me and for helping me get to where I am today.

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AC	Adenylyl cyclase
ADO	Adenosine
AE	Anion exchanger
ASL	Airway Surface Liquid
ATP	Adenosine-5'-triphosphate
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)
BCECF-AM	2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
cAMP	3'-5'-cyclic adenosine monophosphate
Cch	Carbachol
CFTR	Cystic fibrosis transmembrane conductance regulator
CytoD	Cytochalasin D
DAPI	4',6-diamidino-2-phenylindole
db-cAMP	dibutryl-3'-5'-cyclic adenosine monophosphate
DIDS	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid
EGTA	Ethylene Glycol Tetraacetic Acid
EMEM	Eagle's Minimum Essential Medium
ENaC	Epithelial Na^+ channel
ESI-09	3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-Propionitrile

Fsk	Forskolin
Fura-2-AM	2-[6-[Bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-benzofuranyl]-5-oxazolecarboxylic acid (acetyloxy)methyl ester
IBMX	3-Isobutyl-1-methylxanthine
MSD	Membrane spanning domain
mTOR	Mammalian target of rapamycin
NBC	Na^+/HCO_3^- cotransporter
NBD	Nucleotide binding domain
NDCBE	Na^+-dependent Cl^-/HCO_3^- Exchanger
NHE	Na^+/H^+ exchanger
NHERF	Na^+/H^+ Exchange Regulatory Factor 1
OA	Okadaic acid
PBS	Phosphate Buffered Saline solution
PDE	Phosphodiesterase
PFA	Paraformaldehyde
pH_i	Intracellular pH
PKA	Protein kinase A
PKC	Protein kinase C
R	Regulatory domain of CFTR
Rp-cAMP	Rp-adenosine-3',5'-cyclic monophosphorothioate
TBB	4,5,6,7-tetrabromo-benzotriazole

TEER	Transepithelial electrical resistance
tmAC	Transmembrane adenylyl cyclase
VIP	Vasoactive intestinal peptide
β_{HCO3-}	CO_2-HCO_3^- buffer system-dependent buffering capacity
β_i	Intrinsic buffering capacity
β_{tot}	Total buffering capacity
$[cAMP]_i$	Intracellular concentration of cAMP
$[Ca^{2+}]_i$	Intracellular concentration of Ca^{2+}

Chapter 1 Introduction

1.1 HCO_3^- secretion by epithelial cells

The secretion of bicarbonate (HCO_3^-) by epithelial cells is essential for maintaining the normal function of many epithelial tissues as HCO_3^- plays a major role in the regulation of both intracellular and extracellular pH (Durie, 1989; Allen *et al.*, 1993). Extracellular HCO_3^- is also important for many other epithelial functions. As HCO_3^- is a biological buffer, it plays an important role in the process of acid-base homeostasis by preventing metabolic and respiratory disease (Kellum, 2000). The pH of mucosal layers that line all epithelia is also buffered by HCO_3^- which can protect them from injury (Allen *et al.*, 1993). HCO_3^- also plays a crucial role in the process of nutrient digestion and solubilisation of complex mixtures of protein, including digestive enzyme and mucin secreted by epithelial cells (Scratcherd and Case, 1973). Furthermore, HCO_3^- secretion by epithelial cells drives fluid secretion in many epithelial tissues, such as in the gastro-intestinal tract (GIT) and reproductive tracts (Hug *et al.*, 2011). Recently, it has been demonstrated that the local HCO_3^- concentration, which controls the pH and HCO_3^- availability at the plasma membrane surface, plays a critical role in regulating the HCO_3^- transporters involved in fluid and HCO_3^- secretion (McKenna and Frost, 2014). Mutation in HCO_3^- transporters, such as the cystic fibrosis transmembrane conductance regulator (CFTR), Cl^-/HCO_3^- anion exchangers (AEs) belonging to the SLC26 and SLC4 families, as well as Na^+-HCO_3^--cotransporters (NBCe1) (Lee *et al.*, 2012) lead to absence or altered HCO_3^- secretion and development of several diseases such as the cystic fibrosis (CF) (Yang *et al.*, 2009; Quinton, 2010), pancreatitis (Lee *et al.*, 2012; Maleth and Hegyi, 2014), congenital chloride diarrhoea, deafness and hypotension (Durie, 1989; Hoglund *et al.*, 1996; Moseley *et al.*, 1999; Taylor and Aswani, 2002; Wall, 2006; Wangemann *et al.*, 2007) and Sjogren's syndrome (Almstahl and Wikstrom, 2003).

In the GIT, it has been shown that mammalian pancreatic ducts secrete HCO_3^- when stimulated by cAMP agonists such as secretin and forskolin, which is critical for neutralizing the acidic chyme in the small intestine to provide the optimum conditions for activation of pH-sensitive digestive enzymes (Scratcherd and Case, 1973; Ishiguro *et al.*, 1996). In addition, HCO_3^- secretion by epithelial cells of the GIT plays an important role in the protection of the GI mucosa, and the presence of high amounts of HCO_3^-, together with gastric mucins in the stomach, provides a protective barrier for the gastroduodenal mucosa against gastric acid and pepsin (Flemstrom and Isenberg, 2001; Allen and Flemstrom, 2005). Furthermore, it has been found that there is cAMP-dependent HCO_3^- secretion in the duodenum, jejunum and ileum of mice which is critically important to neutralize gastric acid and thereby protect the intestinal mucosa (Seidler *et al.*, 1997; Seidler *et al.*, 2001). HCO_3^- secretion in the GIT also plays a crucial role in mucus release, as HCO_3^- has a critical importance in expansion and solubilisation of secreted mucins to prevent the formation of aggregated mucus (Garcia *et al.*, 2009). Furthermore, it has been demonstrated that reduced HCO_3^- secretion in CF leads to the secretion of aggregated and viscous mucus in affected organs (Chen *et al.*, 2010). A recent study has shown a thick and less penetrable mucus secretion from CF mouse ileum, which adhered to the epithelium (Gustafsson *et al.*, 2012), but importantly the defective properties of this secreted mucus were normalized by adding a high concentration of bicarbonate (100 mM).

As shown in figure 1.1, the mucin glycoproteins inside the intracellular mucin granules are condensed and tightly packed together by the presence of high amounts of Ca^{2+}. As mucin are secreted by exocytosis onto extracellular surface, HCO_3^- plays an essential role in chelation of these cations which causes expansion of the negatively charged mucins chain to form normal mucus (Garcia *et al.*, 2009; Borowitz, 2015). A more recent study has shown that MUC5B, a mucin expressed by respiratory epithelia, bound to Ca^{2+} at its N-terminal D3-domain and formed cross-links which play an essential role in MUC5B condensation and packing inside the secretary granules. The maximal cross-link formation is dependent on the presence of high calcium concentrations and low pH (5-6) in secretory granules. Thus, after MUC5B secretion from secretory

granules, the presence of HCO_3^- allows for efficient mucin expansion and secretion by uncoupling the cross-links between Ca^{2+} and MUC5B (Ridley *et al.*, 2014). Furthermore, HCO_3^- can mimic the effect of EGTA as a Ca^{2+} chelator, to reduce the amount of free Ca^{2+} concentration bound to mucins, which enhances mucin swelling and hydration, thereby dissolving mucin aggregates and likely increasing its transportation (Chen *et al.*, 2010).

Human oesophageal submucosal glands have the ability to secrete sufficient amounts of HCO_3^- that is capable of neutralizing the remaining acid in the esophagous after bolus swallowing which comes near to the HCO_3^- output by salivary glands at rest (Meyers and Orlando, 1992). Another critically important role for HCO_3^- is in the process of fertilisation. It has been shown that HCO_3^- is required for stimulation of sperm motility and spermatogenesis in Leydig cells, and sperm capacitation inside the female reproductive tract (Medina *et al.*, 2003; Hess *et al.*, 2005). Also, it has been reported that defects in CFTR-dependent HCO_3^- secretion leads to reduction in spermatogenesis and thereby azoospermia (Xu *et al.*, 2011).

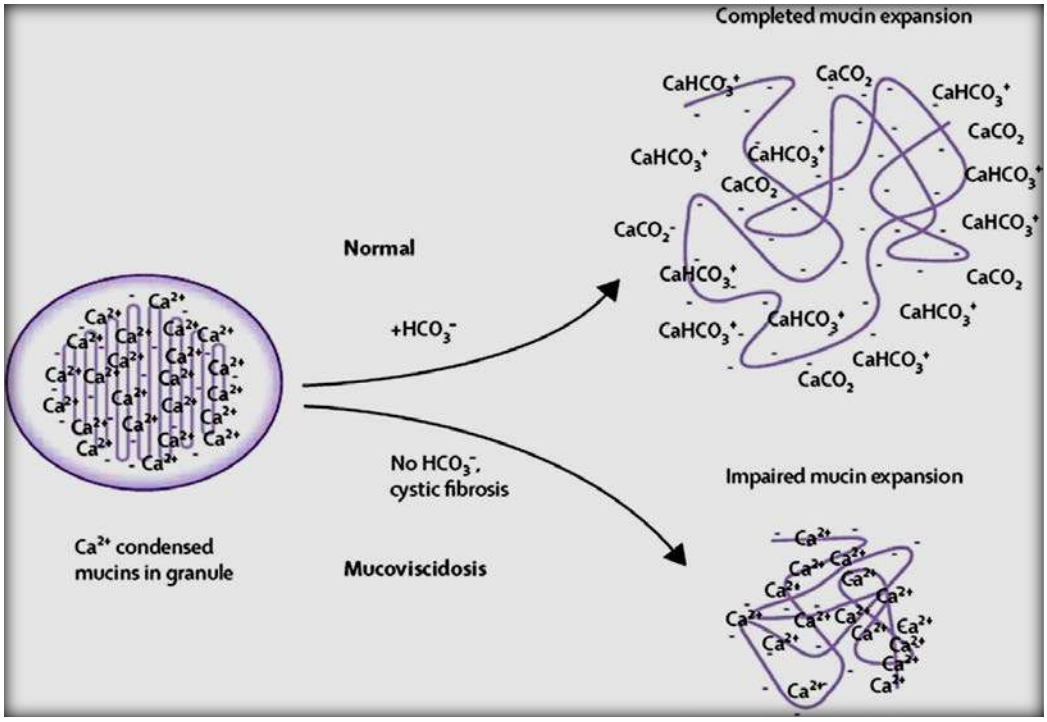


Figure 1. 1: A Schematic diagram of the HCO_3^- dependent mechanism of extracellular mucin expansion and solubilisation from condensed mucin granules. Presence of HCO_3^- in the extracellular surface dissociates the bounded Ca^{2+} with the condensed mucin in granule and therefore rapidly expands the released mucin, and prevents the formation of abnormal mucus. Figure taken from (Borowitz, 2015).

1.1.1 Mechanism of Bicarbonate secretion in epithelial tissues

Although HCO_3^- secretion plays a critical role in the regulation of normal body function, it is still controversial how HCO_3^- secretion is actually achieved in many epithelial tissues, although CFTR appears to be a critical requirement. This may be because different epithelial tissues employ a different complement of transporters, and/or because the mechanism may also be dependent on the agonist employed (Seidler *et al.*, 1997).

The exocrine pancreas is probably the most thoroughly studied tissue, and pancreatic duct cells are capable of secreting several litres per day of a near isotonic $NaHCO_3$ fluid known as pancreatic juice (Ishiguro *et al.*, 2012). In the ductal cells HCO_3^- secretion across the apical membrane is an electrogenic process, but it is still not fully clear whether HCO_3^- exit is mediated solely by

CFTR or by an electrogenic SLC26 anion exchanger (Steward *et al.*, 2005), and/or a combination of the two transporters working together. Furthermore, HCO_3^- secretion is an active process that occurs against an electrochemical gradient, which depends on Na^+, K^+, and Cl^-, and involves a number of proteins including Na^+-K^+ ATPase, Na^+-H^+ exchangers, Cl^-/HCO_3^- exchangers, as well as carbonic anhydrase (CA). Bicarbonate secretion is stimulated by an increase in intracellular cAMP in response to the hormone, secretin, (Argent *et al.*, 2012), and early studies showed that this led to an increase in the Cl^- conductance across the apical membrane of ductal epithelial cell of the rat pancreas (Gray *et al.*, 1988; Novak and Greger, 1988b), which was later identified to be due to activation of CFTR (Gray *et al.*, 1993). These studies together with other electrophysiological data (Novak and Greger, 1988a) and intracellular pH measurements (Stuenkel *et al.*, 1988) led to a model of HCO_3^- secretion in pancreatic duct epithelial cells, as shown in figure 1.2A (Gray *et al.*, 1988; Novak and Greger, 1988b). In this model of HCO_3^- secretion, CO_2 enters the ductal epithelial cells across the basolateral membrane by simple diffusion, where it is then hydrated by the action of cytoplasmic CA to generate HCO_3^-. The protons generated via CA activity are then then actively extruded from the cells across the basolateral membrane by the Na^+/H^+ exchanger, whose activity is maintained by the inward Na^+-gradient, via continuous activity of Na^+-K^+ ATPase. At the apical membrane, HCO_3^- is secreted into the duct lumen by Cl^-/HCO_3^- AE activity, which depends on the luminal availability of Cl^- that is maintained by the activity of CFTR and calcium-activated Cl^- channels, in the case of calcium-stimulated secretion. In later studies, results from Ishiguro *et al*., (2001) led to a modification of this model to account for the ability of ductal cells to maintain the secretion of HCO_3^- against a high concentration of luminal HCO_3^- (>120mM). They showed that bilateral perfusion of high Cl^--low HCO_3^- solutions in pancreatic ducts under resting conditions, lead to HCO_3^- uptake via NBC and HCO_3^- recycling by Cl^-/HCO_3^- exchanger at the basolateral membrane, and Cl^- and HCO_3^- secretion at the apical membrane via Cl^-/HCO_3^- exchanger and CFTR (Figure 1.2B). However, cAMP-stimulation provided more HCO_3^- to accumulate inside the cells, by inhibition of the basolateral Cl^-/HCO_3^- exchanger and activation of the NBC, thereby providing the driving force for HCO_3^- secretion across the apical membrane, where Cl^- exits from the cells via

activated CFTR and HCO_3^- leaves the cells in exchange with Cl^- via Cl^-/HCO_3^- exchange activity (Ishiguro *et al.*, 2001) (Figure 1.2C). However, they also showed that perfusion of a high HCO_3^-, but low Cl^- solution in the lumen of the ducts, under cAMP stimulated conditions, abolished the apical Cl^-/HCO_3^- exchange activity and subsequently reduced the luminal content of Cl^-, which favoured HCO_3^- efflux across the apical membrane solely via CFTR (Figure 1.2D). In addition, Stewart *et al*., (2009) demonstrated that there is a functional interaction between CFTR and the SLC26A6 Cl^-/HCO_3^- AE in HCO_3^- secretion across the luminal membrane of pancreatic interlobular ducts (Stewart *et al.*, 2009). Here, they showed that HCO_3^- secretion was mediated mainly by SLC26A6 Cl^-/HCO_3^- exchanger, which was enhanced by CFTR inhibition. Overall, these studies in the pancreas highlight the dynamic nature of HCO_3^- secretion in this epithelium.

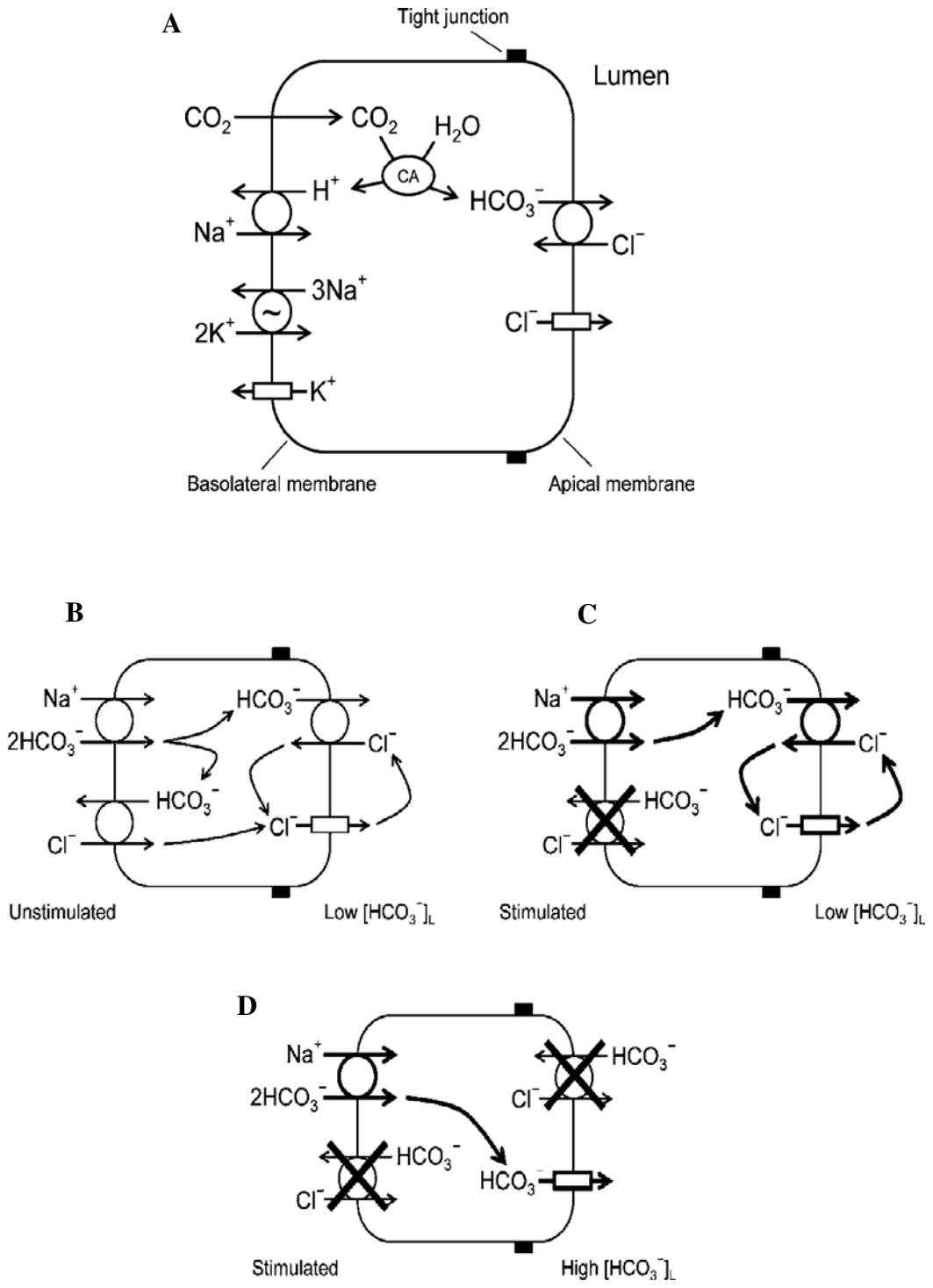


Figure 1. 2: Model of HCO_3^- secretion in pancreatic duct epithelial cells. (A) Proposed cellular mechanism of HCO_3^- secretion in pancreatic duct epithelium. (B) Anion fluxes in unstimulated duct cells with bilateral perfusion of high Cl^- and low HCO_3^- solutions. (C) Anion fluxes in stimulated cells and bilateral perfusion of high Cl^- and low HCO_3^- solutions. (D) Anion fluxes in stimulated cells and bilateral perfusion of low Cl^- and high HCO_3^- solutions. Diagrams taken from (Steward *et al.*, 2005).

1.2 HCO_3^- secretion in the airways

The human respiratory system consists of a series of branching tubes and is composed of two main parts: (i) the conducting zone (larynx, trachea, bronchi and bronchioles) which is lined by a thin layer of fluid (6-10μm) known as the airway surface liquid (ASL; see section 1.4)), and it is the site for gas transportation into the lung; (ii) the respiratory zone that consist of respiratory bronchioles and alveolar ducts terminating in alveoli, which is responsible for gas exchange (Fowler, 1948), as shown in Figure 1.3.

The surface of the conducting airways mainly consists of a ciliated epithelium that is found in the nasal cavity, trachea, and bronchi (Willumsen and Boucher, 1989). The epithelial cells lining the bronchioles are more columnar, with Clara cells interspersed along with ciliated cells. There are also goblet cells which secrete mucus. The ability of the epithelium to maintain different apical and basolateral membranes is determined by the tight junctions (TJs), which play an important role in the regulation of pH by maintaining concentration gradients between the serosa and the ASL that lines the epithelium (Widdicombe, 2002b). The tracheobronchial part of the conducting airways of higher mammals also contain numerous submucosal glands (SMGs) (Goco *et al.*, 1963). These are composed of secretory tubules, a collecting duct, and a ciliated duct that open to the luminal surface of the airway (Meyrick *et al.*, 1969). The glands are lined by ciliated pseudo-stratified columnar epithelial cells, with a small number of goblet cells and brush cells (Carden DL, 2000). SMGs secrete mucins and antimicrobial substances, such as lysozyme, lactoferrin, collectins, and Beta-defensins. Indeed, airway mucus is produced primarily by SMGs. Mucus is a complex mixture of water, salts, mucins and other macromolecules, including antimicrobials, antiproteases and antioxidants (Krouse *et al.*, 2004). Moreover, SMGs are responsible for the secretion of liquid which is important for the transport of macromolecules from the secretory tubules of the glands, as well as producing a significant fraction of the ASL. The foundation of the airway innate host defence mechanism is formed by the concerted action of mucus (which traps pathogens), antimicrobial substances (which eliminate pathogens) and mucociliary transport (which cleans the airways of pathogens) (Verkman *et al.*,

2003; Ballard and Inglis, 2004; Wine and Joo, 2004; Inglis and Wilson, 2005). Studies by Welsh and Smith, (2001) found that cAMP agonists can also provoke HCO_3^- secretion through the apical membrane of airway epithelia (Welsh and Smith, 2001).

The active secretion of both Cl^- and HCO_3^- is responsible for liquid secretion by submucosal glands (see section 1.3 for more details). Bicarbonate performs a wide range of functions, such as the solubilisation and transportation of mucus (Quinton, 2008). Furthermore, the recent finding that mucus secretion is assisted by HCO_3^- secretion (Quinton, 2001; Garcia *et al.*, 2009) and that the viscosity and expansion of mucins are controlled by HCO_3^- (Chen *et al.*, 2010; Muchekehu and Quinton, 2010) at least in the GIT, suggests that a robust secretion of HCO_3^- by SMGs is essential for appropriate mucus homeostasis, which is consistent with a recent study that demonstrated HCO_3^- secretion plays critical role in mucus hydration (Xiao *et al.*, 2012) in the GIT. However, whether this is true for the airways is currently not clear. However,, it has been shown that acidic pH leads to decline in ciliary beating (Clary-Meinesz *et al.*, 1998) and impairs the function of phagocytic cells to destroy pathogenic microorganism (Allen *et al.*, 1997). More recently, Pezzulo (2012) showed that acidic pH in CF human and pig airways led to a marked reduction in bacterial killing, which could be partially recovered after re-alkalinisation of the ASL pH (Pezzulo *et al.*, 2012a). In brief, insufficient HCO_3^- secretion by SMGs is thought to be followed by accumulation of mucus plugs in the airways, bacterial colonisation and biofilm formation, inflammation and pathology characteristic of chronic lung disease such as seen in the lungs of CF patients (Hug *et al.*, 2003). Although it is well known that HCO_3^- has a critical role in a variety of body tissues, the role of HCO_3^- in the lungs is gaining a major interest within the field of airway physiology.

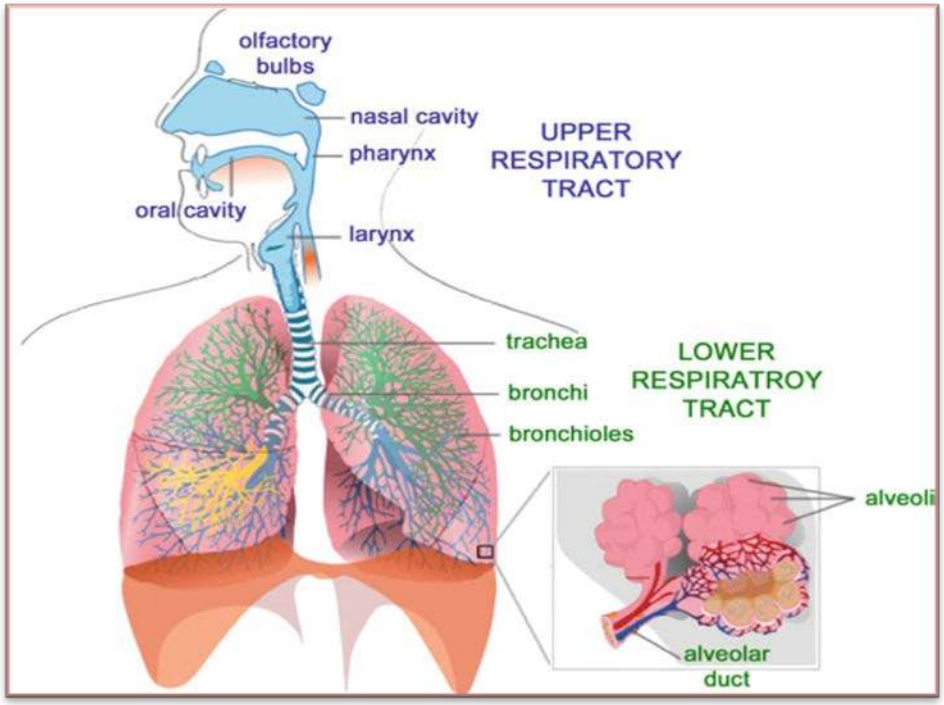


Figure 1. 3: Schematic illustration of the human respiratory system. Diagram shows the conductive airway and the respiratory zone, which is the site of gas exchange. Figure is adapted from (Tu *et al.*, 2013).

1.3 Submucosal glands (SMGs)

SMGs are responsible for the secretion of approximately 95% of upper airway mucus (Reid, 1960). SMGs consist of four distinct regions that play crucial roles in the production and processing of liquid mucus to be secreted onto the airway surface. The first part consists of serous tubules and acini, which are responsible for secretion of salt, water and a wide range of antimicrobial proteins (Tom-Moy *et al.*, 1983; Zhao *et al.*, 1996; Bals *et al.*, 1998; Singh *et al.*, 1998). The second part are the mucus tubules that are lined by epithelia cells which are filled with mucin containing secretory granules (Nadel, 1983). These tubules allow the serous secretion to pass through. The third part, which is lined by non-ciliated columnar epithelial cells, is called the collecting duct. It has been suggested that epithelial cells lining the collecting duct adjust the ionic composition of serous and mucus epithelial cell secretions (Nadel *et al.*, 1979). The last part is the

ciliated duct that is enclosed by cells which line the opening of the gland and an extension of the airway surface epithelium (Figure 1.4A). The majority of gland salt and water, as well as antimicrobial proteins, are thought to be secreted by the epithelial cells lining the serous acini and tubules (Tom-Moy *et al.*, 1983; Zhao *et al.*, 1996; Bals *et al.*, 1998; Singh *et al.*, 1998). The secretion of fluid and antimicrobial factors then become mixed together with mucin, which are then transported onto the luminal surface of the airway through the collecting duct.

1.3.1 Mechanism and regulation of SMG secretion

SMG produce a small amount of liquid secretion at rest, but will secretes copious amount of fluid under synergistic stimulation by either acetylcholine or substance P and vasoactive intestinal peptide (VIP) (Choi *et al.*, 2007; Ianowski *et al.*, 2008). Airway SMG secretions are primarily controlled by two main mechanisms; (i) a non-cholinergic mechanism, which is mediated by airway intrinsic neurons mostly secreting, VIP and tachykinins, and (ii) a cholinergic mechanism, which is under the control of vagal pathways (Wine, 2007); see figure 1.4B)). SMG serous cells are tightly innervated by mutual excitatory intrinsic airway neurons (Widdicombe and Wine, 2015). The neurotransmitters released by intrinsic airway neurons, including VIP and substance P, play an important role in liquid secretion by SMG through activation of VIP receptor 2 (VPAC2) and NK1 tachykinin receptors, respectively, (Groneberg *et al.*, 2001; Phillips *et al.*, 2003). It has been shown that stimulation of tachykinin receptors by substance P leads to an increase in $[Ca^{2+}]_i$, which enhances fluid secretion by SMG in the feline trachea (Nagaki *et al.*, 1994). Stimulation of VPAC receptors by VIP leads to an increase in $[cAMP]_i$ via activation of adenylyl cyclase, which stimulates SMG secretion (Groneberg *et al.*, 2006). The function of SMG serous cells in the secretion of salt and fluid is shown in figure 1.4C. Active transepithelial Cl^- and HCO_3^- secretion plays an important role in fluid secretion by the airway SMG (Ballard and Inglis, 2004). The study by Joo *et al.*, (2001) confirmed the HCO_3^--dependence of fluid secretion from isolated sheep tracheal

SMG where carbachol stimulated fluid secretion was inhibited ~ 67% by replacing HCO_3^- with HEPES (Joo *et al.*, 2001). Moreover, the Cl^- and HCO_3^- dependence of fluid secretion was also observed in porcine SMG, where forskolin or VIP stimulated fluid secretion was inhibited ~50% by either bumetanide or HCO_3^- replacement (Joo *et al.*, 2002). Salt and fluid secretion by serous cells is primarily regulated by the apical anion channel CFTR, which is more strongly expressed in the apical membrane of serous epithelial cells than other types of cells in the airways (Engelhardt *et al.*, 1992; Jacquot *et al.*, 1993; Sehgal *et al.*, 1996), (for more details see section 1.5). In addition, strong evidence has shown that stimulation of CFTR with either forskolin or cholinergic agonists plays an important role in fluid secretion, which was inhibited by the CFTR inhibitor, $CFTR_{inh}$-172, in human and pig airway SMGs (Thiagarajah *et al.*, 2004). Absence of CFTR in the apical membrane of serous epithelial cells leads to a decrease in Cl^- and HCO_3^- secretion onto the airway surface, as well as reduction in ASL, and thereby produces a defect in mucociliary clearance (Boucher, 2007a).

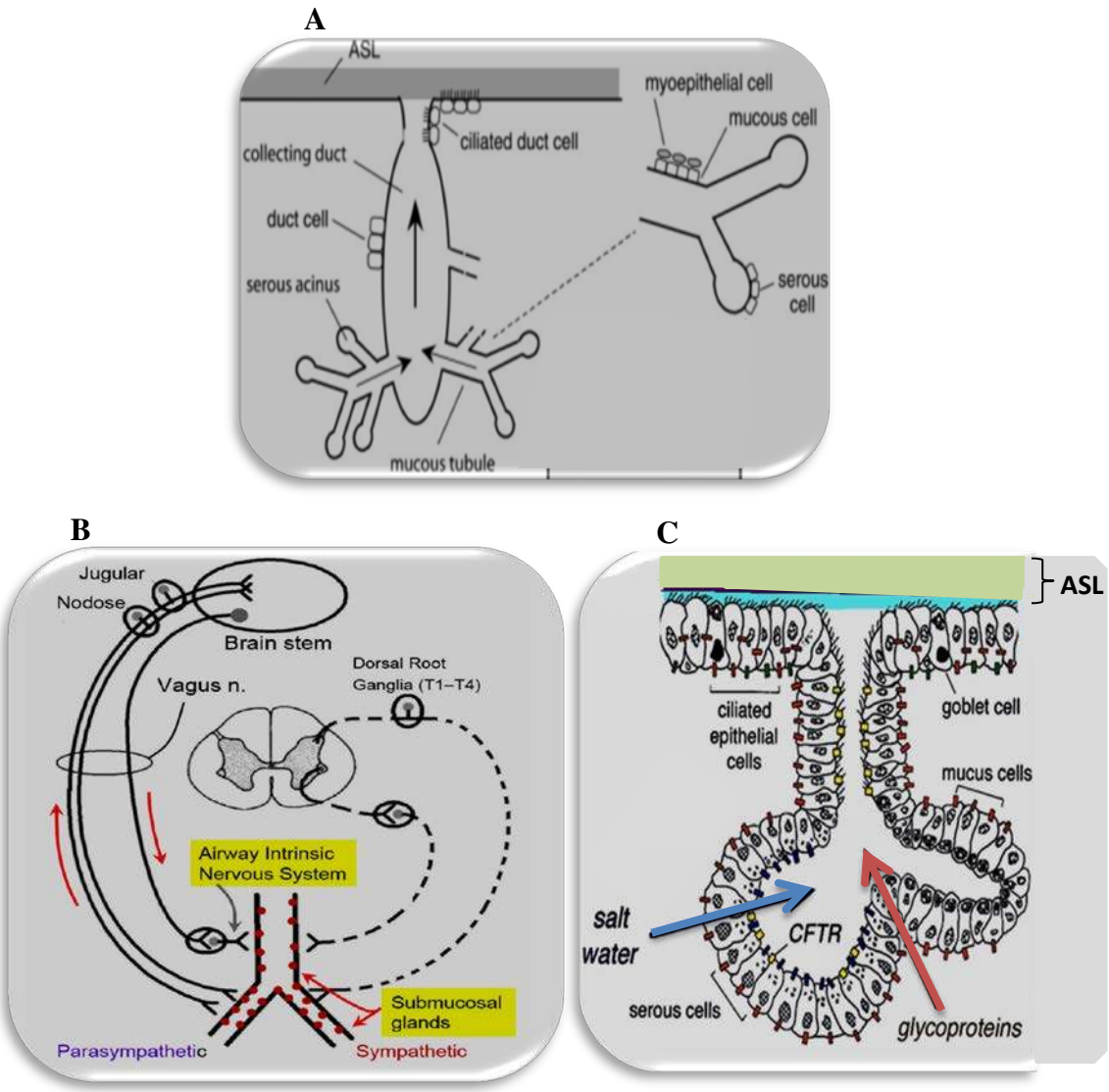


Figure 1. 4: Overview of airway submucosal gland innervation and structures. (A) Schematic presentation of SMG and its four compartments. Reproduced from (Verkman *et al.*, 2003). (B) shows airway innervation by the autonomic nervous system, with parasympathetic (solid lines), and sympathetic system (dashed lines), figure is taken from (Wine, 2007). (C) The extended observation of serous cell of submucosal glands, which plays a major role in the secretion of a watery fluid via the CFTR activity located on the apical surface of the serous cells. Figure adapted from (Salinas *et al.*, 2005).

1.4 Airway surface liquid (ASL)

The ASL is defined as a thin layer of aqueous solution about 10μm deep that lines the airway surface (Widdicombe, 2002a), which contains ions, water, gel-forming glycoprotein (mucin; MUC5B) (Widdicombe and Wine, 2015), and a variety of proteins including lactoferrin, defensins and lysozyme that are considered to play a vital function in airway hydration, natural immunity and antimicrobial defense (Zabner *et al.*, 1998). The majority of the ASL is secreted by SMGs upon stimulation by Ca^{2+} and/or cAMP agonist, that plays an important role in mucociliary clearance and removal of inhaled pathogen from the lung (Cole *et al.*, 1999; Wine and Joo, 2004; Widdicombe and Wine, 2015). It has been suggested by *in vivo* studies of radiotracer particle clearance that normal ASL removes deposited particles, including bacteria, on the airway surface within approximately six hours (Wanner *et al.*, 1996). As shown in Figure 1.5A, the ASL consists of two layers, defined by light and electron microscopy: the periciliary liquid layer (PCL) located next to the airway epithelium, and an overlying thicker gel layer, made up of mucus (Boucher, 2002). Normally, a very thin layer of ASL covers the luminal surface of the airways. Its quantity is accurately regulated to compensate for evaporation. During mucociliary clearance, the ASL facilitates cilia beating, and collectively with antimicrobial factors, plays an important role in the elimination of inhaled pathogens (Boucher, 1999). Deposited particles on the airways surface are effectively removed by the synchronized function of the two-phase gel system on the airway surface as shown in Figure 1.5. The PCL extends from the cell surface to the height of the extended cilia, The mucus layer is situated on the top of the cilia (Knowles and Boucher, 2002). Originally, the PCL was believed to be composed of liquid. However, the polyanionic gel properties of this layer (Randell and Boucher, 2006) give a perfect low resistance environment for cilia beating and also avoids the mucus layers from sticking together with the cell surface. The mucus layer is primarily composed of mucins (Raviv *et al.*, 2003).

Recently, a new model of the periciliary layer has been proposed by Button *et al*., (2012). They showed that a macromolecular meshwork is present in the PCL of primary human bronchial epithelial cell cultures. Here they proposed that the

macromolecular meshwork is occupied by mucins and mucopolysaccharides that are tethered to cilia, microvilli and the epithelial surface to form an extracellular brush that prevents penetration of mucins and inhaled particles deposited on the mucus layer, into the PCL, which is required for effective mucociliary clearance (Button *et al.*, 2012). Inadequate secretion from the SMGs, in combination with improper ion transport activity of the epithelial cells lining the luminal surface of the airways, leads to a reduction in ASL as seen in CF patients. Therefore, regulation of the ASL plays a crucial role in the process of mucociliary clearance, and it is this which is defective in CF, where mucus become more viscous and dehydrated (Matsui *et al.*, 1998). It has been concluded that a reduction in ASL also inhibits neutrophil migration, which prevents the ability of neutrophils to capture and kill bacteria on the surface of airway epithelia (Matsui *et al.*, 2005). In normal airway epithelia (Figure 1.5A), both CFTR and the epithelial Na^+ channel (ENaC) play a critical role in the regulation of ASL volume and composition (see section 1.6 for more detail). As shown in figure 1.0B, defects in CFTR function produces a viscous mucus gel, which adheres to the airway surface and ciliary beating is impaired (Mall *et al.*, 2004), and thereby it becomes the site for chronic bacterial infection (Worlitzsch *et al.*, 2002). Recent studies have utilized Calu-3 cells as a model of airway SMG serous cells (for more details see section 1.11).

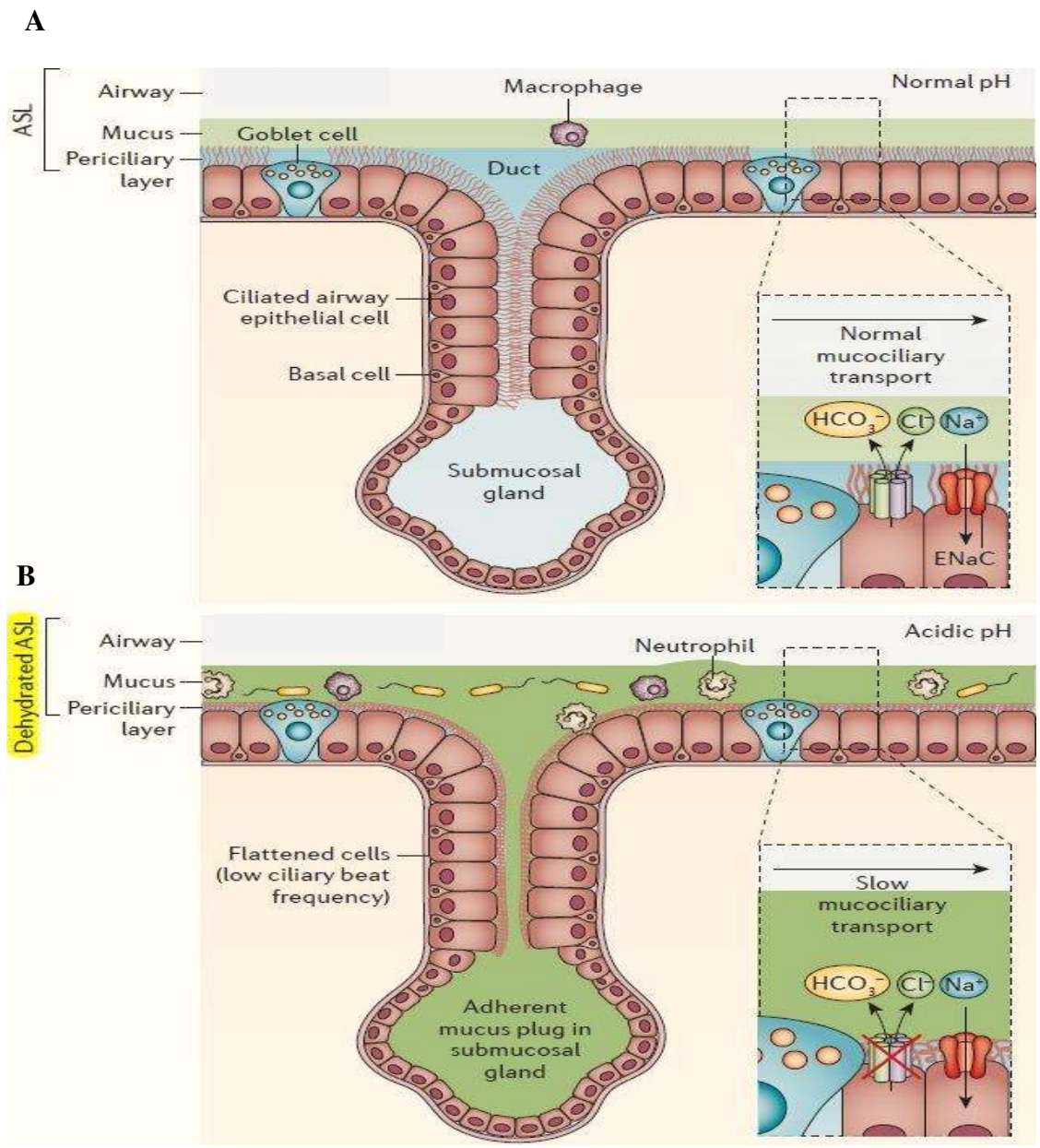


Figure 1. 5: The mechanism of airway mucus ciliary clearance. This illustration shows normal airways (A) in which the process of airways hydration controlled by the coordinated rate of Na^+ absorption and Cl^- secretion to support mucus clearance. It also shows airway surface dehydration (B) due to absence of CFTR function in CF. *ENaC*= epithelial Na^+ channel, *PCL* refers to periciliary liquid, *CFTR*= refers to cystic fibrosis transmembrane conductance regulator. Figure is taken from Ratjen *et al*., (2015) (Ratjen *et al.*, 2015).

1.5 Cystic fibrosis transmembrane conductance regulator (CFTR)

CFTR is a membrane spanning glycoprotein which serves as ion channel to transport anions across the apical surface of epithelial cells, and has an impact on a variety of cellular process (Ratjen *et al.*, 2015). This ATP-binding cassette (ABC) transporter-protein functions primarily as a Cl^- channel on the apical membrane of epithelial cells of various organs, such as liver, pancreas, digestive tract, reproductive tract and the airways (Nagel *et al.*, 1992). CFTR also functions as HCO_3^- transporter in epithelial cells of many organs, including lungs, gastrointestinal tract, and pancreas (Gray *et al.*, 2001). Thus, reduced HCO_3^- secretion has been reported in CF tissue (Ko *et al.*, 2002). Energy is used in the form of adenosine triphosphate (ATP) by all family members of ABC transporters in order to move a wide range of substance through the plasma membrane of cells (Dean *et al.*, 2001). However, CFTR is the only ABC transporter that is not an active pump, and instead uses ATP binding and hydrolysis to induce structural changes in the protein that leads to channel pore opening and closing (channel gating), respectively. CFTR has a significant impact on the amount and composition of epithelial secretions by conducting Cl^- and HCO_3^-, as well as being involved in regulating absorption and secretion of salt across the epithelial cells, which is followed by the creation of osmotic gradients leading to water transport across the epithelial cells to the mucosal surface (Riordan, 2008). Although CFTR serves as an anion channel, it acts also as a regulator, either directly, or indirectly, of a wide range of other ion channels, receptors, and transporters.

As shown in Figure 1.6, CFTR is composed of two repeating units which consist of two domains: a membrane spanning domain (MSD) which is hydrophobic and composed of six transmembrane segments; and a nucleotide binding domains (NBD), which is hydrophilic and is located in the cytoplasm, where its function is to bind and hydrolyse ATP (Naren and Kirk, 2000). The two repeated units are connected to each other through a unique regulatory (R) domain. Phosphorylation of this domain by cAMP-PKA is responsible for the activation of CFTR (Cheng *et al.*, 1991; Gadsby and Nairn, 1999). Activation of the CFTR increases the permeability of the apical membrane to Cl^- ions, which

then flow out from the cells (Figure 1.6). It has been shown that movement of Cl^- via the CFTR is stimulated by ATP binding to the NBDs, which is followed by dimerisation of the NBDs (Vergani *et al.*, 2005). CFTR can also interact via its R-domain with the C and N-terminal domains of a variety of other transporters and regulatory proteins, including members of SLC26 family of anion transporter (see section 1.7) (Ko *et al.*, 2004).

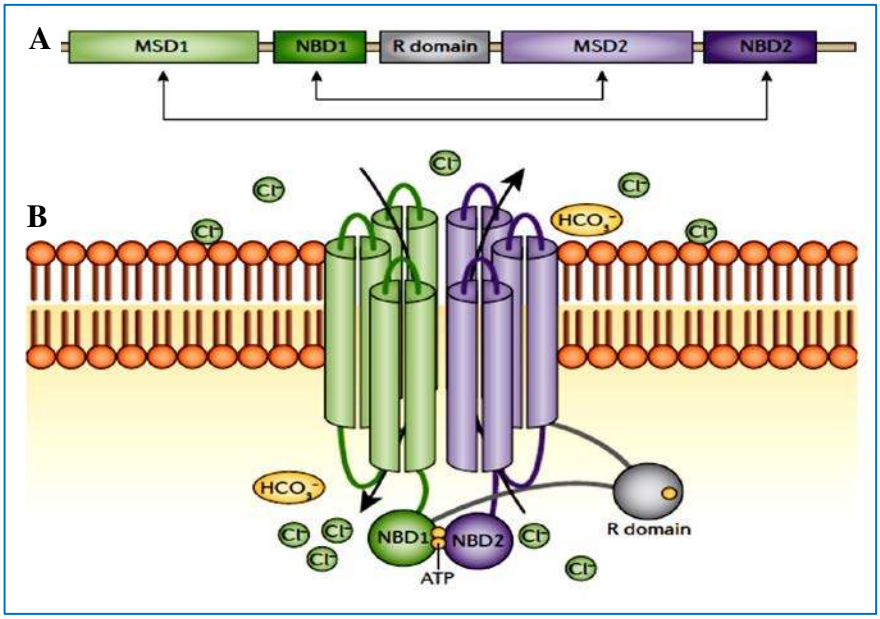


Figure 1. 6: Structures of the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel. (A) Shows the linear structure of CFTR. (B) shows the domains of the CFTR Cl^- channel, which consists of two membrane spanning domain (MSD; MSD1 and MSD1) that function as the ion channel pore through the plasma membrane, each connected with a nucleotide binding domain (NBD; NBD1 and NBD2). The CFTR channel opens when ATP is bound at the NBDs, and when the (R) domain is phosphorylated by PKA. Figure taken from (Ratjen *et al.*, 2015).

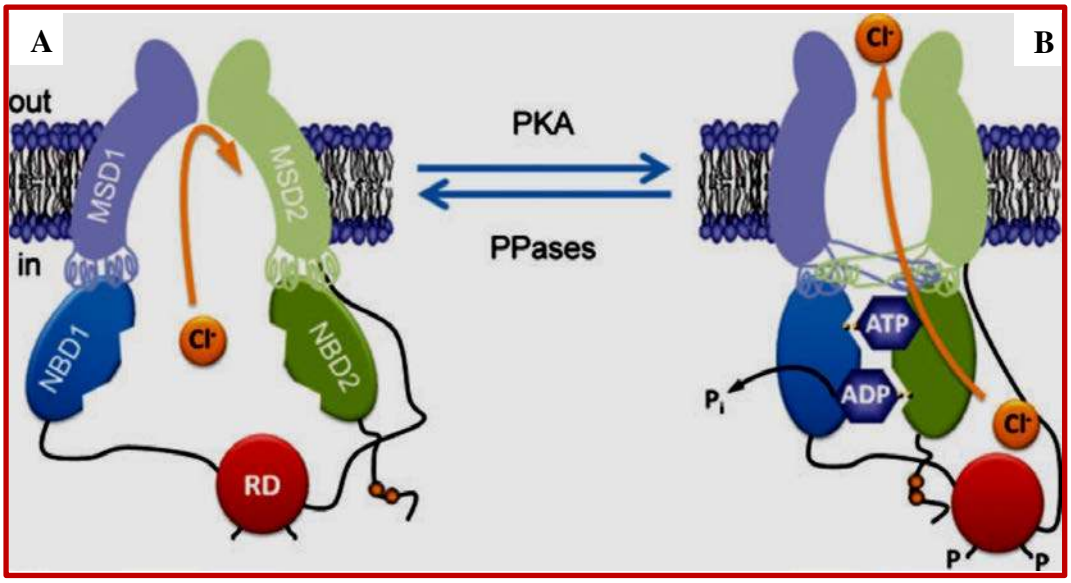


Figure 1. 7: Gating of the CFTR channel. Figure (A) shows the inactive (closed) form of the CFTR Cl^- channel without ATP bound to the NBDs and so Cl^- anions are trapped inside the cell until the process of phosphorylation and ATP binding to the NBDs occurs. (B) Illustrates the active (open) form of CFTR where the R domain is phosphorylated by PKA and ATP is bound to NBD1 and 2 which leads to NBD dimerisation, and channel opening. MSD=membrane spanning protein; NBD= Nucleotide binding membrane; RD= Regulatory domain. Figure taken from (Hwang and Sheppard, 2009).

1.6 Regulation of CFTR

It has been shown that the cAMP/PKA signalling pathway is the major pathway that regulates CFTR activity and which stimulates Cl^- and fluid secretion in CFTR-expressing epithelial cells (Cheng *et al.*, 1991; Haws *et al.*, 1994; Cobb *et al.*, 2002; Cobb *et al.*, 2003; Derand *et al.*, 2004; Hentchel-Franks *et al.*, 2004). In addition to PKA, CFTR has been shown to be phosphorylated by a range of different protein kinases, including Ca^{2+}-independent and Ca^{2+}-dependent isoforms of protein kinase C (PKC), cGMP-dependent protein kinases, PKGI and PKGII (Berger *et al.*, 1993), adenosine monophosphate stimulated kinase (AMPK) (Kongsuphol *et al.*, 2009), transmembrane human lemur tyrosine kinase 2 (LMTK2) (Wang and Brautigan, 2006) and casein kinase 2 (CK2) (Pagano *et al.*, 2008).

More recently, it has been shown that the 'master kinase' CK2 also plays an important role in the regulation of ENaC (Bachhuber *et al.*, 2008) and CFTR activity (Treharne *et al.*, 2009). CK2 is generally composed of two α catalytic subunits and two regulatory β subunits (Litchfield, 2003), which can specifically bind to and phosphorylate the CK2-binding subunits at the C-terminus of the ENaC (Bachhuber *et al.*, 2008). In addition, it has been found that CFTR interacts with CK2 in the apical membrane of human airway epithelia (Treharne *et al.*, 2009). In the NBD1 of CFTR, there is a CK2 binding motif, known as SYDE, which is located close to the F508 residue of CFTR (Venerando *et al.*, 2014). In addition, *in vitro* studies have shown that the α-catalytic subunit of CK2 phosphorylates NBD1 of CFTR (Pagano *et al.*, 2008), and disruption of CK2-CFTR interaction by either CK2 inhibition or ΔF508-CFTR closes the CFTR channel and reduces CFTR-dependent Cl^- transport (Treharne *et al.*, 2009). In the airways, CFTR also functions as a down regulator of the ENaC, and therefore intracellular increases of cAMP also inhibit ENaC, which prevents Na^+ reabsorption from the lumen of the airways, and thereby provides an osmotic driving force for water transport into the ASL (Mall *et al.*, 1999).

1.7 Cystic fibrosis

CF is the most common, life threatening, inherited disease with approximately 9,000 affected people (1 in 2,500 live births) in the UK (Rowntree and Harris, 2003). CF is an autosomal recessive disorder caused by mutations in the CFTR gene which interferes with the functions of a wide range of body organs (Xingshen Sun, 2010). Around 2000 CF mutations in the CFTR gene have been identified (www.genet.sickkids.on.ca). The most common CFTR mutation is deletion of phenylalanine at position 508 (deltaF508), which affects around 70% of CF patients (Davis, 2006). Mutations in the CFTR gene leads to either a reduction in the amount of the CFTR or dysfunction of this protein (Cheng *et al.*, 1990; Welsh and Smith, 1995). DeltaF508 mutated CFTR is unable to reach the proper location in the cell membrane, instead it is retained in the endoplasmic reticulum, via the endoplasmic reticulum quality control (ERQC) and subsequently destroyed by the cytoplasmic proteasome (Cheng *et al.*, 1990; Ward and Kopito, 1994; Ward *et al.*, 1995; Gelman *et al.*, 2002). Therefore, the CFTR protein is not produced, or severely reduced, at the cell membrane, and affected tissues suffer from failure of cAMP dependent Cl^- secretion. Investigation of HCO_3^- secretion in cystic fibrosis (CF) tissue highlighted the importance of CFTR in regulating HCO_3^- secretion. Reduced HCO_3^- secretion has been reported in a wide range of CF tissue, including CF canine airways (Smith and Welsh, 1992), CF murine intestinal epithelia (Xiao *et al.*, 2012), CF human nasal epithelia (Paradiso *et al.*, 2003) as well as in transfected cell lines with a mutant CFTR (Choi *et al.*, 2001). Moreover, it has been shown that ASL $[HCO_3^-]$ in normal cultured primary human bronchial epithelia (HBEs) is higher than CF ASL (Coakley *et al.*, 2003). Here they also found that an increase in $[cAMP]_i$ stimulates HCO_3^- secretion in response to an acidic ASL, thereby causing rapid ASL alkalinisation. However, acidic ASL was not alkalinised in HBEs isolated from CF patients, in resting or cAMP elevated conditions, consistent with lack of CFTR-dependent HCO_3^- secretion by CF airway epithelia (Coakley *et al.*, 2003). Furthermore, similar finding has been demonstrated that the rate of HCO_3^- secretion was markedly decreased in CF human nasal epithelia, compared to non-CF nasal epithelia (Paradiso *et al.*, 2003).

In CF, respiratory failure is the primary cause of death among patients, and occurs as a result of production of a high amount of mucus, and a decrease in the process of mucociliary clearance, which leads to obstructive lung disease and chronic bacterial airway disease (Figure 1.5). It has been shown that over 85% of people with CF, suffer from improper functioning of the pancreas, which is characterised by loss of their ability to produce pancreatic digestive enzyme and subsequently malnutrition occurs (Kerem *et al.*, 1989; Estivill *et al.*, 1995; Mateu *et al.*, 2002).

Current studies have suggested that susceptibility of the airway surfaces to dehydration and failure of mucus clearance in CF lung disease is caused by CFTR gene mutation leading to loss of CFTR dependent Cl^- efflux and control of ENaC activity (Boucher, 2007b). In CF patients, the two main factors that are responsible for chronic bacterial infection and failure of the lung are defects in SMG function and abnormality in the ASL composition, as shown in figure 1. 8. Inadequate secretion of Cl^- across the apical membrane of airway epithelia, together with the absorption of a high amount of Na^+ from the extracellular surface, creates an osmotic gradient and copious amount of water is removed from ASL, this leads to formation of thick, viscous, mucus, which block the airway and then leads to chronic airway infection with bacteria, most commonly *Pseudomonas aeruginosa.* As a result, localized areas of the bronchial tree become irreversibly dilated as its muscle and elastic tissue are destroyed. Moreover, there is progressive destruction of the airway (Welsh, (1995)).

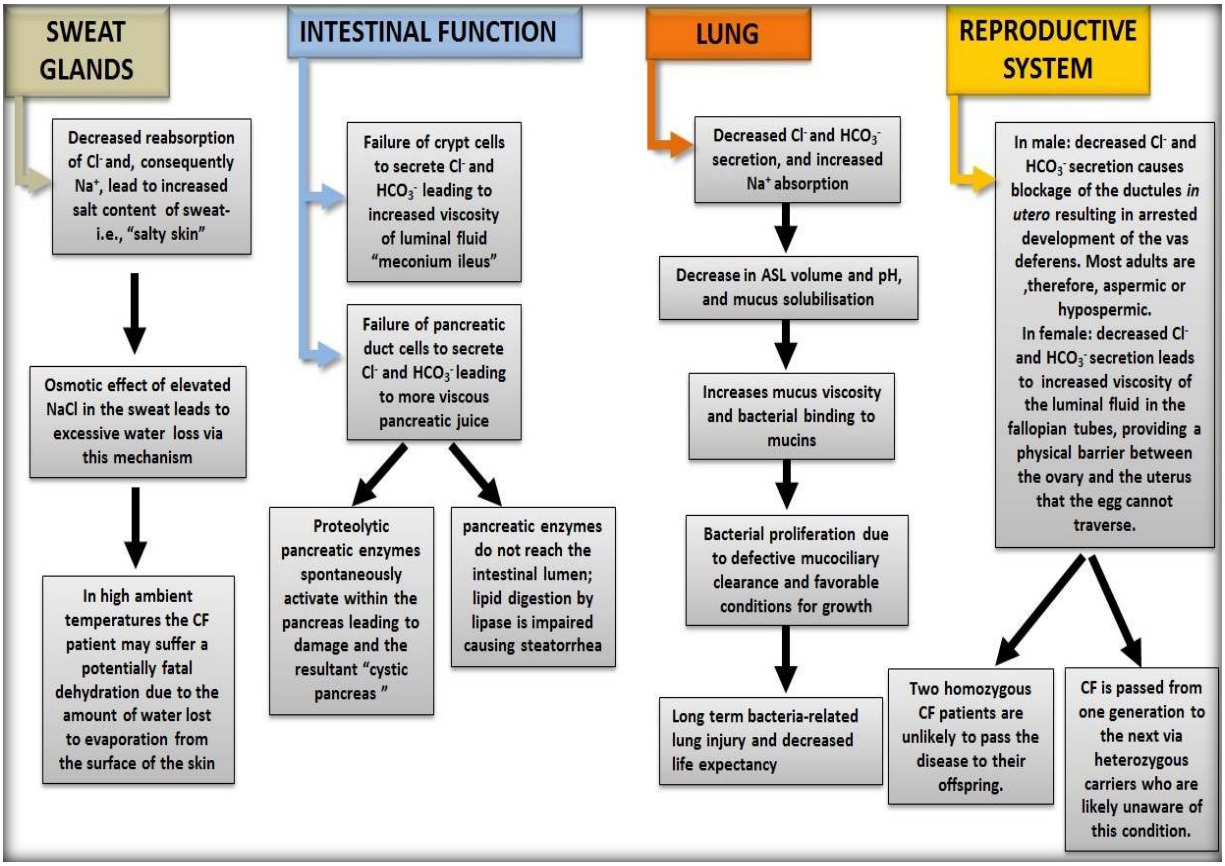


Figure 1. 8: Mutated CFTR Cl^- ion channel and CF of the lung. This figure illustrates the theoretical relationship between defected CFTR protein and several organ systems. Figure is adapted from Goodman and Precy, 2005 (Goodman and Percy, 2005).

1.8 Role of SLC26 Cl^-/HCO_3^- anion exchangers and HCO_3^- secretion in epithelial cells

The human solute carrier (SLC26) gene family are large transmembrane protein anion transporters that consist of 10 members (SLC26A1-A11, SLC26A10 being a pseudogene) (Li *et al.*, 2014), many of which are expressed in epithelial cells where they play an important role in anion secretion and absorption (Ohana *et al.*, 2009). All members of the SLC26 family, except SLC26A5 (Prestin), can function as anion exchangers, and they have a significant role in the transportation of a wide range of monovalent and divalent anions in epithelial cells. For instance, sulphate, chloride, iodide, oxalate, formate, bicarbonate and hydroxyl ion (Bissig *et al.*, 1994; Karniski *et al.*, 1998; Satoh H

and Yadav, 1998; Moseley *et al.*, 1999; Jiang *et al.*, 2002; Xie *et al.*, 2002; Scott DA, (2000) ; Soleimani M and P, (2001)). Most of the SLC26 anion transporters are expressed in the luminal membrane of epithelial cells, and play an important role in epithelial Cl^- and HCO_3^- transportation (Mount and Romero, 2004). However, it has been suggested that SLC26A7 function as Cl^- channel in *Xenopus* oocytes and transfected HEK cells, and it does not conduct HCO_3^- (Kim *et al.*, 2005). Also, a study by Loriol *et al.*, (2008) has shown that SLC26A9 plays an important role in human airway epithelia cells as a Cl^- channel, but was not involved in regulating intracellular pH because it also does not conduct HCO_3^- (Loriol *et al.*, 2008). Recently, it has been demonstrated that SLC26A9 plays an important role in Cl^- secretion in mouse airway epithelial cells, and helps prevent airway mucus obstruction (Anagnostopoulou *et al.*, 2012). In addition, it has been shown that SLC26A9 plays an important role in Cl^- transportation through a cAMP-dependent mechanism when co-expressed with CFTR in HEK-293Tcells, and that SLC26A9 activity was also CFTR-dependent in HBE cells (Bertrand *et al.*, 2009).

The SLC26 family members have an NH_2 terminal domain that encompasses the transmembrane segments, which are thought to be the sites for anion binding, as well as the COOH terminal, which is predicted to contain a sulfate transporter and anti-sigma factor antagonist (STAS) domain (Aravind and Koonin, 2000). It has been described that there is a reciprocal regulation between SLC26 transporters and the CFTR channel, which is facilitated by binding of the STAS-domain of the SLC26 transporters to the R-domain of CFTR, which plays an important role in the regulation of CFTR activity (Ko *et al.*, 2004). It has also been reported that members of SLC26 family are more likely to be involved in Cl^-/HCO_3^- exchange than Cl^-/OH^- exchange (Ko *et al.*, 2002; Wang *et al.*, 2002; Xie *et al.*, 2002). The SLC26 family members of $Cl^-/HCO3^-$ exchangers, SLC26A3, SLC26A4 and SLC26A6, are known to play an important role in $HCO3^-$ secretions in kidney , intestinal and pancreatic duct cells (Soleimani *et al.*, 2001; Stewart *et al.*, 2011).

Several human genetic diseases are associated with mutations in members of the SLC26 family including; chondrodysplasias that is caused by mutation in SLC26A2 (Hastbacka *et al.*, 1994; Superti-Furga *et al.*, 1996); chloride losing diarrhoea (DRA) caused by mutations in SLC26A3 (Mount and Romero, 2004), pendred syndrome and congenital deafness that is linked to mutations in SLC26A4 (Everett *et al.*, 1997; Liu *et al.*, 2003).. It has also been reported that defective SLC26A5 (Prestin) leads to non-syndromic hearing impairment (Liu *et al.*, 2003; Tang *et al.*, 2005). Moreover, it has been reported that absence of SLC26A6 protein in mice produces kidney nephrolithiasis due to abnormal transportation of oxalate (Jiang *et al.*, 2006). SLC26A6 is also involved in HCO_3^- secretion by pancreatic and parotid ducts (Wang *et al.*, 2006; Shcheynikov *et al.*, 2008). Also, SLC26A8 mutation leads to a decrease in the capability of being fertile (Toure *et al.*, 2007). The characteristics of the SLC26 family and diseases linked to their dysfunction are shown in Table 1.01.

Human Gene Name	Aliases	Reported substrates	Tissue Distribution	Disease association(s)
SLC26A1	Sat-1	SO_4^{2-}, oxalate	liver, kidney	
SLC26A2	DTDST	SO_4^{2-}, Cl^-	widespread	Chondrodysplasias
SLC26A3	DRA, CLD	SO_4^{2-}, Cl^-, HCO_3^-, OH^-, oxalate	intestine, sweat gland, pancreas, prostate	Congenital Chloride-losing Diarrhoea
SLC26A4	pendrin	Cl^-, HCO_3^-, I^-, formate	inner ear, kidney, thyroid	Pendred syndrome, Deafness (DFNB4)
SLC26A5	prestin	-	inner ear	Deafness
SLC26A6	CFEX, PAT-1	SO_4^{2-}, Cl^-, HCO_3^-, OH^-, oxalate, formate	widespread	Nephrolithiasis
SLC26A7	none	SO_4^{2-}, Cl^-, oxalate	kidney	
SLC26A8	Tat1	SO_4^{2-}, Cl^-, oxalate	sperm, brain	Asthenozoospermia
SLC26A9	none	SO_4^{2-}, Cl^-, oxalate	lung	
SLC26A10	none	pseudogene		
SLC26A11	none	SO_4^{2-}	widespread	

Table 1.1: The characteristics of the SLC26 gene family. Table adapted from Mount & Romero 2004 (Mount and Romero, 2004).

1.8.1 SLC26A4 (Pendrin)

SLC26A4 (pendrin) is an electroneutral anion exchanger that functions as a Cl^-/HCO_3^- exchanger (Ohana *et al.*, 2009). Pendrin is essential for transcellular movement of monovalent anions, including chloride, iodide and formate, although it doesn't transport divalent anions (sulphate and oxalate) (Scott *et al.*, 1999; Scott DA, (2000)).

Pendrin is highly expressed in the inner ear, thyroid gland and the kidney (Royaux *et al.*, 2000; Lacroix *et al.*, 2001). It has been shown that human pendrin expressed in *Xenopus* oocytes functions as a Cl^-/formate exchanger,

similar to the renal Cl^-/formate exchanger, which is thought to play a critical role in transepithelial Cl^- transport by renal proximal tubule (Scott and Karniski, 2000). However, immunolocalisation studies have shown pendrin expression on the apical surface of renal β-intercalated cells of collecting ducts, but failed to detect any expression in the proximal tubules (Royaux *et al.*, 2001). Here, they also showed that pendrin plays an important role in HCO_3^- secretion in the kidney. In addition, a study by Amlal *et al.*, (2010) showed that genetic deletion of SLC26A4 (pendrin) in mice impaired HCO_3^- secretion by renal collecting ducts and these animals displayed significantly acidic urine, along with elevated HCO_3^- concentration in the serum, compared to WT mice (Amlal *et al.*, 2010). Furthermore, pH_i measurements in β-intercalated cells of renal collecting ducts showed that the alkalinisation in pH_i due to Cl^- removal in the extracellular solution, was markedly decreased in pendrin knock out mice, compared to wild type mice, suggesting that pendrin functions as a Cl/HCO_3^- anion exchanger (Amlal *et al.*, 2010). It has been also reported that pendrin can be found in other organs, such as epithelial cells of the airway (Pedemonte *et al.*, 2007; Di Valentin E, 2009), mammary gland (Rillema and Hill, 2003), testis (Lacroix *et al.*, 2001), placenta (Bidart *et al.*, 2000), endometrium (Suzuki *et al.*, 2002) and liver (Alesutan *et al.*, 2011). Mutations in the pendrin gene lead to pendred syndrome which is an autosomal recessive illness in which the anion exchanger does not function properly. Pendred syndrome is distinguished by loss of hearing in newborns and enlargement of the thyroid gland, due to a defect in the formation of thyroglobulin in the thyroid gland, that leads to development of goiter (Morgans and Trotter, 1958; Dror *et al.*, 2010).

A recent study showed that there is cooperation between CFTR and pendrin in the process of Cl^- and HCO_3^- secretion by serous cells of airway submucosal glands (Garnett *et al.*, 2011; Garnett *et al.*, 2013). Abnormality in the regulation of pendrin function by CFTR, as would occur in CF, would lead to reduced Cl^-/HCO_3^- exchange. This would be predicted to lead to a decrease in the quantity of bicarbonate secreted into the luminal airway surface liquid (Mount and Romero, 2004). Moreover, it has been shown that pendrin plays an important role in thiocyanate secretion into the ASL, which provides ability for scavenging oxidants and innate defense against bacterial infection, in human bronchial

epithelial cells under resting and IL-4 stimulated conditions (Pedemonte *et al.*, 2007).

Furthermore, it has been shown that pendrin plays an important role in the regulation of ASL thickness and asthma exacerbation induced by rhinovirus infection or allergic airway diseases (Nakagami *et al.*, 2008). Here, they showed that pendrin expression was significantly increased about 5 fold in infected human with common colds caused by rhinovirus. They also showed that pendrin deficiency in mice caused a significant decrease in airway hyperactivity and inflammation by improving ASL hydration**.** Thus pendrin might be a potential therapeutic target in asthma exacerbations.

1.9 Regulation of SLC26 anion exchangers by CFTR

CFTR plays an important role in the activation of SLC26 Cl^-/HCO_3^- exchangers, indicating the importance of SLC26 anion exchangers in HCO_3^- transportation in many epithelial cells (Mount and Romero, 2004). In unstimulated cells, unnecessary fluid and electrolyte secretion is avoided through interaction of the unphosphorylated R-domain with NBD1 that blocks its binding with NBD2 (Baker *et al.*, 2007) and thus leads to inhibition of CFTR Cl^- channel activity. However, phosphorylation of the R domain by PKA is followed by the detachment of the R domain from NBD1, which stimulates its interaction with the STAS domain of SLC26 anion transporters (Lamprecht *et al.*, 2002; Ko *et al.*, 2004). CFTR-associated protein 70 (CAP70) which is one of the PDZ proteins that are primarily located in the apical membrane of epithelial cells, has been reported to play an important role in the opening of CFTR in Calu-3 cells of airway SMG via its binding to the C-terminal of CFTR (Wang *et al.*, 2000; Raghuram *et al.*, 2001). This interaction leads to the combined activation of CFTR and SLC26 anion transporters (Ko *et al.*, 2002; Ko *et al.*, 2004) and subsequently electrolyte and fluid efflux (figure 1.9). In addition, it has been found that this type of regulatory activation is present for SLC26A3, SLC26A4

and SLC26A6, while other types of SLC26 transporters might be involved in this regulatory process (Wang *et al.*, 2006).

Recently, a clinical study has confirmed the functional cooperation between SLC26 AE and the CFTR channel (Dirami *et al.*, 2013). Here, they showed that SLC26A8 strongly stimulates CFTR channel activity in human sperm, which plays an important role in anion exchange activity that is required for sperm motility and capacitation, and subsequently fertilization. The study by Dirami *et al.*, (2013) also suggested that SLC26A8 mutations impair the formation of the SLC26-CFTR complex, and thereby leads to human asthenozoospermia (Dirami *et al.*, 2013). The SLC26 anion exchangers also play an essential role in the activation of CFTR channel in vivo (Dirami *et al.*, 2013). It has also been shown that salivary and pancreatic ductal fluid and HCO_3^- secretion involves basolateral HCO_3^- influx by NBCe1 to provide more HCO_3^- to accumulate inside the cells, and luminal HCO_3^- efflux and Cl^- absorption across the apical membrane by coordinated activity of the CFTR and SLC26A6 Cl^-/HCO_3^- AE (Lee *et al.*, 2012; Ahuja *et al.*, 2014).

Moreover, a recent study in primary cultures of human nasal cells from normal and patients carrying mutations in pendrin (DFNB4) showed that mutation in SLC26A4 lead to reduced anion exchange activity in the apical membrane of IL-13 treated cells, but enhanced ASL height. In addition, there was as a decrease in expression of CFTR and short circuit current in the pendrin mutant airway cells (Lee *et al.*, 2015). Furthermore, preliminary co-immunoprecipitation experiments suggested that pendrin may be coupled to CFTR, with the two proteins working in concert to transport Cl^- and HCO_3^- across the apical membrane of human airway epithelial cells (Lee *et al.*, 2015). However, based on the observed changes in ASL height, the authors concluded that this was evidence that pendrin actually works in an absorptive, rather than a secretory mode, to regulate ASL volume. Overall, it is clear that regulation of these anion exchangers is an important area of research, and might be a novel target for future therapy of CF.

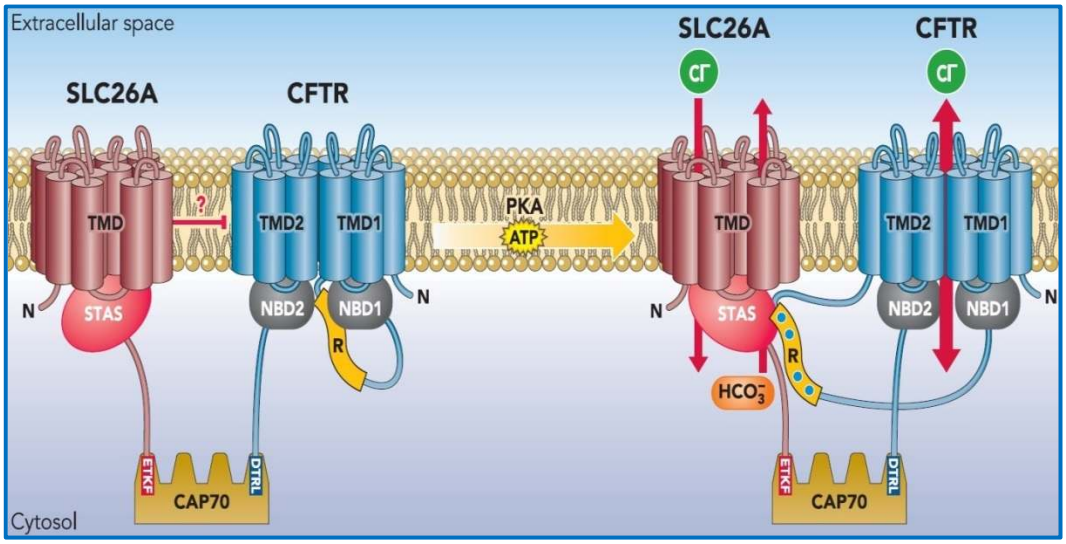


Figure 1. 9: The physical and functional interaction between CFTR and SLC26 transporters. The CFTR plays a crucial role in the regulation of SLC26A transporters. An increase in intracellular cAMP leads to activation of PKA, resulting in phosphorylation of R-domain of CFTR, which binds to SLC26 transporters *via* their STAS domain to enables CFTR to directly regulate the SLC26 anion exchanger activity. Figure taken from (Dorwart *et al.*, 2008).

1.10 The SLC4 family of anion transporters

In addition to the SLC26 family that code for Cl^-/HCO_3^- exchangers, there is another family which is called solute carrier 4 (SLC4) family of transporters that consist of ten genes, and all SLC4 family members encode plasma membrane spanning proteins (Romero *et al.*, 2004). The SLC4 family members play an important role in HCO_3^- transportation across the mammalian plasma membrane to regulate intracellular and extracellular pH, as well as transportation of Na^+ and/or Cl^-, and potentially water (Bevensee *et al.*, 2000; Kurtz, 2014). It has been shown that mutations in SLC4 transporter proteins lead to a wide range of human genetic diseases (Cordat and Casey, 2009), such as haemolytic anaemia and distal renal tubular acidosis by SLC4AE1 mutation (Schofield *et al.*, 1992; Bruce *et al.*, 1997), generalized epilepsy by SLC4AE3 mutation (Sander *et al.*, 2002), glaucoma, cataract and bad keratopathy by NBCe1 mutation (Usui *et al.*,

1999; Demirci *et al.*, 2006) and hypertension by NBCe2 mutation (Barkley *et al.*, 2004; Hunt *et al.*, 2006).

Most recently, it has been shown that eight members of the SLC4 family encode proteins that are involved in the process of HCO_3^- transportation, and functionally they are classified into two main groups: three electroneutral Cl^-/HCO_3^- exchangers, AE1, AE2 and AE3 (SLC4A1, SLC4A2, SLC4A3, respectively) and five Na^+-coupled HCO_3^- transporters (NBC) (Romero *et al.*, 2013; Kurtz, 2014). NBCe1 (SLC4A4), and NBCe2 (SLC4A5) are two electrogenic NBCs, while NBCn1 (SLC4A7), NBCn2 (SLC4A10), and Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE; SLC4A8) are three electroneutral transporters (Romero *et al.*, 2013; Kurtz, 2014). Although SLC4A9 (AE4) was initially reported to perform anion exchange activity, it mostly resembles Na^+-coupled SLC4 transporters (Kurtz, 2014). Electrogenic NBCe's plays an important role in HCO_3^- secretion in the pancreatic duct, and HCO_3^- reabsorption in the proximal renal tubules. However, the electroneutral NBCn and Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE's) play a vital role in the regulation of pH_i in vascular smooth muscle and CNS neurons, respectively (Boron, 2001). Furthermore, the products of the SLC4 gene family perform a vital role in the process of CO_2 transportation from the systemic circulation to the lungs by red blood cells, H^+ or HCO_3^- absorption or secretion by a wide range of epithelial cells, as well as controlling intracellular pH and cell size (Romero *et al.*, 2004).

The gene products of SLC4 transporters are comprised of three domains: (i) N-terminal cytoplasmic domain comprising of 400–700 amino acids, (ii) a transmembrane domain (TMD) of 500 amino acids, and (iii) a short C-terminal tail that binds to carbonic anhydrase II. The amino acid sequences of AE1–3 TMDs are ~65% identical, while the homologous cytoplasmic N-terminal domains are only ~35% identical in amino acid sequence identity (Vince and Reithmeier, 2000). AE1 is the most numerous membrane protein in erythrocytes (Hunter, 1977) and α-intercalated cells of renal collecting duct (Verlander *et al.*, 1988). AE2 is highly expressed at the basolateral membrane of most epithelia including stomach mucosa (Stuart-Tilley *et al.*, 1994), colon (Gawenis *et al.*,

2010), collecting duct of the kidney (Alper *et al.*, 1997), pancreatic duct epithelial cells (Rakonczay *et al.*, 2008; Ishiguro *et al.*, 2009) and respiratory airway (Dudeja *et al.*, 1999). AE3 is mostly expressed in excitable tissues, including brain (Kudrycki *et al.*, 1990), retina (Kobayashi *et al.*, 1994), heart (Yannoukakos *et al.*, 1994) and smooth muscle (Brosius *et al.*, 1997), which plays an important role in the regulation of intracellular pH by exporting HCO_3^- during intracellular alkalinisation (Sander *et al.*, 2002). Most, if not all, of the SLC4 family members are functionally inhibited by 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (DIDS) (Romero *et al.*, 2013). It has been shown that DIDS, used extensively to characterize the HCO_3^- transport activity of the SLC4 family members, covalently interacts with a lysine residue in the protein (Landolt-Marticorena *et al.*, 1995). The characteristics of the SLC4 family members are shown in figure 1.10.

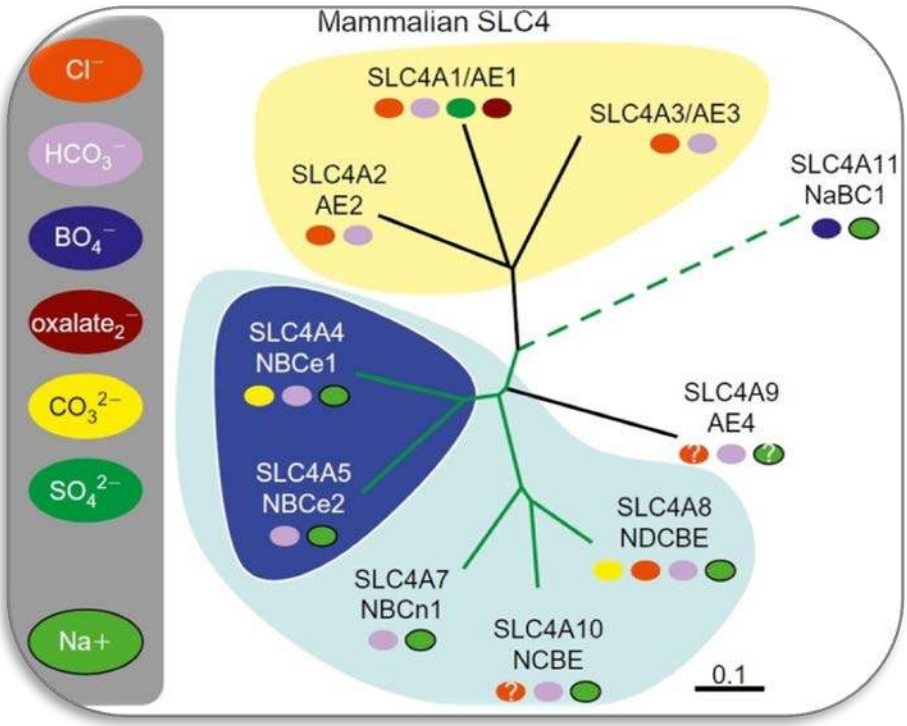


Figure 1. 10: SLC4 family members and their transport activity. The SLC4 gene family contains 10 members; all are involved in HCO_3^- transportation apart from one member, SLC4A11. Figure taken from (Alvarez-Leefmans and Delpire., 2009)**.**

1.10.1 Anion exchanger 2 (SLC4A2 or AE2)

The electroneutral Cl^-/HCO_3^- anion exchanger 2 (AE2) (Huang *et al.*, 2012) is a trans membrane protein that plays an important role in the regulation of intracellular pH and cell volume (Turi *et al.*, 2003). It has also been shown that SLC4A2 function as a Na^+-independent Cl^-/HCO_3^- anion exchanger in polarized epithelial cells that plays a pivotal role in the regulation of intracellular pH, intracellular Cl^- concentration and cell volume by excretion of HCO_3^- from cells, and loading of cells with Cl^- (Alper, 2002; Romero *et al.*, 2004). In addition, it has been assumed that expression of AE2 in most epithelial cells plays important role in the regulation of intracellular pH by outward transportation of HCO_3^- during intracellular alkalinisation, and /or control of cell volume by Cl^- uptake (Stewart *et al.*, 2002). AE2 plays an important role in the regulation of intracellular pH and transepithelial acid-base transportation in biliary epithelial cells (Concepcion *et al.*, 2013). Also, it has been reported that AE2 is expressed on the apical surface of both biliary epithelial cells and hepatocytes and is critically involved in pH_i regulation and HCO_3^- secretion into the bile (Martinez-Anso *et al.*, 1994; Medina *et al.*, 1997). Furthermore, it has been shown that AE2 is expressed in the airways (Al-Bazzaz *et al.*, 2001) as well as Calu-3 cells at the basolateral membrane by immunofluorescence (Loffing *et al.*, 2000) More recently, it has been confirmed that AE2 mRNA and protein are expressed in the basolateral membrane of Calu-3 cells, which plays an important role in basolateral Cl^- loading and HCO_3^- recycling under cAMP stimulated condition in Calu-3 cells (Huang *et al.*, 2012). Moreover, the intracellular alkalinisation produced by basolateral Cl^- removal was inhibited by 80% in AE2 knock down Calu-3 cells (Huang *et al.*, 2012). Basolateral AE2 is thought to plays an important role in the regulation of intracellular pH in Calu-3 cells by participating in HCO_3^- absorption, and also it facilitates the inward movement of Cl^- through the basolateral membrane, together with the sodium-potassium chloride cotransporter NKCC1 (Loffing *et al.*, 2000; Inglis *et al.*, 2002). In contrast to results by Huang *et al.*, (2012), most recent results by Garnett *et al*, have demonstrated that a functional AE was present in the basolateral surface of Calu-3 cells under resting, non-stimulated conditions, with properties that are

consistent with AE2, and which was almost completely inhibited by intracellular elevation of cAMP (Garnett *et al.*, 2011; Garnett *et al.*, 2013). In addition to regulating pH_i and cell volume, AE2 also plays a vital role in superoxide (O_2^-) transportation into the extracellular space in exchange for extracellular HCO_3^- (Turi *et al.*, 2002). AE2 has been shown to be involved in hyperoxia-induced oxidative lung injury, which was markedly reduced by inhibition of AE2 by DIDS or perfusion with a bicarbonate free buffer solution (Nozik-Grayck *et al.*, 1997). However, the mechanisms that regulate AE2 activity in epithelia are not well understood. This gives much interest to further investigate the mechanism that regulates this anion exchanger in airway epithelia.

1.11 Calu-3 Cells as a model of human submucosal gland serous cells

There was an excitement about discovering a human cell line that has many characteristics resembling serous cells, as primary human serous cells are difficult to isolate and grow in culture (Shen *et al.*, 1994). The Calu-3 cell line was derived from human lung adenocarcinoma cells (Shen *et al.*, 1994) and was introduced as a serous cell model based on the presence of many properties of serous cells, such as high CFTR expression, production of antimicrobials, including lysozyme and lactoferrin (Haws *et al.*, 1994; Duszyk, 2001a; Dubin *et al.*, 2004). Also, Calu-3 cells form polarized monolayers (Garnett *et al.*, 2011; Shan *et al.*, 2011) capable of anion secretion in response to secretagogues that increase intracellular cAMP concentration (Shen *et al.*, 1994; Devor *et al.*, 1999; Garnett *et al.*, 2011; Garnett *et al.*, 2013). cAMP stimulation of Calu-3 cells leads to CFTR-dependent electrogenic Cl^- secretion (Cobb *et al.*, 2002), as well as HCO_3^- secretion (Shan *et al.*, 2012; Garnett *et al.*, 2013; Kim *et al.*, 2014). Also, stimulation of Calu-3 cells with cAMP agonist, VIP, caused a CFTR-dependent I^- transport through a DIDS insensitive but PKA-dependent mechanism (Derand *et al.*, 2004). Based on the presence of these features, Calu-

3 cells are a good model to use for studying transepithelial HCO_3^- secretion, and the importance of CFTR and anion exchangers in serous cell anion secretion.

A current model for mechanism of anion secretion in Calu-3 is summarised in figure 1.11. At the basolateral membrane of the Calu-3 cells, NBC is responsible for HCO_3^- influx into the cells due to the positive regulatory effect of cAMP on NBC, with an inwardly-directed Na^+ gradient maintained by Na^+-K^+ ATPase (not shown in the figure). Interestingly, recent work in our laboratory has shown that increases in intracellular cAMP leads to inhibition of basolateral Cl^-/HCO_3^- (AE) activity, which is followed by stimulation of NBC and HCO_3^- transportation into the cell (M. Turner, PhD thesis 2014). Cl^- uptake across the basolateral membrane is regulated by Na^+-K^+-Cl^- cotransporter (NKCC) and potentially AE2 (Devor *et al.*, 1999). At the apical membrane, Cl^- exits from the cells by the CFTR channel. It has been discovered for the first time that there is a cAMP/PKA activated Cl^-/HCO_3^- exchanger on the apical surface of Calu-3 cells, and its functional properties are similar to those of pendrin (SLC26A4) that play a crucial role in HCO_3^- secretion across the cell membrane of airway epithelia Calu-3 cells, while Cl^- is recycled by coordinated activity between CFTR and pendrin (Garnett *et al.*, 2013). An inwardly-directed Cl^- concentration gradient acts as the main driving force for HCO_3^- secretion by luminal Cl^-/HCO_3^- exchangers. In addition an apical Cl^- conductance is essential for efficient operation of apical Cl^-/HCO_3^- exchangers (Dorwart *et al.*, 2008). Intracellular elevation of cAMP leads to stimulation of CFTR Cl^- efflux and HCO_3^- secretion across the apical membrane via pendrin, while inhibiting basolateral AE2 (Garnett *et al.*, 2011; Garnett *et al.*, 2013). Paradoxically, recent studies by Shan *et al*., (2012) and Kim et al. (2014) found that CFTR was the predominant pathway for HCO_3^- secretion in Calu-3 cells, and it worked independently of an apical Cl^-/HCO_3^- exchanger (Shan *et al.*, 2012; Kim *et al.*, 2014). In addition HCO_3^- secretion was blocked by CFTR inhibitors or genetic knock down of CFTR (Shan *et al.*, 2012). Furthermore, basolateral AE2 was not abolished during cAMP stimulation in Calu-3 cells (Huang *et al.*, 2012; Shan *et al.*, 2012; Kim *et al.*, 2014). The reason for these different findings is not clear but may be related to different growth conditions or batches of Calu-3 cells.

However, we don't have a full understanding how CFTR regulates HCO_3^- secretion in the airways, or the molecular mechanisms that regulate both the apical and basolateral Cl^-/HCO_3^- AE in airway HCO_3^- transportation. Thus, regulation of CFTR-dependent apical Cl^-/HCO_3^- AE activity, as well as the basolateral AE is an important area of research, and forms the basis of this thesis.

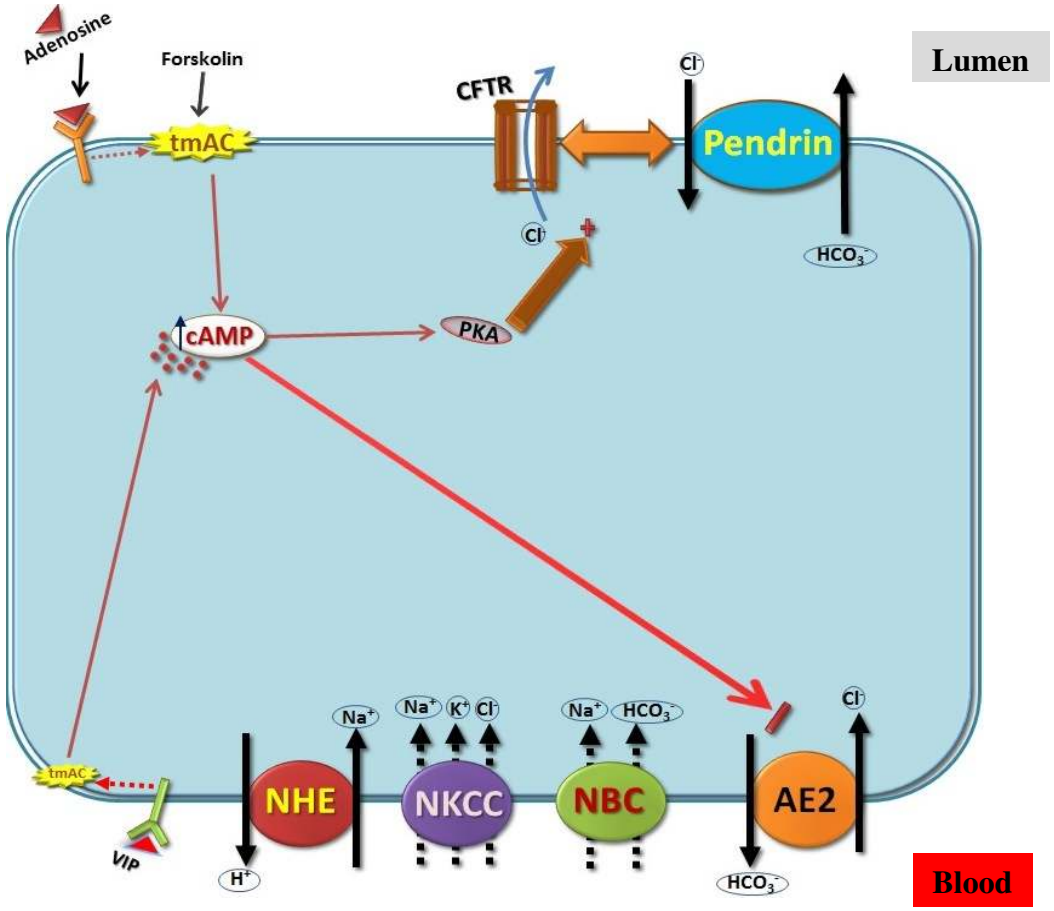


Figure 1. 11: Mechanism of anion secretion in the Calu-3 cells. This new model shows HCO_3^- transportation across the apical and basolateral membrane in cAMP-stimulated Calu-3 cells. tm*AC*= transmembrane adenylyl cyclase, *CFTR*= Cystic fibrosis transmembrane conductance regulator, *NKCC*= Na^+-K^+-$2Cl^-$ cotransporter, *NBC*= Na^+-HCO_3^- cotransporter, *NHE*= Na^+-H^+ exchanger, *AE2*= anion exchanger 2, VIP= Vasoactive intestinal peptide. (+) denotes stimulation, (-) denotes inhibition.

Aims

The major aim of the current study was to investigate the signalling mechanisms that regulate the CFTR-dependent apical Cl^-/HCO_3^- anion exchanger (AE), as well as the basolateral Cl^-/HCO_3^- AE, to better understand their role in bicarbonate secretion in a model human airway epithelial cell line.

The detailed aims of this study were to:

- Study the functional properties of the basolateral Cl^-/HCO_3^- AE in polarised cultures of Calu-3 cells
- Investigate the effect of cAMP agonists on the activity of the apical and basolateral Cl^-/HCO_3^- AEs in polarised cultures of Calu-3 cells
- Investigate the role of intracellular and extracellular Ca^{2+} on the regulation of the apical and basolateral Cl^-/HCO_3^- AE activity in polarised cultures of Calu-3 cells
- Identify the protein kinases and phosphatases that regulate the apical and basolateral Cl^-/HCO_3^- activity under resting and cAMP-stimulated conditions.
- Study the role of the actin cytoskeleton and dynamin in the regulation of the apical and basolateral Cl^-/HCO_3^- AE activity in polarised cultures of Calu-3 cells
- Compare the properties of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells to those of mouse AE2, after transient transfection in HEK-293T cells.

Chapter 2 Methods

2.1 Cell culture

Routine cell culture was carried out in a vertical laminar flow hood (Jouan Ltd, UK). The human adenocarcinoma-derived cell line, Calu-3 (ATCC HTB-55) (Shen *et al.*, 1994) were grown in T75 Costar cell culture flasks (75cm^2) with 30 mls of Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 Um1^{-1} penicillin and 100µgml^{-1} streptomycin, 1% non-essential amino acids, 2mM L-Glutamine (Sigma) and incubated at 37ºC in a humidified air containing 5% CO_2. Culture media and solutions were pre-warmed to 37˚C in a water bath prior to use. All culture media, supplements and consumables were sterile, and all the equipment was sterilized before use in the hood using ethyl alcohol (70%). Cells were initially seeded at 3 x 10^6 cells per flask, and then incubated in humidified air at 37°C containing 5% (v/v) CO2. Cells became confluent after 7 days, and then passaged once per week. Frozen stocks were stored in liquid nitrogen, and were only used between passages 20-50. To subculture Calu-3 cells, the media was removed from the flask and cells were washed 3 times with sterile phosphate buffered saline (PBS). Cells were trypsinized in 5 mls of trypsin solution (0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Earle's balanced salt solution) for 20 minutes in a humidified incubator at 37ºC. After the first 20 minutes, the detached cells (approximately 50% of the cells) were added to 10 mls pre-warmed culture medium, and then another 5 mls of trypsin solution was added to the flask and incubated for another 20 minutes to ensure detachment of the remaining cells, and these cells were added to the culture medium. The resulting cell suspension was agitated gently, and then centrifuged at 1500g for 3 minutes. The supernatant was discarded but the cell pellet was re-suspended in 10 mls of culture media before seeding the cells into a new flask. For pH_i experiments, cells were seeded at 250,000 cells cm^2 initial seeding density onto semi-permeable Transwell supports (0.4 µm pore, polyester membrane insert, 1.12 cm^2 surface area, (Corning, UK)). Calu-3 cells generally produced a polarized

monolayer after 7 days. All experiments described in this thesis were carried out 8-14 days after seeding.

Human Embryonic Kidney-293T cells (HEK-293T) and CK2 (alpha and alpha prime) knock out HEK-293T cells were cultured in flasks ($75cm^2$) with 30 mls of Dulbecco's Modified Eagle's Medium (DMEM), supplemented as for Calu-3 cells. HEK-293T cells were initially seeded at 1 x 10^6 cells in a T75 flask, and then incubated at 37°C in a 5% (v/v) CO_2 atmosphere. Cells became confluent after 7 days, and were only used between passage 35 and passage 50. HEK-293T cells were subcultured once per week, using the same protocol as Calu-3 cells apart from the fact that HEK-293T cells were much more easily detached after incubation in 5 mls of trypsin solution for 1 minute in a humidified incubator at 37ºC. For pH_i experiments, HEK-293T cells were seeded onto the glass coverslips (25mm diameter) at 100,000 cells cm^2 initial seeding density, and then transiently transfected with cDNA coding for mAE2, CFTR and/or various CK2 constructs, 1 day post seeding.

The knockout CK2 (alpha and alpha prime) cells were a kind gift from Dr. Salvi and were generated in Dr. Salvi lab (University of Padova, Italy) in collaboration with Horizon Discovery, using the CRISPR/CaS9 method. The absence of the catalytic subunits kinase was confirmed by western blotting (see figure 2.1)

For transient transfections, DNA was pre-complexed with Lipofectamine-2000 (Manufacturers) at a ratio of 1: 2.28 respectively. After that, Opti-MEM media with GlutaMax was added for 15 minutes at room temperature, and then diluted in culture media to 1µgDNA/ml prior to adding to the cells. The transfected cells were incubated for 6 hours at 37°C, and then the complex media was removed and cells incubated with OptiMEM+10% FCS overnight, after which the cells were returned back to normal culture media. pH_i experiments were performed on the transfected cells 48 hours post transfection.

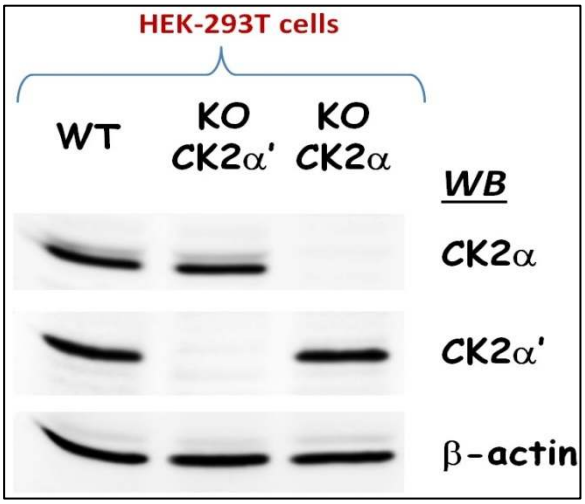


Figure 2. 1: Western blotting against CK2 alpha subunits. The absence of both CK2 alpha (α) and alpha prime (α') subunits of CK2, in the knockout cells, was confirmed by western blot analysis, which was performed in Dr. Salvi lab in collaboration with Horizon Discovery.

2.1.1 Expression constructs:

Mouse AE2 cDNA (HA-tag) was a kind gift from Beth Lee and Ron Kopito's lab, for more detail see Lee et al., (1991) (Lee *et al.*, 1991). Empty plasmid (pcDNA 3.1 myc/His), human WT-CK2 (CK2-alpha), and double mutant CK2 (DM-CK2; less sensitive to TBB) cDNA were a kind gift from Dr. M Salvi Lab, see reference (Salvi *et al.*, 2006), DNA sequencing analysis to confirm the constructs. Human CFTR was a kind gift from Dr. Paul Linsdell, and was inserted into the pIRES2-EGFP vector, which allows strong expression of green fluorescent as well as CFTTR. For more detail see (Zhou *et al.*, 2010). CFTR transfected cells were visualized under the fluorescent microscope before doing the pH_i experiments and the GFP did not interfered with pH_i measurements in BCECF-loaded cells.

2.2 Transepithelial Resistance Measurements

The trans-epithelial electrical resistance (TEER) of Calu-3 cells grown on Transwell supports was used to determine whether cells had formed a polarized, resistive monolayer, and was measured using an Epithelial Voltohmmeter (World Precision Instruments, UK).

Resistance measurements were corrected for the resistance of an empty transwell which was calculated as 111± 4 Ω cm^{-2}. Cells were only used for experiments when TEER had reached a stable resistance of above 600 Ω cm^{-2}.

2.3 Measurement of intracellular pH

Real-time intracellular pH measurements (pH_i) were performed to investigate the H^+/HCO_3^- transport in Calu-3 and HEK-293T cells.

(i) Calu-3 cells. Following culture of Calu-3 cells on Transwell supports, the cells were first washed with NaHEPES, and then the cells were loaded with 10μM of the pH sensitive dye, 2'-7'-bis (carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) in NaHEPES. 0.5 ml of the dye solution was added to the apical chamber and 1 ml of dye-free NaHEPES was added to the basolateral compartment. Cells were then incubated for 60 mins at 37 ºC. After dye loading, transwells were placed in a perfusion chamber and then mounted onto the stage of a Nikon inverted microscope. Cells were viewed at x60 magnification using a long working distance objective (N.A 0.6) and perfused with a high chloride KREBS solution at 37 ºC gassed with 5% CO_2/95%O_2, in order to adjust the pH of the solution to 7.4. Perfusion of the apical and basolateral side of the cells was at a rate of 3 and 6 ml per minute, respectively.

(ii) HEK-293T cells. Following culture and transient transfection of HEK-293T cells on glass coverslips, the cells were washed with NaHEPES, and then were loaded with 10μM of the pH sensitive dye, BCECF-AM in NaHEPES solution. 1 ml of the dye solution was added onto the coverslip inside the well plate, and then incubated for 10 mins at 37 ºC. After dye loading, coverslips were placed in

a perfusion chamber and then mounted onto the stage of a Nikon inverted microscope. Cells were viewed at x100 magnification using an oil immersion objective (N.A 1.2) and perfused with a high chloride KREBS solution at 37 ºC gassed with 5% CO_2/95%O_2, in order to adjust the pH of the solution. Cells were then perfused with the solution at a rate of 3 ml per minute.

To measure pH_i, a Life Sciences Microfluorimeter system (Life Science Resources, UK) was used. Cells were excited by two different wavelengths, 490 nm (proton-bound BCECF-AM; pH_i-dependent) and 440 nm (proton-free BCECF-AM; pH_i-independent) with emitted light collected at 510 nm every 1.024 s using a photomultiplier tube. The ratio (R) of 490 nm emission to 440 nm emission was recorded using the PhoCal 1.6b software and used to calculate pH_i using the intracellular pH calibration (see section 2.4).

2.4 Intracellular pH Calibration

The intracellular pH calibration was performed by using the high K^+/nigericin method as described by James-Kracke, (1992) (James-Kracke, 1992). In brief, Calu-3 cells were prepared in the same way as intracellular pH measurements and high K^+/nigericin solutions of set pH were perfused across the cells without gassing. A standard curve was generated by plotting the resulting mean R value calculated over a 60 s period against the pH of the perfused solutions. (pH_i calibration data is shown in Figure 2.2). During the period of my research, several calibrations were performed to calibrate pH_i in different batches of Calu-3 cells. The experimental R value was converted to pH_i by using the following equation:

$$pH_i = R - (\text{Y-axis intercept}) / \text{slope}$$

Where:

R = 490/440 ratio value

Slope = slope of the linear regression fit to the standard curve

Y-intercept = point at which the line crosses the X-axis of a graph, when X= 0.

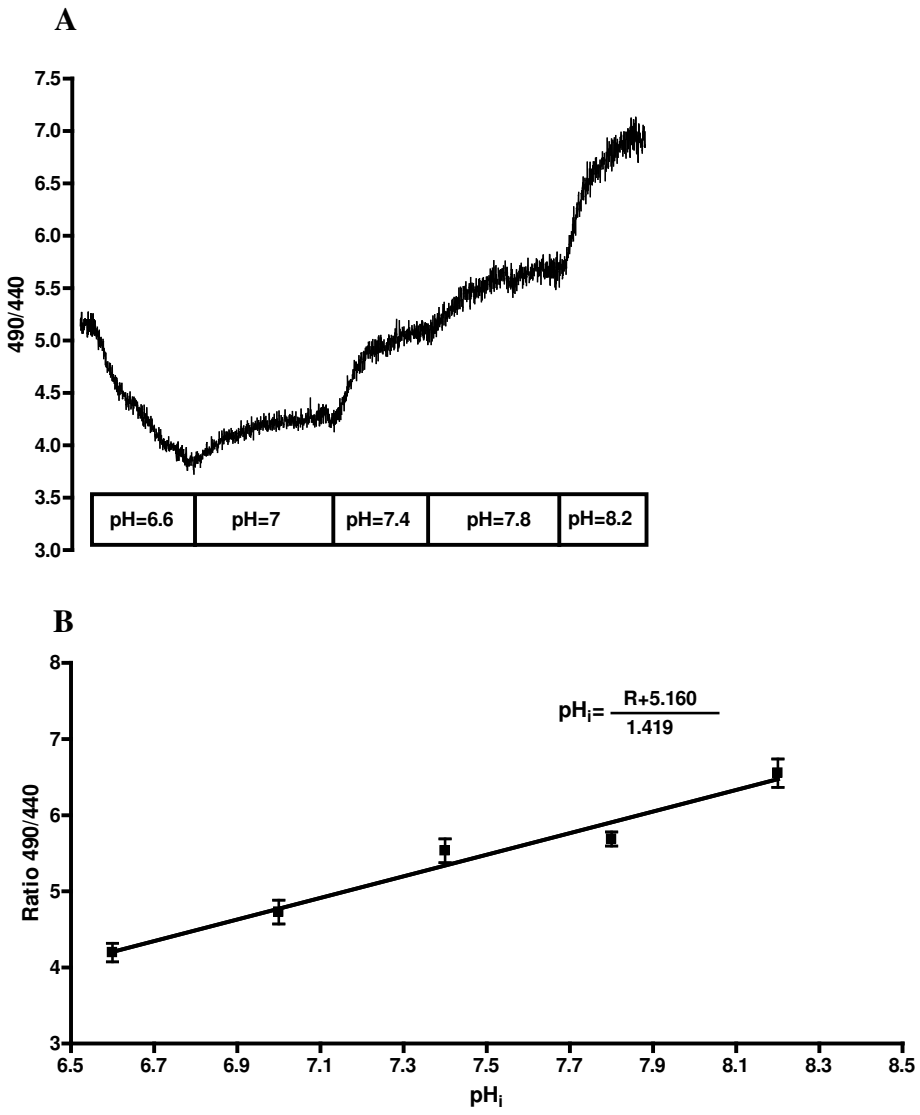


Figure 2. 2: Intracellular pH calibration using the high K+/nigericin technique. (A) Shows a representative trace of BCECF-AM (10µM) loaded Calu-3 cells illustrating how the R value alters when cells are perfused with high K^+/nigericin solutions of different pH. (B) Shows the standard curve generated from the pH_i calibration and is used for the calculation of pH_i from the 490/440 ratio data. Data represents mean ± S.E.M.; n = 4.

2.5 Data analysis of pH_i measurements

As shown in figure 2.3, estimation of the mean change in pH_i (ΔpH_i) was calculated by taking the average pH_i over 60 s before (labelled A) and after (labelled B) the new solution was applied, in order to establish a basal pH_i. This value was then subtracted from the mean pH_i obtained in the presence of test solution after the pH_i had reached a new steady state (labelled C). Linear regression was used to calculate the initial rate of re-acidification ($\Delta pH_i/\Delta t$) following Cl^- re-addition (labelled D) over a period of no less than 30 s of data. The $\Delta pH_i/\Delta t$ was converted to transmembrane HCO_3^- efflux by multiplying $\Delta pHi/\Delta t$ by the total buffering capacity of the cell (see section 2.6).

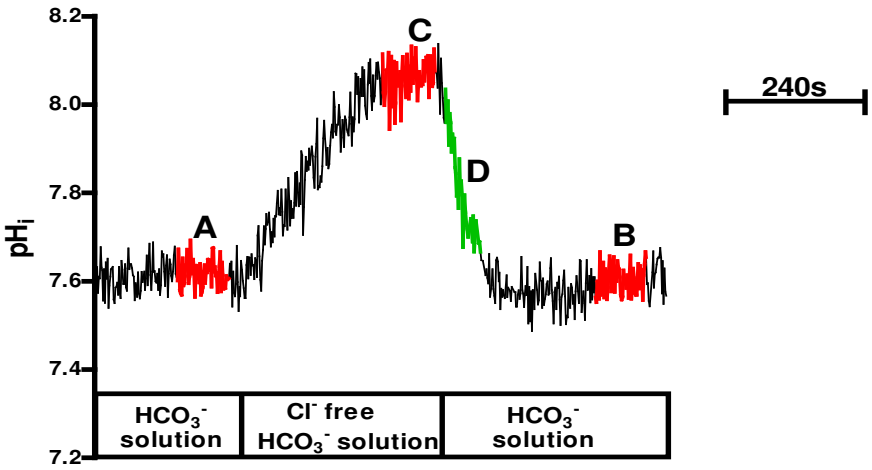


Figure 2. 3: Data analysis of intracellular pH measurements. Standard response to Cl^- free solution showing the effect of high Cl^- and free Cl^- buffer solution on pH_i in Calu-3 cells. Red areas represent data used to calculate mean pH_i change, and the green line represents the data points for linear regression used to calculate the rate of pH_i change (reacidification) by linear regression.

2.6 Determination of Total and Intrinsic Intracellular Buffering Capacity of cells

In order to determine the total buffering capacity (βtot) of both cell lines used in this study (Calu-3 cells and HEK-293T cells), the intrinsic buffering capacity (β_i) was first obtained by experimentation and then added to the buffering capacity of the CO_2-HCO_3^- buffer system (βHCO_3^-). Calu-3 cells and HEK-293T cells βi was calculated using the NH_4^+ pulse technique (Roos and Boron, 1981). Cells were exposed to solutions containing differing amounts of NH4Cl (0, 2.5, 5, 10, 20, 30mM NH4Cl). The perfused ammonium solution was free from Na^+ and HCO_3^- to block Na^+ and HCO_3^--dependent pH regulatory mechanisms, respectively (Figure 2.4). NH3 enters the cell and subsequently binds to free protons to form $NH4^+$ which causes an alkalinisation of pH_i. β_i is then calculated *via* the following equation:

$$\beta_i = \frac{\Delta[NH4+]_i}{\Delta pH_i}$$

Where $\Delta[NH4+]_i = [NH4+]_{out} \times 10^{(pHout-pHin)}$

Where $[NH4+]_{out} = [NH4Cl]/(1+10^{(pH\ out\ -\ pKa)})$

pH_{out} =7.4, pKa = 8.9

So for 30 mM NH_4Cl, $[NH_4^+]_o$ = 29.08mM at pH 7.4

β_{HCO3^-} is calculated using the formula: $\beta_{HCO3^-} = 2.3 \times [HCO_3^-]_i$,
in which the Henderson Hasselbach equation states:
$[HCO_3^-]_i = pCO2 \times 10^{(pH - pK)}$
where pK= 6.1

The total buffering capacity is then calculated by the following equation;

$\beta_{tot} = \beta_i + \beta_{HCO3-}$ where βi represents the intrinsic buffering capacity and β_{HCO3-} is the CO_2-HCO_3^--dependent buffer capacity.

The buffering capacity for Calu-3 cells and HEK-293T cells at 5% CO_2 are displayed in figure 2.5.

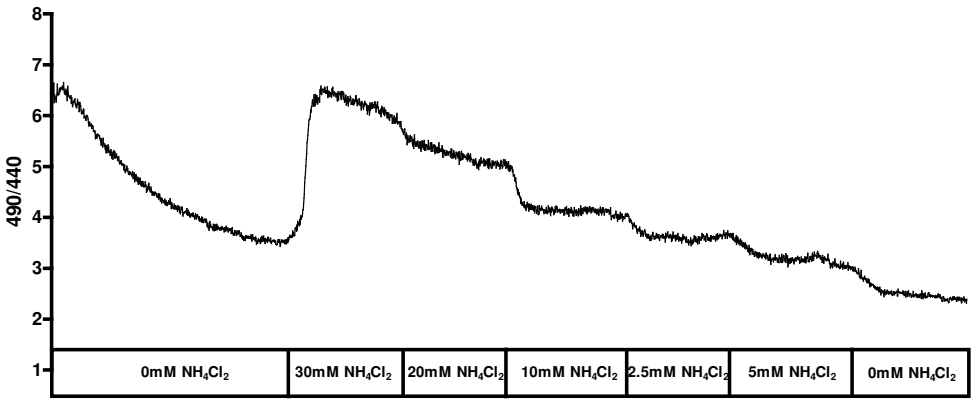


Figure 2. 4: Determining of total intrinsic buffering capacity in Clau-3 cells. Experimental pH trace showing the changes in ratio in Calu-3 cells in response to differing concentration of NH4Cl solutions containing of (0, 2.5, 5, 10, 20, 30 mM/L NH4Cl).

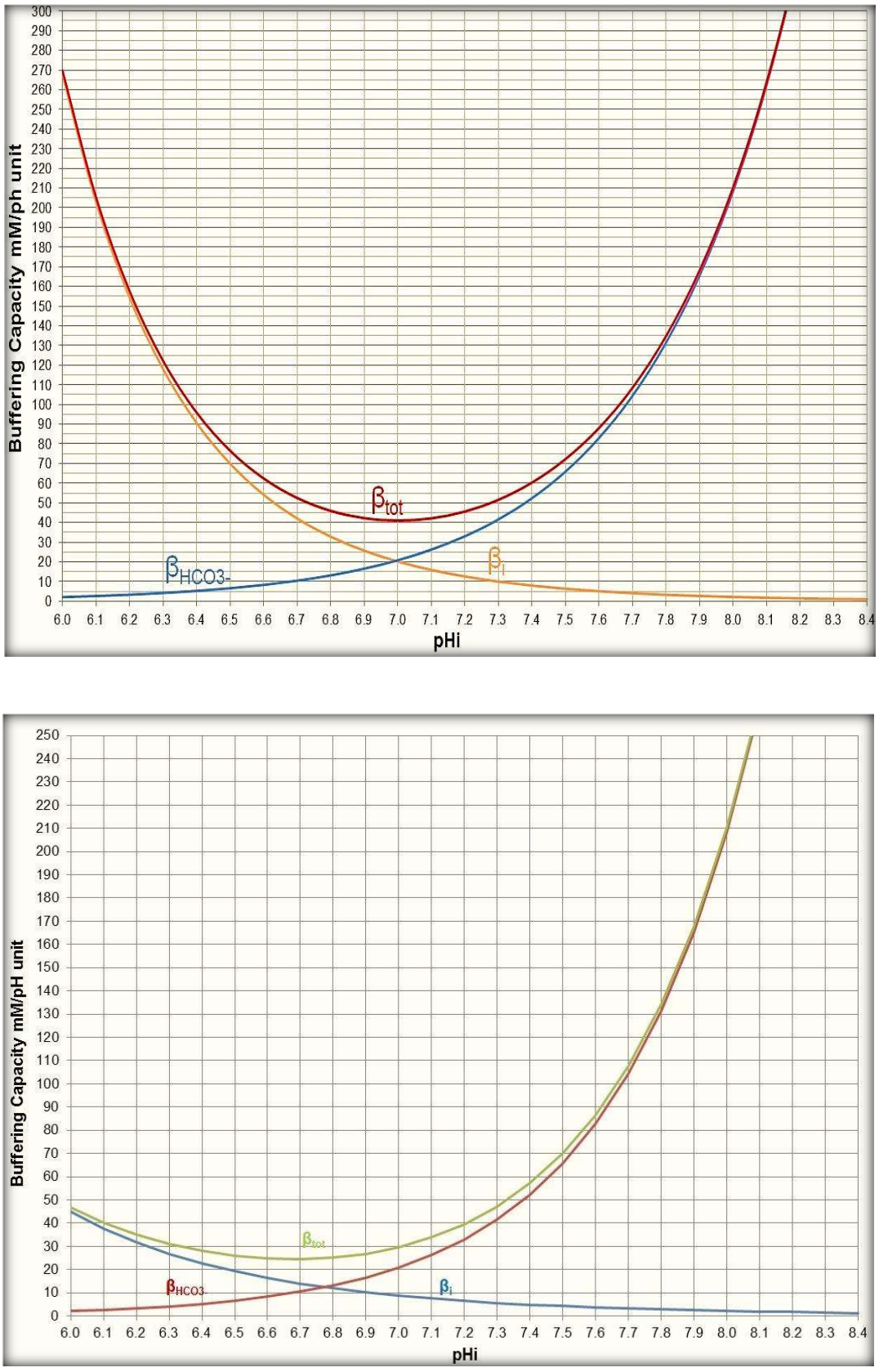


Figure 2. 5: Buffering capacity at various pH values in Calu-3 cells (A) and HEK-293T cells (B). Total buffering capacity (β_{tot}) was calculated by adding the intrinsic buffering capacity of Calu-3 cells (βi) to the buffering capacity of the CO_2/HCO_3^- buffer system (βHCO_3^-). β_{tot} was calculated for cells exposed to 5% CO_2, n = 6.

2.7 Fluid Secretion Assays

Polarized monolayers of Calu-3 cells grown on permeable Transwell supports for 7-10 days, were first washed three times with PBS (1ml to apical and basolateral compartments) in order to remove any mucus from the apical surface of cells that may have accumulated over time. All PBS was then aspirated from the apical and basolateral sides with extra care to ensure no residual fluid remained on both sides of the transwell at the end of the washes. To determine the rate and pH of the secreted fluid, 200 µl and 1ml of KREBS/HCO_3^- solution was added to the apical and basolateral compartments respectively, as well as the desired pharmacological agonist or inhibitor. Cells were then incubated at 37˚C in humidified air containing 5% (v/v) CO_2 over a 24 hour period. The volume of apical fluid was then measured using an appropriate Gilson pipette by removing the first 180µl of the fluid and placing this in an eppendorf. Then the remaining fluid was removed, 1µl at a time, to ensure high accuracy and combined with the first 180µl.

For pH measurements, the collected samples were incubated at 37˚C in humidified air containing 5% (v/v) CO_2 for at least 5 mins for pH to equilibrate, and then pH measured using a micro pH electrode attached to a pH meter (pH meter 240, Corning).

2.8 Confocal Microscopy

(i) Actin-cytoskeleton. Confluent Calu-3 cell monolayers, grown for 8-12 days on 12 mm transwell supports, were used to visualise the actin cytoskeleton network using confocal microscopy. After removing the culture media from the transwells, 10µM cytochalasin-D or solvent control was added to the cells and incubated at 37˚C in for 1 h in humidified air containing 5% (v/v) CO_2. Following treatment, cells were washed with PBS three times and then fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cells were then washed three times with PBS (each for 5 minutes), and then washed with 50mM NH4Cl to

quench any remaining PFA. After washing, cells were permeabilized by Triton X-100 (1% in PBS) for 5 minutes at room temperature and then rinsed with PBS three times for 5 minutes. Blocking buffer containing PBS with 3% FCS and 0.1% azide was added to the cells to block non-specific binding. At this point, the transwell membrane was cut out gently and placed in a 12 well plate with the apical side facing upwards. Cells were then treated with 200µl of PBS containing 3% fetal calf serum, 0.1% azide and 0.25% fluorescent Phalloidin–Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC) (Sigma) for 30 minutes at room temperature, and subsequently cells were washed twice with PBS to remove unbound phalloidin conjugate. 0.2µg/ml 4',6-diamidino-2-phenylindole (DAPI) was then added for two minutes in order to stain nuclei, and then cells were rinsed with PBS, and then mounted on slides using 1-2 drops of Vectashield mounting medium (Vector Laboratories, UK).

(ii) Detection of HA-tagged mAE2. Control, untransfected and transfected HEK-293T cells expressing HA-tagged mAE2, grown on glass coverslips for 2 days, were fixed with 4% PFA for 10 mins at room temperature. Cells were then washed with PBS three times for 5 minutes, and then washed with 50mM NH4Cl to quench any remaining PFA. After washing, fixed cells were then incubated in Triton X-100 (1% in PBS) for 5 minutes at room temperature to permeabilize cells, and then washed in PBS three times for 5 minutes. To block non-specific binding, cells were incubated with blocking buffer containing 1% Na-azide and 5% Goat serum in PBS at room temperature for 30 minutes. Blocking buffer was removed and cells were then incubated in diluted primary antibody (Anti-HA16B12, 1/1000 in blocking buffer, Abcam) overnight at 4°C on a shaker. Cells were then rinsed in PBS three times for 15 minutes to remove any residual unbounded primary antibody. At this point, cells were incubated with the diluted FITC-conjugated secondary antibody (Goat-anti mouse antibody, 1/100 in blocking buffer) for 1hour at room temperature away from light by covering with aluminium foil. Cells were then washed with PBS three times for 15 minutes to remove any unbounded secondary antibody. After washing, DAPI dye (1µg/ml) was added onto coverslips for 2 minutes, at room temperature, away from light, to stain the nucleus and then gently washed in PBS to remove any remaining DAPI. Coverslips was then mounted onto a

labelled microscope slide, using mounting medium, with the cell-side faced down. Some silicone gel was added along the edge of coverslip to seal it to the slide to prevent drying and movement of the coverslip under the microscope.

Cells were observed under Nikon A1R Confocal microscope at x60 magnification (0.1 DIC lens) with a numerical aperture of 1.4. Cells were excited with the DAPI excitation wavelength of 405nm to visualize DAPI stained specimens, and imaged at the emission wavelength of 450nm. TRITC stained specimens was visualized with 595nm excitation wavelength and 561nm emission wavelength. To visualize FITC stained specimens, cells were excited with the FITC-excitation wavelength of 495nm and imaged at the emission wavelength of 517nm.

2.9 Solutions and reagents

All reagents and inhibitors were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., UK), apart from forskolin, BAPTA-AM, and TBB (R & D Systems); BCECF-AM, DIDS, Lipofectamine 2000, WGA (Invitrogen), GlyH-101, CFTRinh-172, J-8 (Santa Cruz), Okadaic acid (Calbiochem), RpcAMP (Enzo life science), and Anti-HA antibody (Abcam). Gas cylinders were purchased from BOC and consisted of the following mixtures: 5% CO_2 /95% O_2. CX-4945 was a kind gift from Dr. Andrea Venerando (University of Padova, Italy).

All stock solutions of agonists and inhibitors used for pH_i experiments and fluid secretion assay were made in DMSO, apart from nigericin (made in 100% ethanol), and 8CPT-2Me-cAMP, carbachol and adenosine (dissolved in deionised water).

The HCO_3^- free Na-HEPES buffer solution consisted of 130mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM NaHEPES and 10mM D-Glucose. The Cl^--free HEPES buffer solution consisted of 130mM Na-gluconate, 2.5mM

$K2SO_4$, 6mM Ca-gluconate, 1mM Mg-gluconate, 10mM HEPES (free acid) and 10mM D-Glucose. All HEPES-buffered solutions were calibrated to pH 7.6 at room temperature (7.4 at 37˚C) by addition of 1M HCl.

High Cl^-- Krebs solution consisted of $NaHCO_3^-$ (25mM), NaCl (115mM), KCl (5mM), $CaCl_2$ (1mM), $MgCl_2$ (1mM) and D-Glucose (10mM). In the high K^+/high Cl^- Krebs solution, the KCl concentration was increased to 115mM and NaCl decreased to 5mM to maintain osmolarity. In the Ca^{2+}-free high Cl^-- Krebs solution, the NaCl concentration was increased to 116mM, and $CaCl_2$ was replaced with $MgCl_2$ and 0.5mM EGTA was added to chelate any remaining Ca^{2+}. In the Cl^--free HCO_3^- solution, Cl^- was substituted for gluconate, and consisted of $NaHCO_3^-$ (25mM), Na-Gluconate (115mM), K_2SO_4 (2.5mM), Ca-Gluconate (6mM), Mg-Gluconate (1mM) and D-Glucose (10mM). In the Ca^{2+}-free Cl^--free Krebs solution, the Na-Gluconate concentration was increased to 124mM, and 0.5mM EGTA was added to chelate any remaining Ca^{2+}. The solution was adjusted to pH 7.4 at 37°C by bubbling with $\%5CO_2/\%95O_2$.

The intracellular pH calibration solutions consisted of (in mM) 5 NaCl, 130 KCl, 1 $CaCl_2$, $1MgCl_2$, 10 D-Glucose, 10 HEPES (for solutions set at pH 7.6 or below) or 10 TRIS (for solutions set at pH 7.8 or above) as well as 10µM nigericin. This was added just before the start of experiments. Solutions were set to the desired pH by using either 1M HCl or 1M NaOH.

The ammonium pulse solutions used to determine intracellular buffering capacity consisted of (in mM) 4.5 KCl, $1MgCl_2$, 2 $CaCl_2$, 5 BaCl, 10 HEPES, 10 D-Glucose as well as varying concentrations of NH_4Cl/NMDG-Cl, ranging from 0 NH_4Cl/145 NMDG-Cl to 30 NH_4 Cl/115 NMDG-Cl. All solutions were titrated to pH 7.4 at 37˚C using 1M CsOH.

2.10 Statistical analysis

All results are presented as mean ± S.E.M. where *n* is the number of experiments. For data presentation and statistical analysis, GraphPad Prism 4 software (GraphPad Software, USA) was used for statistical analysis and either a Student's *t* test (paired or unpaired), one-way ANOVA (with Tukey's multiple comparison *post*-test) or two-way ANOVA (with Bonferroni's *post*-test) used where applicable. P values of <0.05 were considered statistically significant.

Chapter 3 Regulation of the apical Cl^-/HCO_3^- anion exchange activity in polarised cultures of Calu-3 cells

3.1 Introduction

As detailed in the Introduction (section 1.1.1) it has been shown that transepithelial HCO_3^- secretion in many epithelial tissues is dependent on CFTR. However, there is still some uncertainty about the exact role that CFTR plays in this process, particularly in the airways. Evidence from our own laboratory (Garnett *et al.*, 2011), and more recently from (Lee *et al.*, 2015) have demonstrated that SLC26A transporters are present in the airways, suggesting that airway HCO_3^- secretion could involve the combined activity of CFTR and Cl^-/HCO_3^- exchange. However, this conclusion is in marked contrast to previous work from Calu-3 cells, which suggested that both Cl^- and HCO_3^- secretion was mediated only by CFTR (Poulsen *et al.*, 1994; Illek *et al.*, 1997; Lee *et al.*, 1998; Krouse *et al.*, 2004; Shan *et al.*, 2012). While the reasons for these different conclusions are still not resolved, it is clear that the molecular mechanisms that orchestrate HCO_3^- secretion in these cells is still incompletely understood (Garnett *et al.*, 2013), and requires further investigation. Although, our previous work showed that stimulation of HCO_3^- secretion was clearly cAMP/PKA-dependent (Garnett *et al.*, 2011), little is known about the contribution of other cAMP-dependent binding proteins, or indeed other second messengers (such as intracellular calcium), and associated protein kinase signalling pathways, in regulating this process. This chapter details the investigations into the effects of non-cAMP/PKA dependent pathways in the regulation of CFTR-dependent apical anion exchange activity in human Calu-3 cells. Furthermore, it also describes the role of the actin cytoskeleton and dynamin in regulating apical AE activity.

3.2 Role of cyclic nucleotides (cAMP and cGMP) in the regulation of apical Cl^-/HCO_3^- AE activity

3.2.1 Role of cAMP/PKA in the regulation of apical Cl^-/HCO_3^- AE activity

Apical Cl^-/HCO_3^- AE activity in Calu-3 cells was assessed by measuring the change in intracellular pH (pH_i) in response to the removal and readdition of Cl^- from the apical perfusate, using HCO_3^- containing buffers (see Methods) in the absence and presence of different treatments. Figure 3.1A shows a typical pH_i response trace to these manoeuvres in Calu-3 cells, in the absence and presence of the cAMP agonist forskolin (Fsk), which increases cAMP by directly stimulating transmembrane adenylyl cyclise (tmAC). Under non stimulated conditions, perfusion of a Cl^- free solution to the apical side had little or no effect on pH_i. Exposing cells to Fsk (5μM) in symmetrical high Cl^- conditions, caused a significant acidification of 0.17±0.02 pH units, at a rate of 0.27±0.05 pH units min^{-1} ($P<0.05$, n=10). Subsequent apical Cl^- removal now produced an alkalinisation in pH_i of 0.48 ± 0.03 pH units. Following Cl^- readdition pH_i reacidified at a rate of 0.86 ± 0.15 pH unit min^{-1} ($P<0.05$, n=10, Figure 3.1C and D). It was noticeable that the alkalinisation caused by apical Cl^- removal was biphasic, with a fast initial rate of change, followed by a slower increase in pH_i to a new plateau level, consistent with previous results published by our group (Garnett *et al.*, 2011).

In order to further investigate the dependency of apical Cl^-/HCO_3^- AE activity on intracellular cAMP, the physiological cAMP agonist adenosine (ADO) was also tested on apical Cl^-/HCO_3^- AE activity (Figure 3.1B). Adenosine can stimulate adenylyl cyclase activity via binding to adenosine type 2B receptors ($A_{2B}R$) , and thus increase intracellular cAMP (Clancy *et al.*, 1999). It has also been shown by short circuit measurements that A_{2B} rare expressed on both the apical and basolateral membranes of Calu-3 cells (Szkotak *et al.*, 2003). My results showed that apical Cl^- removal under bilateral ADO stimulation (10μM) produced a significant alkalinisation in pH_i of 0.22 ± 0.04 pH units, which reacidified at a rate of 0.09 ± 0.01 pH unit min^{-1} ($P<0.05$, n=3, Figure 3.1E and F) upon Cl^- readdition. However, Fsk caused a significantly larger increase in apical Cl^-/HCO_3^- AE activity, both in the magnitude of alkalinisation produced

by apical Cl^- removal, and the rate of reacidification following Cl^- readdition, compared to ADO stimulated cells. Also, the rate of HCO_3^- efflux upon Cl^- readdition under Fsk stimulation was significantly greater compared to ADO stimulation (Figure 3.1G). Furthermore, in different experiments, stimulation of Calu-3 cells with 5μM forskolin and incubated in 5% CO_2 (v/v) in air for 24 h in high Cl^- Krebs solution at 37˚C, produced a significant increase in both volume and the pH of the secreted fluid, compared to untreated cells (Figure 3.22A and B). These results indicate that Calu-3 cells have little AE activity in the apical membrane under resting conditions, while increasing $[cAMP]_i$ by Fsk (and ADO) caused activation of apical Cl^-/HCO_3^- AE activity, as well as an increase in net HCO_3^- secretion.

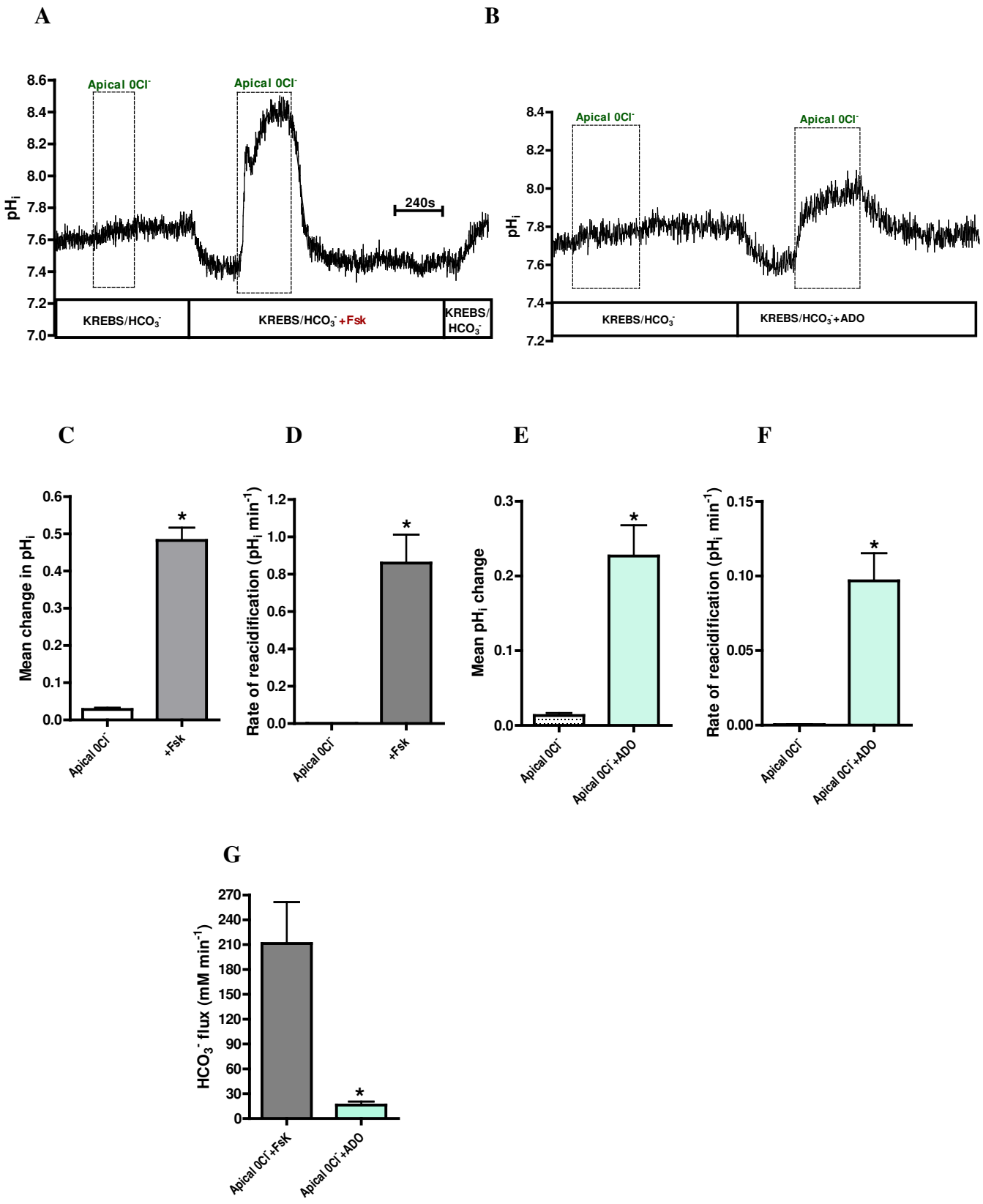
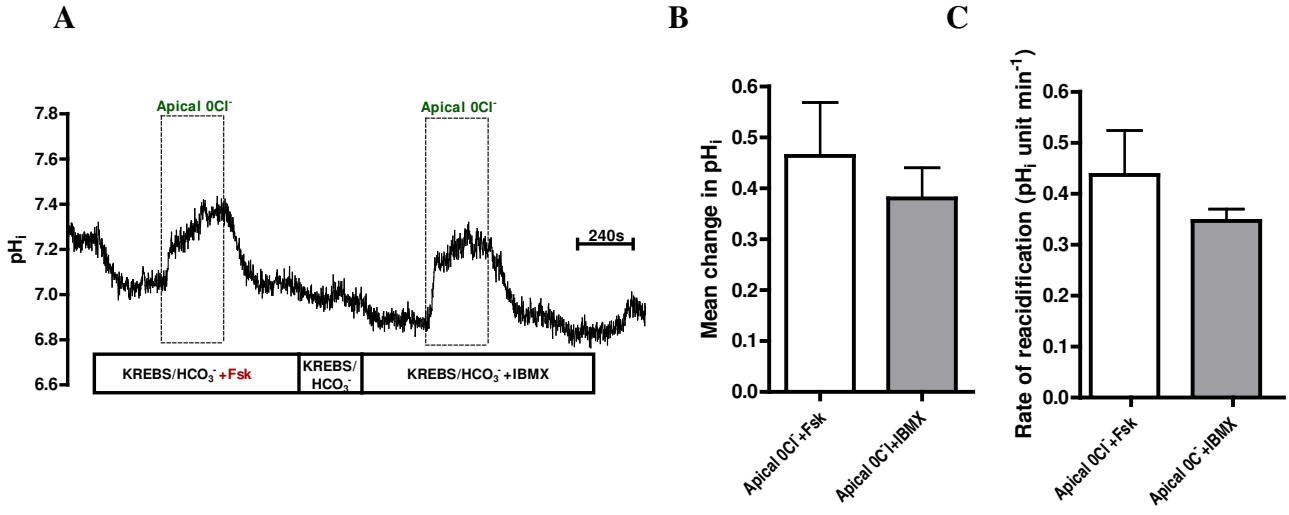


Figure 3. 1: Apical Cl^-/HCO_3^- AE activity in Calu-3 cells before and after stimulation with cAMP agonists. Representative pH_i trace showing the effect of Cl^- removal in the apical perfusate on pH_i under basal and Fsk-stimulated conditions (A) or ADO-stimulated conditions (B). Summary of mean pH_i change (alkalinisation) caused

by Cl^- removal (C), and the rate of reacidification following Cl^- readdition (D), in the presence and absence of forskolin (5µM), paired observations, n=10 for each condition. *P<0.05 compared to apical $0Cl^-$ (Basal). (E) mean pH_i change (alkalinisation) caused by Cl^- removal, and the rate of reacidification (F) following Cl^- readdition, in the presence and absence of ADO (10µM), n=3 for each condition. *P<0.05 compared to apical $0Cl^-$ (G). The rate of HCO_3^- flux under both Fsk and ADO stimulated conditions *P<0.05 compared to apical $0Cl^-$ + Fsk. Fsk denotes forskolin (5µM), ADO denotes adenosine (10µM), Apical $0Cl^-$ refers to the removal of apical Cl^-. Data are shown as mean ±SEM

Another way to increase cAMP inside cells, aside from Fsk and ADO, but which is downstream of tmAC, was to expose cells to the general phosphodiesterase (PDE) inhibitor 3-Isobutyl-1-methylxanthine (IBMX) or to use a membrane permeable analogue of cAMP, such as dibutrylyl-cAMP (db-cAMP). My results showed that both these cAMP agonists caused stimulation of apical Cl^-/HCO_3^- AE activity (Figure 3.2) which was not significantly different to the results obtained with Fsk. These results provide further support that stimulation of apical Cl^-/HCO_3^- AE activity is cAMP dependent.



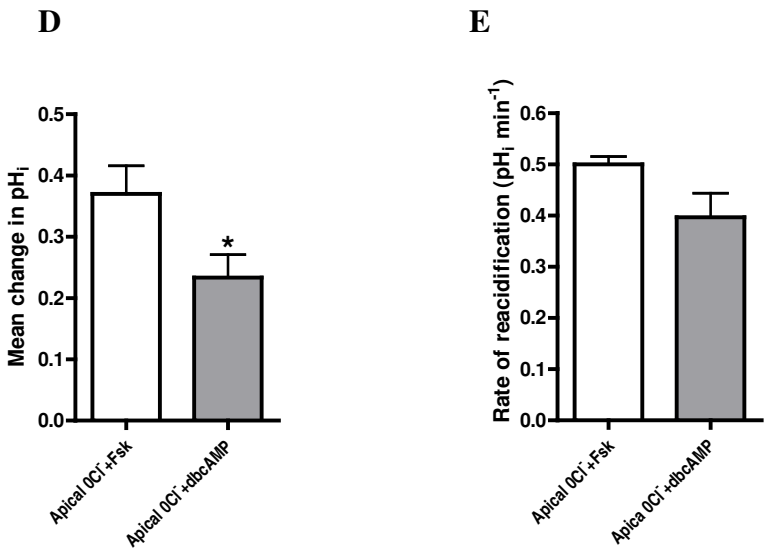


Figure 3. 2: The PDE inhibitor, IBMX, and the membrane permeable analogue of cAMP, dbcAMP, mimic the effect of Fsk on apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing the effect of Cl^- removal in the apical perfusate on pH_i under Fsk and IBMX (1.0mM added apically) stimulated conditions. Summary of mean pH_i change (alkalinisation) caused by Cl^- removal (B), and the rate of reacidification (C) following Cl^- readdition in Fsk and IBMX stimulated conditions; no significant difference ($P>0.05$) compared to apical $0Cl^-$+Fsk. Mean pH_i change (alkalinisation) caused by Cl^- removal (D), and the rate of reacidification (E) following Cl^- readdition in Fsk stimulated cells compared to dbcAMP stimulation (800µM, added bilaterally). No significant difference ($P>0.05$) compared to apical $0Cl^-$+Fsk, although mean pH_i change was significantly ($P<0.05$) lower with dbcAMP compared to apical $0Cl^-$+Fsk. Data are shown as mean ±SEM (n=3), paired observations.

It was of interest to investigate the mechanism responsible for cAMP stimulation of apical Cl^-/HCO_3^- AE activity, and to understand whether cAMP-induced activation of apical Cl^-/HCO_3^-AE was direct or through a downstream target of cAMP, including PKA and exchange protein directly activated by cAMP (Epac) (Schmidt *et al.*, 2013). Therefore, I performed a series of experiments to assess the effect of two different protein kinase A (PKA) inhibitors, H-89 and Rp-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMP) on the Fsk response, in Calu-3 cells. Results showed that preincubating

cells with H-89 (50μM for 60 min) significantly reduced Fsk activation of apical Cl^-/HCO_3^- AE activity. Figure 3.3A and B shows that both the magnitude of alkalinisation, produced by apical Cl^- removal, and the rate of reacidification, following Cl^- readdition, were decreased in H-89 treated Calu-3 cells compared to untreated control cells (Figure 3.3A and B). This is consistent with previous results from our laboratory that showed inhibition of PKA, using H-89, markedly decreased the Fsk-induced apical Cl^-/HCO_3^- AE activity in Calu-3 cells (Garnett *et al.*, 2011). However, it was found that the resting pH_i was significantly decreased in H-89 treated cells (7.14± 0.10, n=4), compared to control cells (7.60± 0.02, n=10, $P<0.05$).

Since H-89 is a non-specific inhibitor of PKA, an alternative PKA inhibitor, Rp-cAMP was also tested. Rp-cAMP has a different structure to H-89 and acts as a specific competitive antagonist of the cyclic nucleotide–binding domains on PKA (de Wit *et al.*, 1984). Calu-3 cells were preincubated with 1mM Rp-cAMP on the apical side for one hour, before apical Cl^-/HCO_3^- AE activity was measured. Results also showed that PKA inhibition significantly reduced Fsk stimulated alkalinisation in pH_i produced by apical Cl^- removal, as well as the rate of reacidification following Cl^- readdition, compared to untreated control cells (Figure 3.3C and D). These results clearly indicate that stimulation of apical Cl^-/HCO_3^- AE activity by cAMP is through a PKA-dependent mechanism in Calu-3 cells. However, it was evident that neither PKA inhibitor completely abolished Fsk stimulated AE activity.

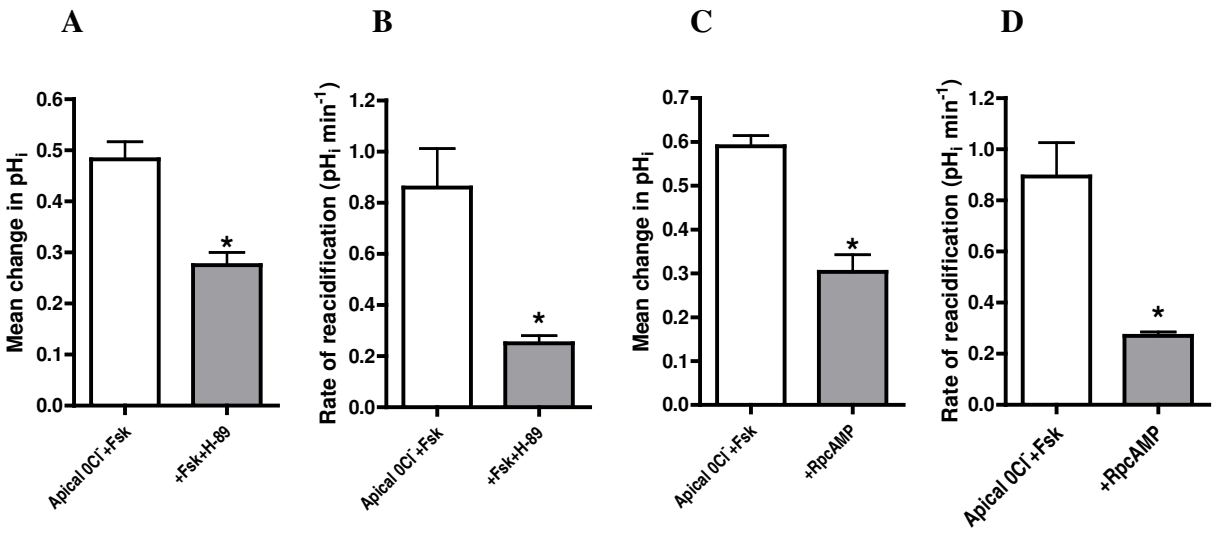


Figure 3. 3: PKA inhibitors, H-89 and Rp-cAMP decrease forskolin activated apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the impact of PKA inhibitors, H-89 and Rp-cAMP, on apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) mean alkalinisation in pH_i in response to Cl^- free solution, and the rate of reacidification (B) in Calu-3 cells pre-treated with 50µM H-89 for 60 min, n=10 Apical $0Cl^-$+Fsk, n=4 for +Fsk+H-89. (C) mean alkalinisation in pHi in response to Cl^- free solution, (D) the rate of reacidification in Calu-3 cells pre-treated with 1mM Rp-cAMP for 60 min, n=6 Apical $0Cl^-$+Fsk, n=3 for +Fsk+ Rp-cAMP. *P<0.05 compared to control apical 0Cl+Fsk. Data are shown as mean ±SEM. Control cells run in parallel.

3.2.2 Role of exchange protein directly activated by cAMP (Epac) in the regulation of apical Cl^-/HCO_3^- AE activity

Based on the fact that both PKA inhibitors, H-89 and RpcAMP, did not completely abolish the Fsk stimulated apical Cl^-/HCO_3^- AE activity, my hypothesis was that other potential downstream targets of cAMP would be involved in regulating apical anion exchange activity, such as the exchange protein directly activated by cAMP (Epac) (Tsalkova *et al.*, 2012). Although a variety of cAMP dependent cellular processes were previously thought to be solely regulated by PKA, extensive studies have now shown that many of these are also controlled by Epac proteins (Tsalkova *et al.*, 2012). Moreover, it has been found that Epac has novel cAMP properties that are independent of PKA

(Schmidt *et al.*, 2013), and it has been reported that Epac1 plays a critical role in the regulation of intestinal Cl^- secretion by Fsk, via a PKA independent mechanism (Hoque *et al.*, 2010). Thus, I investigated whether Epac is involved in the regulation of apical Cl^-/HCO_3^- AE activity by first preincubating Calu-3 cells with the membrane permeable Epac agonist, 8CPT-2Me-cAMP-AM (Lamyel *et al.*, 2011), for 60 mins during dye loading. Results showed that the Fsk-induced activation of the apical Cl^-/HCO_3^- exchanger was not affected in Calu-3 cells exposed to the Epac agonist, nor did the Epac agonist stimulate apical Cl^-/HCO_3^- AE activity itself, under basal conditions (Figure 3.4A and B). This suggests that Epac stimulation by cAMP is not involved in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cell.

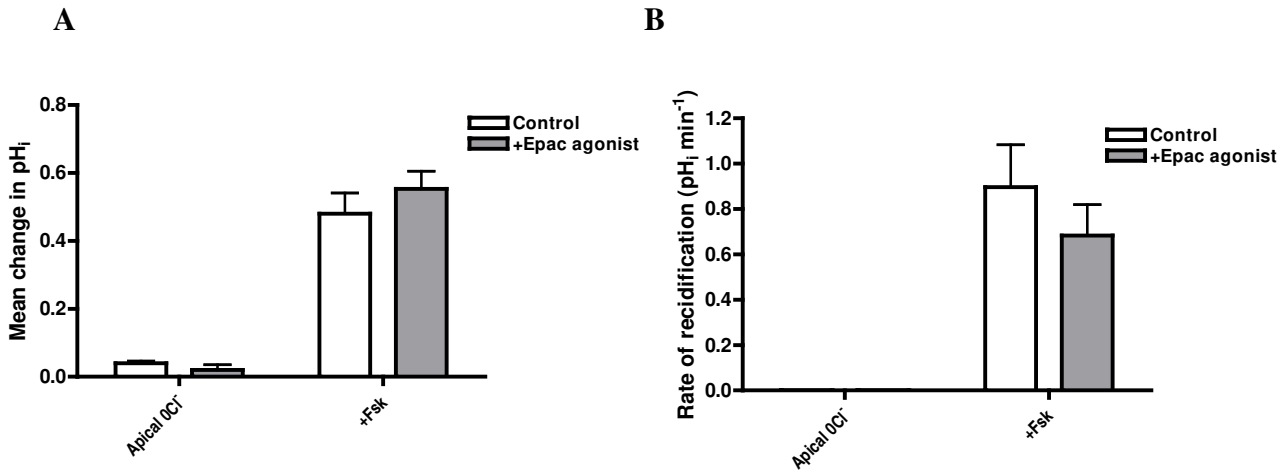
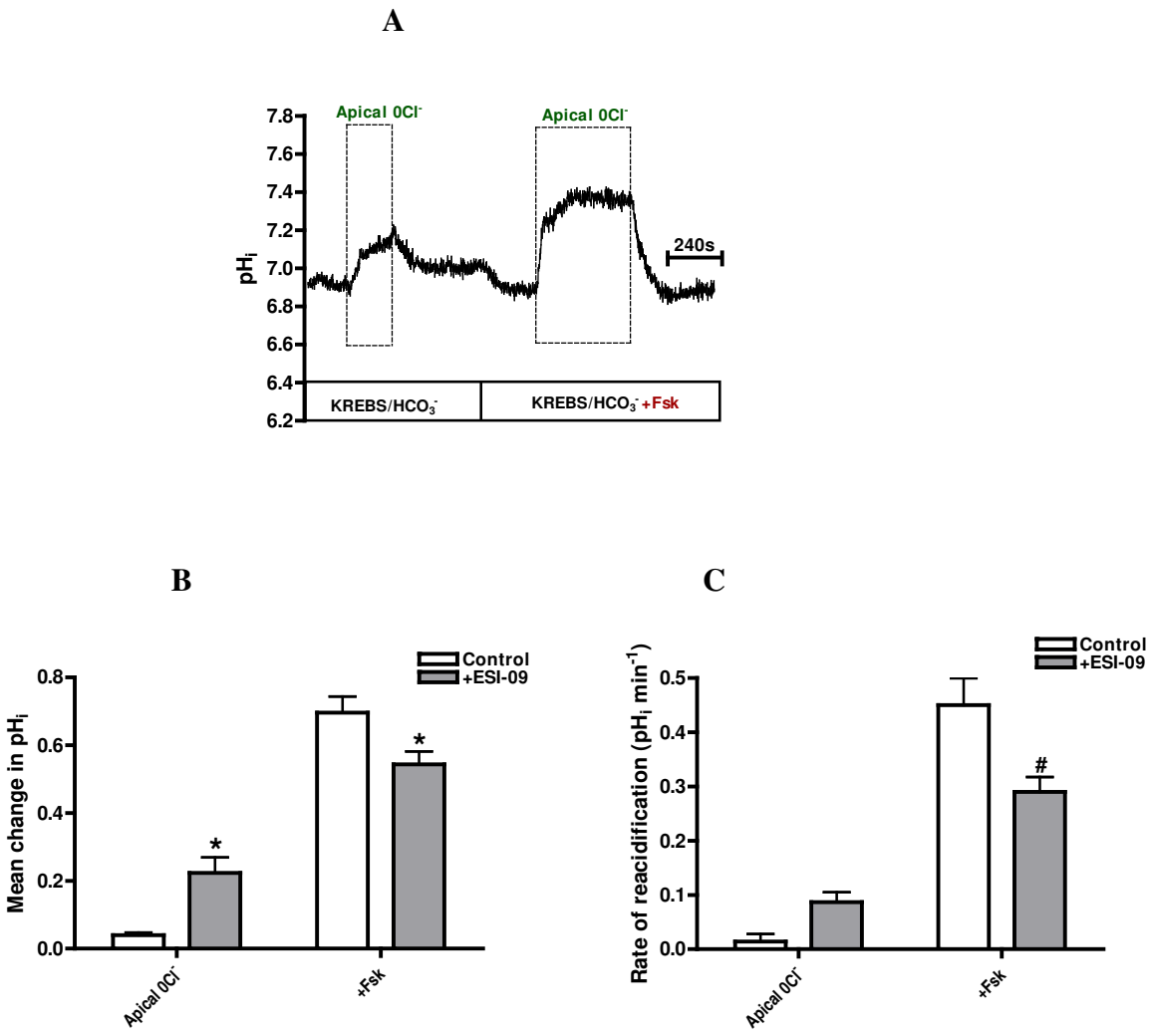


Figure 3. 4: **Apical Cl^-/HCO_3^- AE activity is not affected by the Epac agonist 8CPT-2Me-cAMP in Calu-3 cells.** Summary of the impact of the Epac agonist on apical Cl^-/HCO_3^- AE activity. Calu-3 cells were pre-treated with 10µM 8CPT-2Me-cAMP-AM for 60 min. (A) mean alkalinisation in pH_i in response to Cl^- free solution. (B) the rate of reacidification upon Cl^- readdition. No significant difference ($P>0.05$) compared to control responses, n=3 for each condition. Control cells run in parallel.

However, in another series of experiments, Calu-3 cells were preincubated with the specific Epac inhibitor, ESI-09 (Almahariq *et al.*, 2013), (Figure 3.5A). In the ESI-09 treated cells it was noted that the resting pH_i was significantly reduced compared to untreated cells (6.90±0.04, n=6, compared to 7.45±0.03,

n=5, respectively; P <0.05). Surprisingly, Epac inhibition caused a partial, but significant, stimulation of apical Cl^-/HCO_3^- AE activity in the absence of any cAMP agonist (Figure 3.5A,B and C), as well a marked stimulation in HCO_3^- efflux (Figure 3.5D), compared to untreated cells. Furthermore, Epac inhibition caused a significant decrease in Fsk stimulated apical Cl^-/HCO_3^- AE activity (Figure 3.5B and C) as well as the rate of HCO_3^- efflux (Figure 3.5E). These results suggest that Fsk-induced stimulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells is not only through PKA, but involves an Epac-dependent mechanism. They also suggest that keeping Epac active is essential to maintain apical Cl^-/HCO_3^- AE activity under resting conditions.



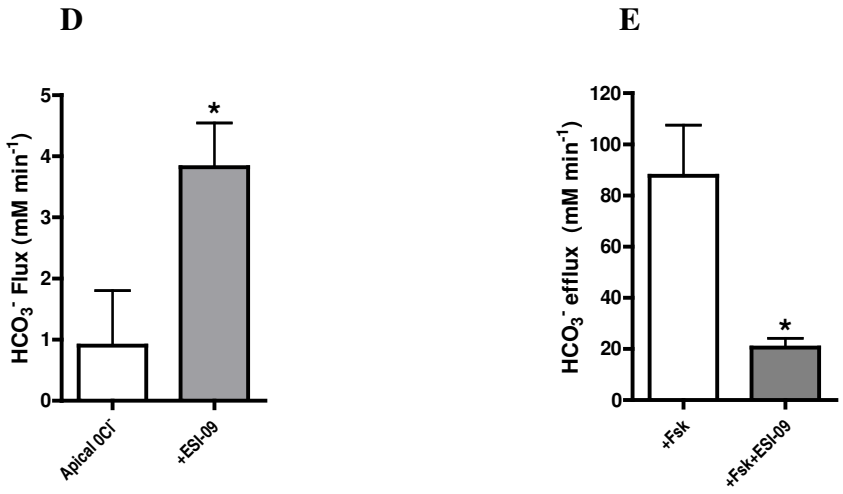


Figure 3. 5: **Effect of the Epac inhibitor, ESI-09, on the apical Cl^-/HCO_3^- AE activity in Calu-3 cells.** (A) pH_i experimental trace showing the effect of preincubation of Calu-3 cells with 10µM Epac inhibitor ESI-09 for 60 min on the apical Cl^-/HCO_3^- AE activity after Cl^- removing in the apical perfusate under basal and Fsk stimulated conditions. Summary of the effect of 10µM ESI-09 preincubation on mean alkalinisation in pH_i in response to a Cl^- free solution (B), and the rate of reacidification after Cl^- readdition (C) in unstimulated and Fsk stimulated Calu-3 cells. (D) The rate of HCO_3^- flux during apical Cl^- removal in control and +ESI-09 treated cells. (E) The rate of HCO_3^- flux during apical Cl^- removal in control +Fsk and +FsK+ESI-09 treated cells. Data are shown as mean ±SEM. B, C, and E: *P<0.05 compared to control apical 0Cl^- under basal and stimulated conditions, n=5 for control, n=6 for +ESI-09. D: *P<0.05, #P<0.01 compared to control apical 0Cl^-, n=8 for apical 0Cl^-, and n=5 for +ESI-09. Control cells run in parallel.

Since apical Cl^-/HCO_3^- exchange activity is markedly affected by CFTR (Ko *et al.*, 2002), and our laboratory has recently found that CFTR knock-down, or the CFTR inhibitor, GlyH-101, reduced the rate of pendrin-mediated anion exchange in Calu-3 cells (Garnett *et al.*, 2011), I wanted to investigate if the ESI-09-induced apical Cl^-/HCO_3^- AE activity observed under basal conditions was also dependent on anion transport by CFTR. To do this, Calu-3 cells were preincubated with the Epac inhibitor and apical Cl^-/HCO_3^- AE activity measured under basal conditions in the absence and presence of the CFTR pore blocker GlyH-101. Results showed that the apical Cl^-/HCO_3^- AE activity induced by

Epac inhibition was completely abolished by GlyH-101 (Figure 3.6), suggesting that this apical Cl^-/HCO_3^- AE activity was entirely dependent on CFTR anion transport, or that it was in fact due to CFTR itself.

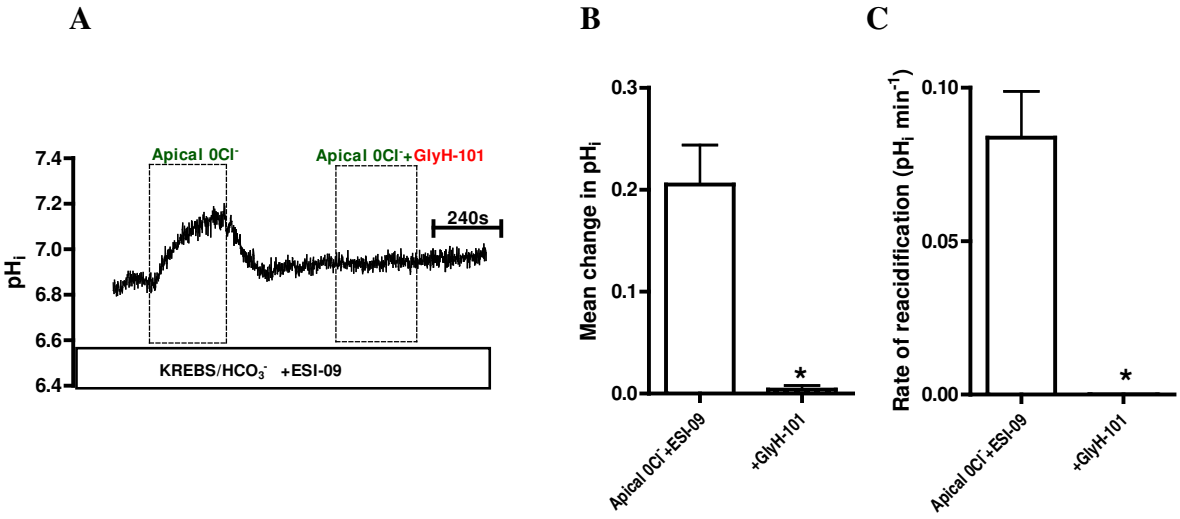
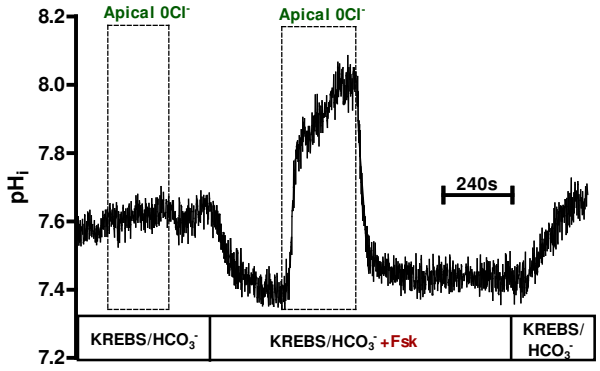


Figure 3. 6: The ESI-09-induced apical Cl^-/HCO_3^- AE activity is abolished by the CFTR pore blocker GlyH-101 under basal conditions in Calu-3 cells. (A) Representative pH_i trace showing the effect of Cl^- removal in the apical perfusate on pH_i under basal condition in cells preincubated with Epac inhibitor, ESI-09, which was inhibited by the CFTR inhibitor GlyH-101. Calu-3 cells were pre-treated with 10µM ESI-09 for 60 min. (B) mean alkalinisation in pH_i in response to Cl^- free solution (C) the rate of reacidification.*P<0.05 compared to control apical 0Cl under basal condition. Data are shown as mean ±SEM, n=8 for Apical $0Cl^-$+ESI-09, and n=5 for +GlyH-101.

It has been reported that, in addition to cAMP, cyclic 3',5'-guanosine monophosphate (cGMP) also plays an important role in regulating a variety of anion transporters (Barnes, 1995), and that both cGMP-dependent PKG I and PKG II regulate the activation of CFTR in Calu-3 cells (Chen *et al.*, 2008), and stimulate anion secretion (Duszyk, 2001b). Also Wit *et al*, (1994) showed that compounds responsible for increasing cGMP and cAMP can act synergistically to relax microvascular smooth muscle in vivo (de Wit *et al.*, 1994), and that a

combination of cAMP and cGMP plays an important role in increasing cilia beat frequency in bovine bronchial epithelial cells (Wyatt *et al.*, 2005). However, it was unknown whether cGMP was involved in the regulation of the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Thus, experiments were done to test the effect of 8-Bromo cyclic guanosine mono phosphate (8Br-cGMP), a membrane permeable cGMP agonist on apical Cl^-/HCO_3^- AE activity. Results showed that a 60 min preincubation of Calu-3 cells with 1mM 8Br-cGMP did not change apical Cl^-/HCO_3^- AE activity under resting or after Fsk stimulation (Figure 3.7B and C). This suggests that cGMP dependent protein kinase is not involved in the regulation of the apical anion exchangers in Calu-3 cells.

A



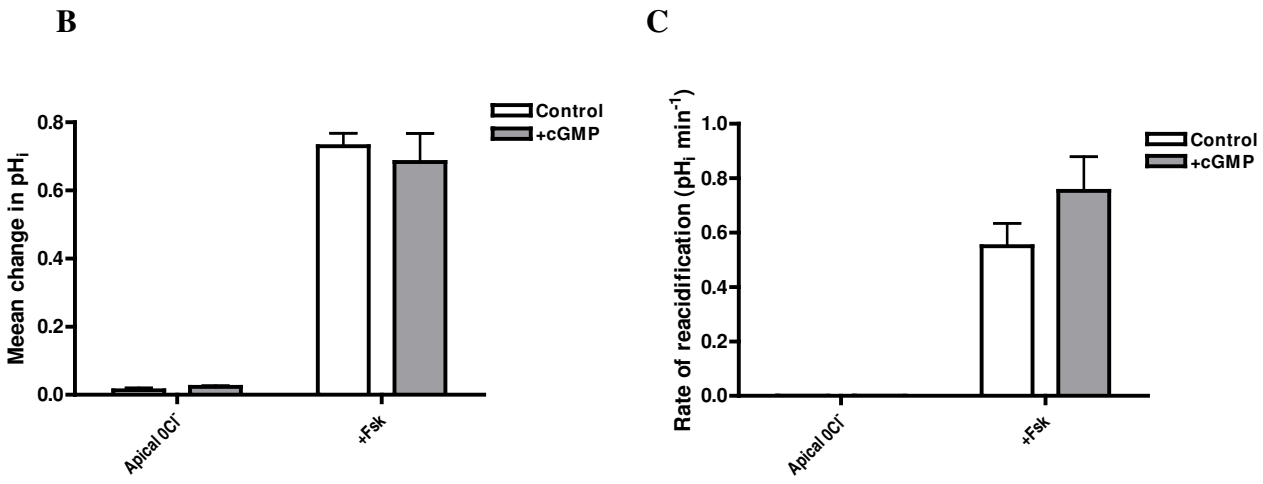


Figure 3. 7: cGMP does not activate apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pHi trace showing the effect of Cl^- removal in the apical perfusate on pH_i before and after Fsk stimulation in 8Br-cGMP preincubated (1.0mM for 60 mins) Calu-3 cells. Summary of the impact of 8Br-cGMP preincubation on mean pHi change (alkalinisation) caused by Cl^- removal (B) and the rate of reacidification (C) following Cl^- readdition, in the presence and absence of forskolin (5µM) in Calu-3 cells. Data are shown as mean ±SEM (n=3). No significant difference (P>0.05) compared to apical $0Cl^-$. Control cells run in parallel.

3.3 Role of CFTR and basolateral transporter in the regulation of apical Cl^-/HCO_3^- exchange activity

Previous work by Garnett *et al*., (2011) showed that addition of the CFTR inhibitor, GlyH-101 during the alkalinisation caused by apical Cl^- free exposure in fsk-stimulated Calu-3 cells, produced a very fast intracellular acidification which could be prevented by pre-exposing the cells to 500 µM basolateral H_2DIDS. This result suggested two things; (1) that CFTR regulates apical anion exchange activity and (2) that a H_2DIDS-sensitive basolateral HCO_3^-/H^+ transporter also influences events at the apical membrane, and therefore could be involved in modulating transepithelial HCO_3^- secretion. In order to investigate the identity of the putative basolateral HCO_3^-/H^+ transporter further, different concentrations of DIDS were applied to the basolateral side of Calu-3 cells and

the response to apical GlyH-101 measured. Figure 3.8 illustrates the effect of adding GlyH-101 on the alkalinisation produced by apical Cl^- removal in fsk-stimulated Calu-3 cells not exposed to basolateral DIDS. It can be seen that GlyH-101 significantly reduced the magnitude of the alkalinisation produced by Cl^- removal (Figure 3.8A and B) and that the rate of the GlyH-101 induced 'acidification' was 0.35 ± 0.03 pH unit min^{-1} (n=3), which is similar (not significantly different, P>0.05) to that observed when Cl^- was reintroduced in control cells 0.45 ± 0.12 pH unit min^{-1} (n=3). These experiments were then repeated but cells were first exposed to different concentrations of DIDS in the basolateral perfusate prior to apical Cl^- removal or GlyH-101 addition. Overall, it was found that only 100µM DIDS affected the magnitude of the GlyH-101 induced acidification, which was inhibited by 70±20% (Figure 3.9B). In addition, only 100 µM DIDS affected the GlyH-101 induced rate of 'acidification', which decreased from 0.35 ± 0.03 pH unit min^{-1} (n=3) in untreated cells to 0.04 ± 0.03 pH unit min^{-1} (P<0.05, n=5, Figure 3.9C) in DIDS-treated cells., which equated to an 88.5±8.3% inhibition. Based on this limited dose-response to DIDS (and the data presented in Chapter 4) these results suggest that the basolateral anion exchanger SLC4A2 (or AE2) could be the transporter involved in modulating the pH_i response to apical GlyH-101 addition. However, previous work by our group (Garnett et al., 2011), and result presented in chapter 4, showed that cAMP stimulation leads to the inhibition of a DIDS-sensitive basolateral Cl^-/HCO_3^- AE, most probably AE2.

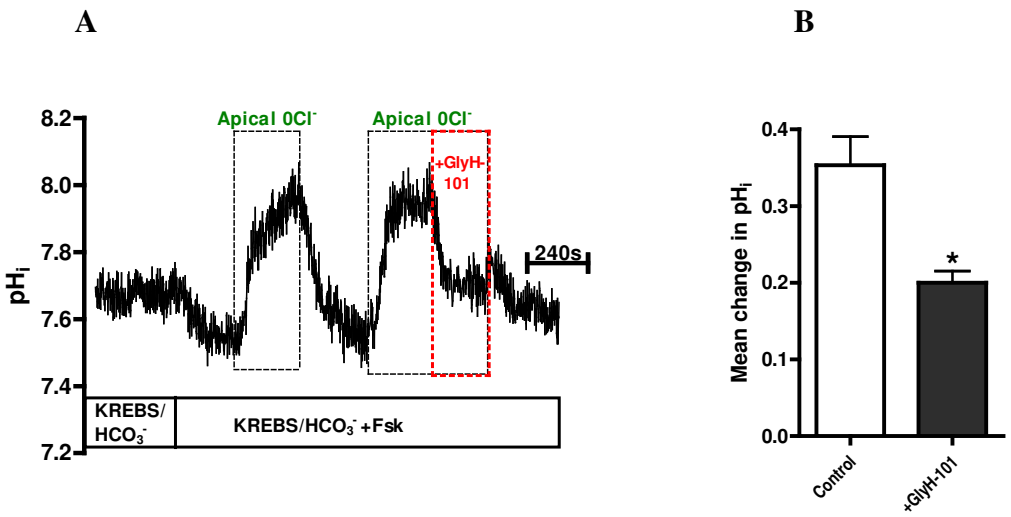
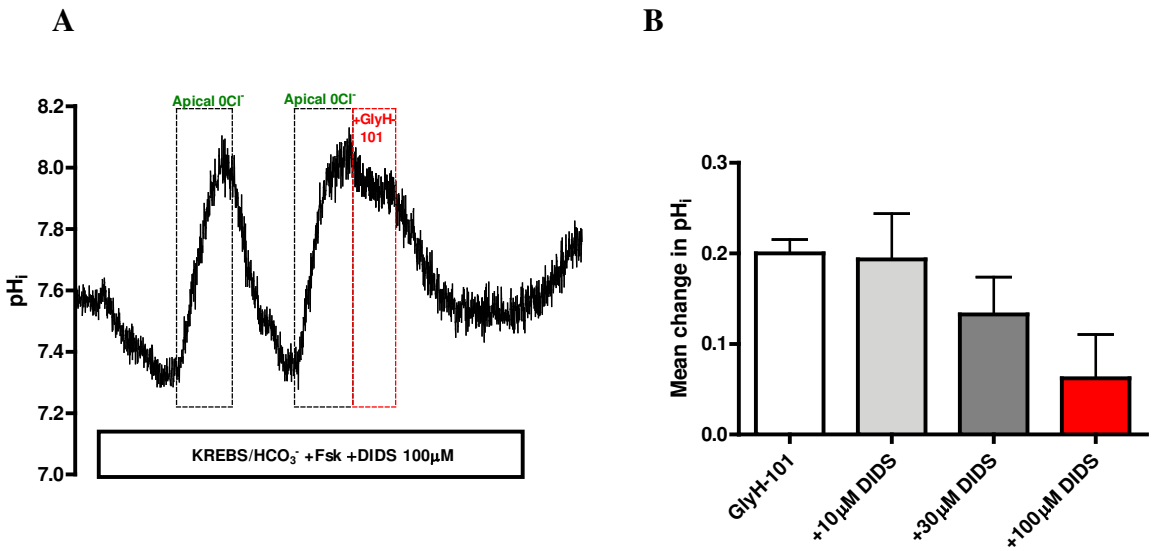


Figure 3. 8: CFTR inhibitor GlyH-101 reacidifies pHi following an apical Cl^- free induced alkalinisation in Calu-3 cells. (A) Representative pHi trace illustrating the effect of adding the CFTR inhibitor GlyH-101 (10 µM) after cells were first exposed to a Cl- free solution. Note that the CFTR inhibitor caused pHi to rapidly acidify to a new steady-state. (B) Summary of the effect of CFTR pore blocker GlyH-101 on the mean alkalinisation in pHi following apical Cl^- removal, in the absence and presence of GlyH-101, in Fsk stimulated Calu-3 cells. *P<0.05 compared to control. Data are shown as mean ±SEM, n=3, paired observations.



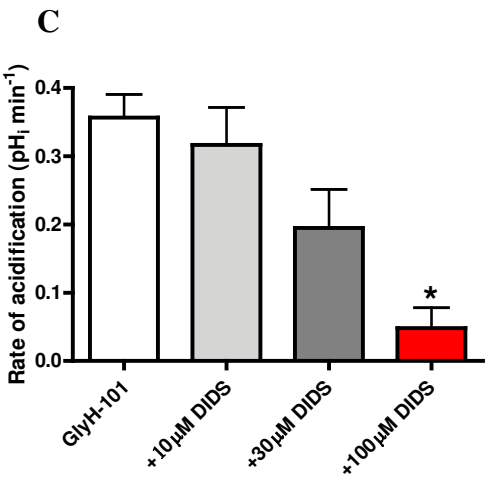


Figure 3. 9: Basolateral DIDS blocks the GlyH-101 induced intracellular acidification under apical Cl^- free conditions in Fsk treated Calu-3 cells. (A) Representative pHi trace illustrating the effect of adding the CFTR inhibitor GlyH-101 (10µM), in the presence of basolateral DIDS (100µM), after cells were first exposed to a Cl- free solution, under Fsk stimulated conditions. Summary of the effect of different concentrations of DIDS on the magnitude (B), and the rate of acidification caused by GlyH-101 (10 µM) in Cl- free conditions in the presence of Fsk compared to control response. Data shown as mean ± SEM, *P< 0.05 compared to control, n=3 for control and 10µM DIDS; n=4 for 30µM DIDS, and n=5 for 100 µM DIDS.

3.4 Role of intracellular and extracellular Ca^{2+} in the regulation of apical Cl^-/HCO_3^- anion exchanger activity

It has been demonstrated that both cAMP and Ca^{2+} play an important role in stimulation of HCO_3^- secretion in Calu-3 cells (Krouse *et al.*, 2004). It has also been shown that an increase in intracellular Ca^{2+} $[Ca^{2+}]_i$ in epithelial cells regulates a wide range of cellular process, such as the activation of Ca^{2+} activated Cl^- channels and Cl^- secretion, (Paradiso *et al.*, 1991; Grubb *et al.*, 1994), as well as stimulation of CFTR dependent Cl^-/HCO_3^- exchange activity in many CFTR-expressing epithelia cells (Namkung *et al.*, 2003). Furthermore, a recent study has shown that elevation of intracellular cAMP caused activation of adenosine A_{2A} receptors in human pulmonary epithelial cells and a subsequent increase in $[Ca^{2+}]_i$, which played a crucial role in mediating signalling pathways inside the cells (Ahmad *et al*., 2013). Also, an increase in $[Ca^{2+}]_i$ plays an important role in stimulation of ciliary beat frequency in airway epithelial cells (Delmotte and Sanderson, 2006). Most recently, it has been suggested that cAMP and Ca^{2+} signaling are the most prominent regulators of HCO_3^- secretion in epithelial cells (Jung and Lee, 2014), and that there is a synergistic interaction between Ca^{2+} and cAMP signaling pathways to control electrolyte and fluid secretion (Lee *et al.*, 2012). Therefore, it was possible that there was a cross-talk mechanism between cAMP and Ca^{2+} signaling pathways in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Thus, another series of experiments were done in order to assess the role of Ca^{2+} signalling in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells.

3.4.1 Role of intracellular Ca^{2+}

In order to assess if intracellular Ca^{2+} modulates the cAMP-stimulated apical Cl^-/HCO_3^- AE activity, Calu-3 cells were pre-treated with BAPTA-AM, a chemical which has been widely used in studies to control intracellular Ca^{2+} signals that regulate many cellular functions (Bissonnette *et al.*, 1994). Calu-3 cells were preincubated with 50μM BAPTA-AM (both apical and basolateral sides) for one hour and then the pH_i response to apical Cl^- free solution was measured after forskolin stimulation. As shown in Figure 3.10, BAPTA-AM significantly

reduced Fsk stimulated apical Cl^-/HCO_3^- AE activity, and reduced both the mean change in pH_i produced by apical Cl^- removal, and the rate of reacidification upon readdition of Cl^- to the apical side, compared to control cells not preincubated with BAPTA-AM. Note that the resting pH_i was not significantly changed in BAPTA-AM treated cells (7.70±0.02, n=5 compared to control cells 7.60±0.06, n=7, P>0.05). These results suggest that resting Ca^{2+} levels play an important role in regulating the cAMP stimulated apical Cl^-/HCO_3^-AE activity in Calu-3 cells, and that intracellular chelation of Ca^{2+} by BAPTA-AM might disrupt the synergistic cross-talk mechanism between Ca^{2+} and cAMP, and thereby regulate Cl^- and HCO_3^- transport in Calu-3 cells.

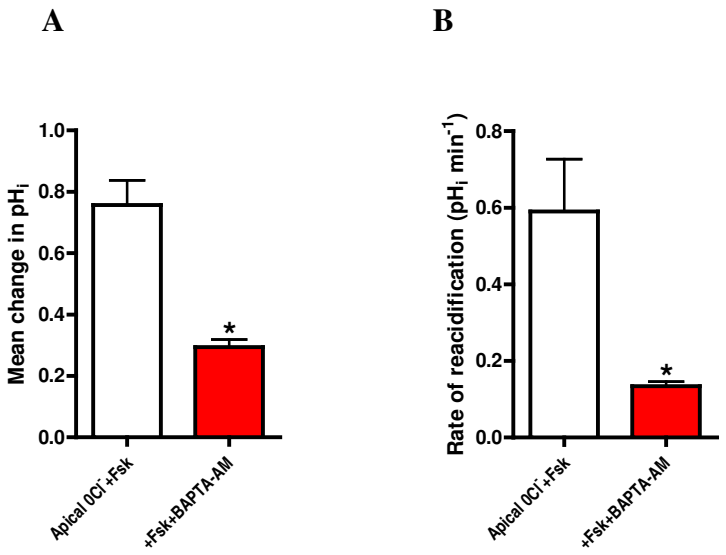


Figure 3. 10: BAPTA-AM reduced apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of the preincubation of Calu-3 cells with BAPTA-AM for 60 min. on apical Cl^-/HCO_3^- AE activity (A) mean alkalinisation in pH_i in response to Cl^- free solution (B) the rate of reacidification upon Cl^- readdition in Fsk stimulated condition. *P<0.05 compared to control. Data are shown as mean ±SEM, n=7 for control, and n=5 for treated cell with BAPTA-AM. Control cells run in parallel.

Moreover, I have also used a Ca^{2+} agonist to investigate if an increase in resting Ca^{2+} could alter the cAMP stimulated apical Cl^-/HCO_3^- AE activity, because it has been shown that CFTR mediated Cl^- current is stimulated by Ca^{2+} agonists through a Ca^{2+} dependent activation of adenylyl cyclase I (ACI) and cAMP/PKA signaling pathway in primary cultures of human bronchial epithelial cells (Namkung *et al.*, 2010). Thus, I studied the effect of Ca^{2+} release from the endoplasmic reticulum (ER), by exposing Calu-3 cells to the selective SERCA pump inhibitor thapsigargin (Tg), which causes an increase in $[Ca^{2+}]_i$ by inhibiting Ca^{2+} uptake via the Ca^{2+} ATPase, back into the ER (Thastrup *et al.*, 1990). Calu-3 cells were exposed to 200nM Tg for 5 mins, as previous work from our group has shown that this leads to calcium elevation in these cells (Garnett *et al.*, 2011). Responses to apical Cl^- removal were measured in the presence of Tg under Fsk stimulated conditions. Figure 3.11 shows that Tg caused a significant decrease in the mean change in intracellular pH and rate of reacidification in response to Cl^- removal, suggesting that an increase in $[Ca^{2+}]_i$ does play an important, but negative, role in the regulation of the apical transporter (CFTR/Pendrin) in Calu-3 cells. However, as shown in Figure 3.11, the effect of thapsigargin was not prevented by BAPTA-AM preincubation. Indeed, the presence of thapsigargin and BAPTA-AM caused a further, and significant, reduction in apical Cl^-/HCO_3^- AE activity, under Fsk stimulated conditions. The percent inhibition of Fsk stimulated apical Cl^-/HCO_3^- AE activity (as measured by the change in pH_i) by thapsigargin alone (Figure 3.12A) was significantly less than the percent inhibition produced by BAPTA-AM and thapsigargin+BAPTA-AM. However, the percent inhibition of the rate of reacidification was not significantly different between the 3 treatments (Figure 3.12B).

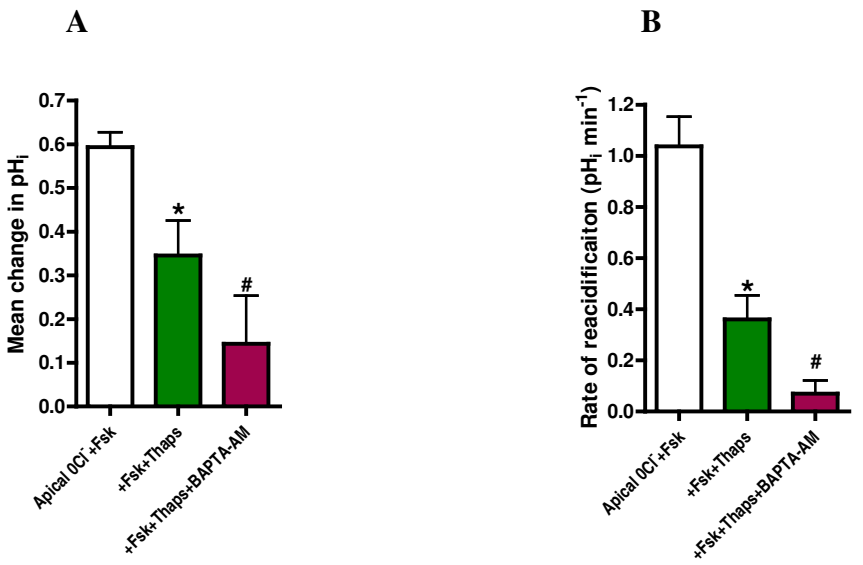


Figure 3. 11: Thapsigargin reduced forskolin-stimulated apical Cl^-/HCO_3^- AE activity in Calu-3. Summary of the effect of thapsigargin (200nM) on the Fsk-stimulated apical Cl^-/HCO_3^- AE activity measured in the absence and presence of BAPTA-AM. (A) Mean alkalinisation in pH_i produced by apical Cl^- removal and the rate of reacidification upon Cl^- readdition (B) under Fsk stimulation. Data are shown as mean ±SEM. A: *P<0.05, compared to apical Cl^-+Fsk, #P>0.05 compared to Fsk+Thaps. B: *P<0.01, #P<0.001 compared to apical Cl^-+Fsk, #P>0.05 compared to Fsk+Thaps, n=12 for control; n=7 for Fsk+thapsigargin, and n=3 for Fsk+thapsigargin+BAPTA-AM.

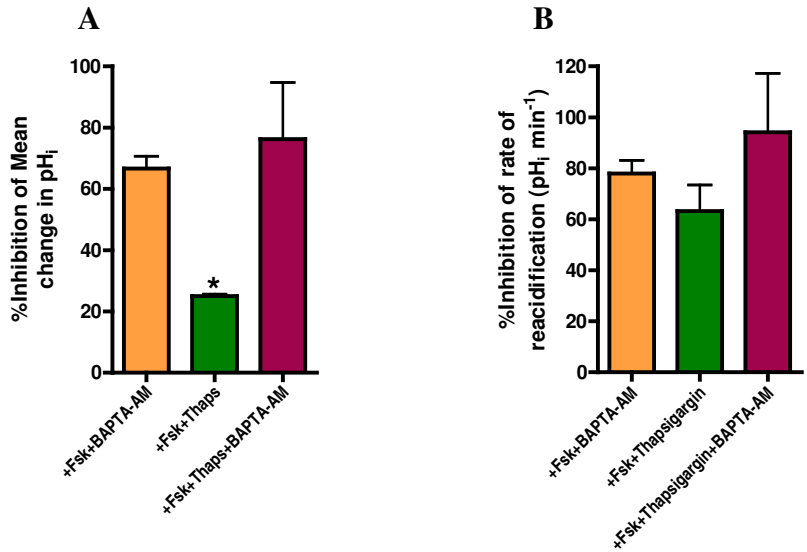


Figure 3. 12: Percent inhibition of apical Cl^-/HCO_3^- AE activity by thapsigargin and BAPTA-AM in Calu-3. Summary of the % inhibition of the apical Cl^-/HCO_3^- AE activity caused by thapsigargin (200nM) and BAPTA-AM (50µM) exposure. (A) Effect on mean alkalinisation in pH_i produced by apical Cl^- removal and (B) the rate of reacidification upon Cl^- readdition under Fsk stimulated conditions. Data are shown as mean ±SEM. A: *P<0.001 compared to +Fsk+BAPTA-AM and Fsk+thapsigargin+BAPTA-AM. B: no significant difference (P>0.05), n=5 for +Fsk+BAPTA-AM; n=7 for Fsk+thapsigargin, and n=3 for Fsk+thapsigargin+BAPTA-AM.

3.4.2 Role of extracellular calcium concentration:

My previous experiments showed that chelation of intracellular Ca^{2+} and Ca^{2+} store depletion both significantly reduced the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. To date, however, there were no available data on the effect of changing extracellular Ca^{2+} on the apical Cl^-/HCO_3^- AE activity under Fsk stimulated conditions in Calu-3 cells. It has been demonstrated that changes in extracellular free calcium concentration can be 'sensed' by the extracellular calcium-sensing receptor (CaSR), which is a G protein-coupled receptor expressed in many epithelial tissues, including human bronchial epithelial cells, where this receptor is linked to intracellular calcium signaling. Activation of CaSR by increases in extracellular Ca^{2+} ions also plays an important role in the regulation of intracellular cAMP signalling cascades (Ward, 2004; Milara *et al.*, 2010). In order to test the effect of changing extracellular Ca^{2+} on the apical Cl^-/HCO_3^- AE activity, cells were bilaterally perfused with Ca^{2+} free Krebs prior to Fsk stimulation. Figure 3.13 shows that the absence of extracellular Ca^{2+} did not affect apical Cl^-/HCO_3^- AE activity in Calu-3 cells, compared to the control response. This implies that extracellular Ca^{2+} is not involved in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells.

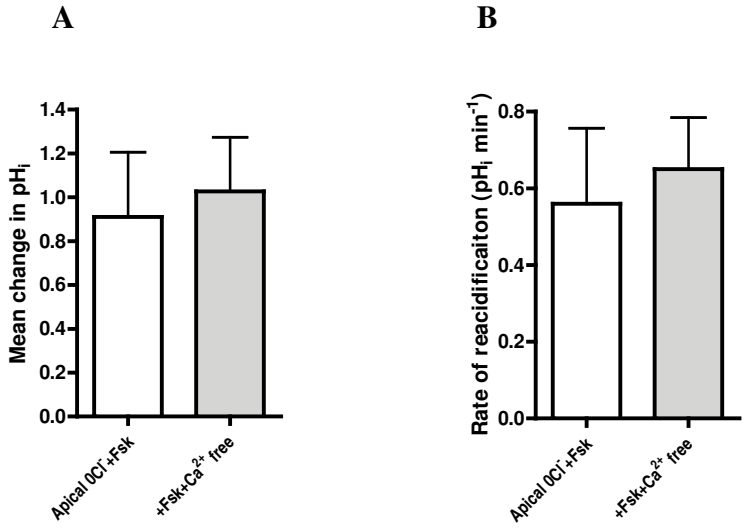


Figure 3. 13: Removal of extracellular Ca^{2+} did not affect the Fsk stimulated apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of removal of extracellular Ca^{2+} on the mean alkalinisation in pH_i in response to apical Cl^- removal (A), and the rate of reacidification upon Cl^- readdition (B) under Fsk stimulation. Data are shown as mean ±SEM. No significant difference (P>0.05) between control and treated cells, n=3 for each condition, paired observation.

3.5 Role of calmodulin and Ca^{2+}/calmodulin-dependent protein kinases in the regulation of apical Cl^-/HCO_3^-AE activity

It has been shown that there is an extensive cross-talk between Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK) and some cAMP-dependent protein kinases that could be involved in the regulation of signalling cascades inside the cytoplasm of cells (Soderling, 1999). In addition, it has been shown that the activity of calcium–activated Cl^- channels was abolished by inhibition of the CaMK pathway (using STO-609, a CaMKK inhibitor) in *Xenopus oocytes*, while it did not affect CFTR activity (Faria D., 2012 PhD thesis, University of Lisbon). Moreover, it has been reported that adenylyl cyclase I (ACI), which is responsible for elevation of intracellular cAMP, is a Ca^{2+}/calmodulin stimulated adenylyl cyclase that colocalized with CFTR in the apical membrane of human bronchial epithelial cells (Namkung *et al.*, 2010). It has been found that CaMK

kinase (CaMKK) is an upstream regulator of CAMK (Soderling, 1999) which is a Ser/Thr kinase whose activation is through a Ca^{2+}/CaM dependent mechanism (Swulius and Waxham, 2008). Moreover, CAMK kinase-alpha (CaMKK-α) and CAMK kinase-beta (CaMKK-β) have been purified and cloned from rat brain and shown to function as upstream regulators of both CaMKI and CaMKIV (Tokumitsu *et al.*, 1997). Therefore, I tested whether the CaMKK pathway contributes to the regulation of the apical Cl^-/HCO_3^- AE in Calu-3 cells.

3.5.1 Inhibition of CaMKK

Preincubation of cells with the CaMKK inhibitor STO-609 (inhibitor of CaMKK-α and CAMKK-β) for 60 min (Figure 3.14A) had no effect on basal AE activity, but did cause a small, but significant, reduction in intracellular alkalinisation produced by apical Cl^- removal following Fsk stimulation (Figure 3.14B). However, it did not affect the rate of reacidification compared to control cells (Figure 3.14 C). Also, inhibition of the CaMK pathway produced no significant effect, on the amount of secreted fluid that collected after 24 hours preincubation of Calu-3 cells with CaMKK inhibitor STO-609, and the pH of secreted fluid did not change (see Figure 3.22A and B). These results indicate that the CaMK pathway does not play a significant role in regulating the activity of the apical anion exchanger or fluid secretion in Calu-3 cells.

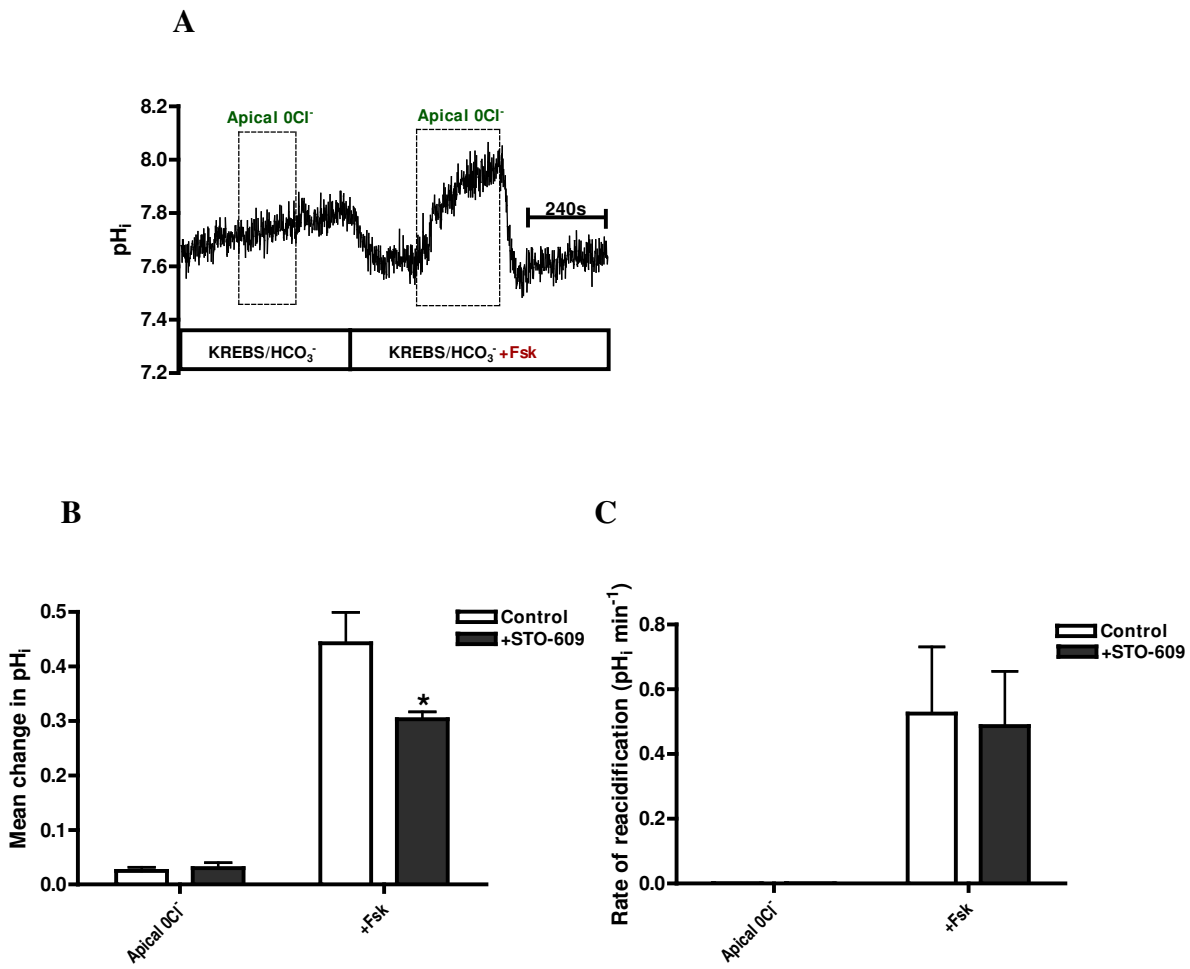


Figure 3. 14: CAMKK inhibitor, STO-609, reduced the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing that STO-609 preincubation (20μM) for 60 min modestly reduced the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of apical preincubation of STO-609 on (B) mean alkalinisation in pH_i in response to Cl^- free solution, and (C) the rate of reacidification under basal and stimulated condition. Data are shown as Mean±SEM.*P<0.05 compared to control, n=3 for each condition. Control experiments were run in parallel.

3.5.2 Inhibition of CaMKII

Although my results showed that CaMKK inhibition did not markedly affect apical Cl^-/HCO_3^- AE activity, it has been found that CaMKII plays an important role in the regulation of membrane Cl^- permeability through Cl^- channels in a variety of epithelial cells (Hartzell *et al.*, 2005). Therefore, in another series of experiments, Calu-3 cells were preincubated with 5μM CaMKII inhibitor, KN-93, for 60 min, and then apical Cl^-/HCO_3^- AE activity was measured in Cl^- free solutions (Figure 3.15A). Similar to the results with CaMKK, inhibitor KN-93

had no effect on either basal or Fsk stimulated apical Cl^-/HCO_3^- AE activity (Figure 3.15B and C).

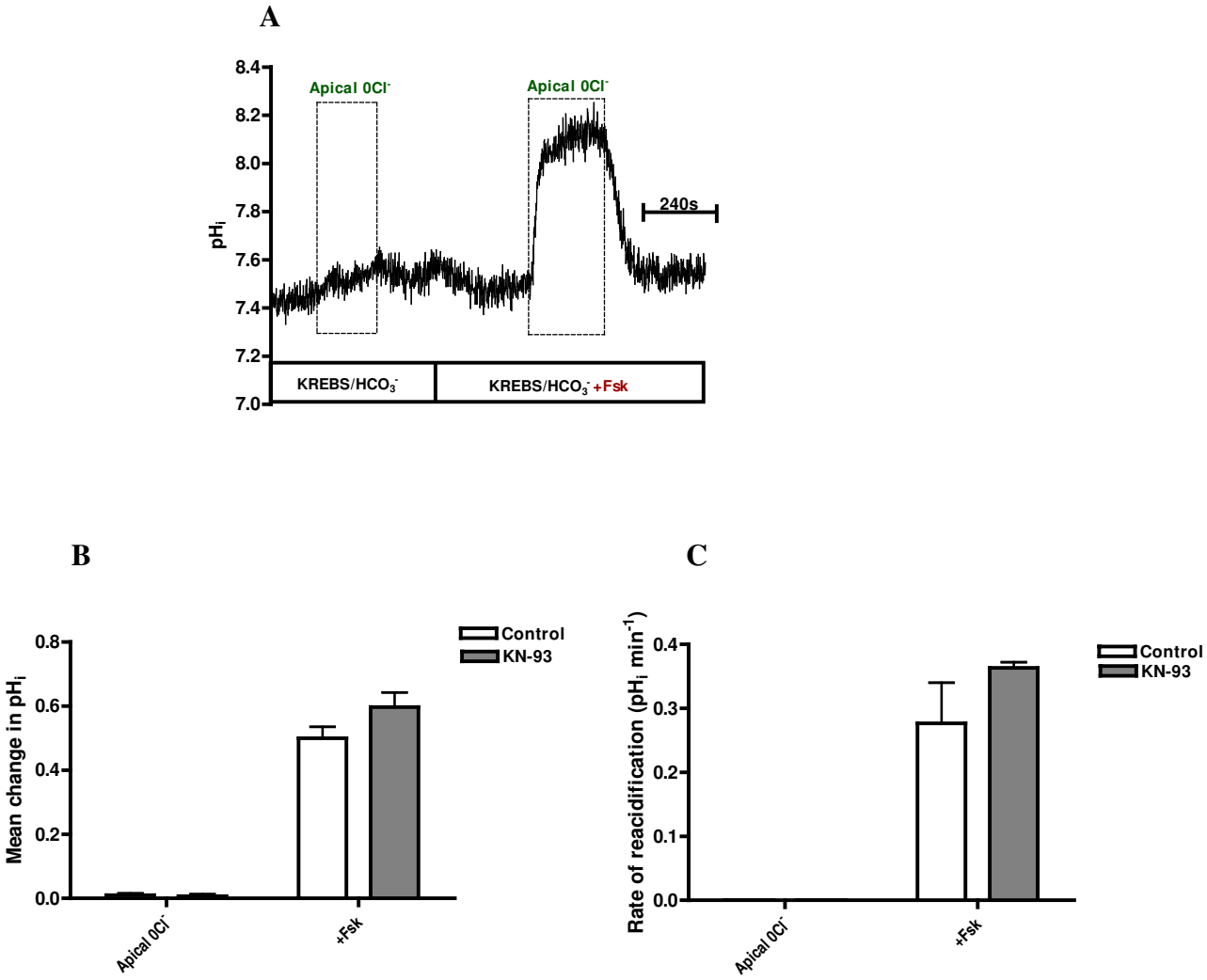


Figure 3. 15: Inhibition of CaMKII had no effect the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing that KN-93 preincubation (5µM) for 60 min did not affect the apical Cl^-/HCO_3^- AE activity under basal and Fsk stimulated condition. Summary of the effect of apical preincubation of KN-93 on mean alkalinisation in pH_i in response to Cl^- free solution (B), and the rate of reacidification (C) under basal and stimulated condition. Data are shown as Mean±SEM. No significant difference (P>0.05) compared to control, n=3 for each condition. Control cells run in parallel.

3.5.3 Role of CaM in the regulation of apical Cl^-/HCO_3^- exchange activity

In order to further investigate the mechanism of regulation of the apical Cl^-/HCO_3^- AE activity by intracellular Ca^{2+}, the role of CaM was tested under basal and cAMP stimulated conditions, because it has been found that binding of the Ca^{2+} /CaM complex enhances cAMP synthesis, via stimulation of membrane adenylyl cyclase activity (Ferguson and Storm, 2004). I therefore preincubated Calu-3 cells with N-(8-aminooctyl)-5-iodonaphthalene-1-sulfonamide (J-8, 50µM), which is a highly specific CaM inhibitor (Tian *et al.*, 2011), for 60 min and then apical Cl^-/HCO_3^- AE activity was measured in Cl^- free solutions (Figure 3.16A). Results showed that CaM inhibition had little effect on apical Cl^-/HCO_3^- AE activity under cAMP stimulated conditions, compared to untreated cells (Figure 3.16B and C). This suggests that CaM is not involved in the regulation of the apical Cl^-/HCO_3^- AE activity, and is also consistent with the results obtained for CaMKK and CaMK inhibition.

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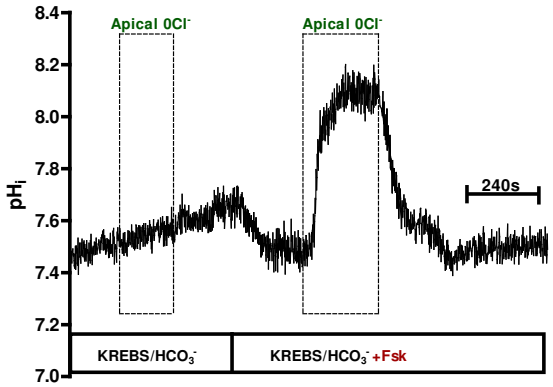


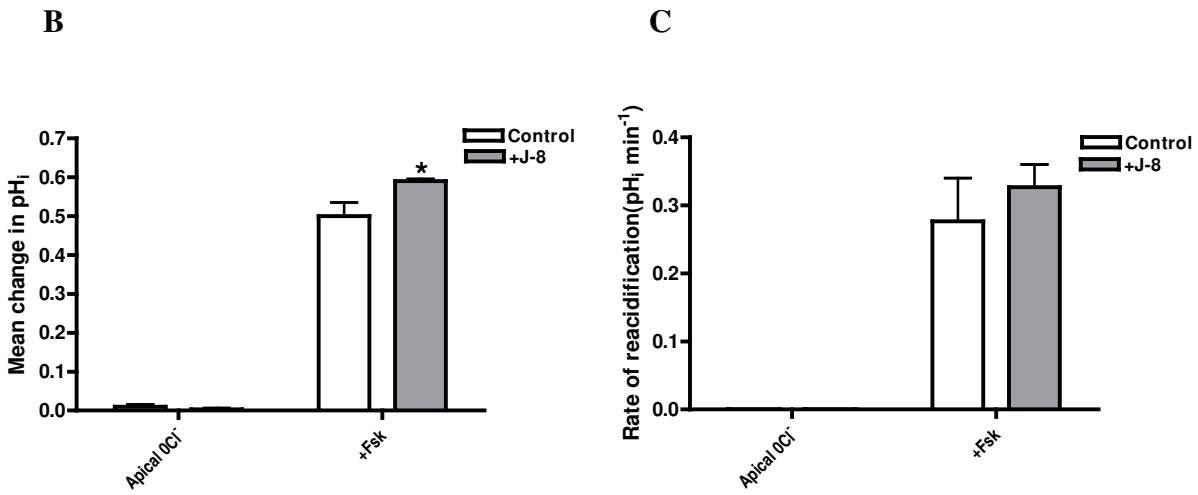


Figure 3. 16: Calmodulin does not regulate the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing the effect of calmodulin inhibition by preincubation with 50µM J-8 for 60 min, on the apical Cl^-/HCO_3^- AE activity under basal and Fsk stimulated conditions. Summary of the effect of J-8 on the mean alkalinisation in pH_i in response to Cl^- free solution (B), and the rate of reacidification upon Cl^- readdition (C) under basal and Fsk stimulated condition in Calu-3 cells. Data are shown as Mean±SEM. *P<0.05 compared to control, n=3. Control experiments run in parallel.

3.6 Regulation of apical Cl^-/HCO_3^- exchange activity by dynamin

Dynamin is a GTPase and plays an essential role in the release of newly formed clathrin-coated vesicles during endocytosis, by causing scission of the neck of budding vesicle at the last stage of vesicle formation (Macia *et al.*, 2006). Dynamin activity is enhanced by elevation of $[Ca^{2+}]_i$ (Liu *et al.*, 1994). It has been reported that CFTR internalization from the cell surface is mediated by clathrin-coated vesicles (Lukacs *et al.*, 1997), and that siRNA knock down, and pharmacological inhibition of dynamin by the cell-permeable inhibitor, dynasore, increased CFTR accumulation on the surface of Hela cells by inhibiting CFTR endocytosis (Young *et al.*, 2009). It has also been found that inhibition of dynamin, by dynasore, significantly increased the steady-state surface level of the apical $Na^+/K^+/2Cl^-$ cotransporter (NKCC2) by reducing

NKCC2 endocytosis in rat renal thick ascending limb epithelial cells (Ares and Ortiz, 2012). However, it was unknown whether dynamin regulates the surface levels the apical Cl^-/HCO_3^- AE in Calu-3 cells. Thus, Calu-3 cells were preincubated with 80μM dynasore on the apical side, and then apical Cl^-/HCO_3^- AE activity measured (Figure 3.17A). Interestingly, in dynamin treated cells, Fsk stimulation in high Cl^- conditions caused a larger acidification in pH_i (0.42±0.04 pH unit for dynasore treated, n=3 *vs*. control of 0.28±0.01 pH unit, n=4, P<0.05) which was also at faster but not significant rate (0.43±0.07 pH_i min^{-1}, n=3 for dynasore treated *vs*. control of 0.23±0.03 pH_i min^{-1} n=4, P<0.05). Moreover, measuring apical Cl^-/HCO_3^- AE activity in Cl^- free solutions showed that dynamin inhibition had no effect on apical Cl^-/HCO_3^- AE activity under resting conditions, but did induce a significant reduction in Fsk-stimulated apical Cl^-/HCO_3^- AE activity. Both the magnitude of alkalinisation produced by apical Cl^- removal, and the rate of reacidification upon Cl^- readdition, were significantly lower compared to the control response (Figure 3.17C and D). In addition, the rate of HCO_3^- flux produced by Fsk stimulation was significantly decreased in dynasore treated Calu-3 cells compared to the control response (Figure 3.17E).

In a separate set of experiments, the effect of dynamin inhibition on transepithelial electrical resistance (TEER) in Calu-3 cells was also determined to see if dynamin affects monolayer integrity. Thus, Calu-3 cells were treated with dynasore and then TEER was measured every 20 min in control and dynamin-treated cells. Figure 3.19 shows that dynamin inhibition did not affect TEER in Calu-3 cells, over the two hour period, suggesting that tight junction integrity in Calu-3 cells was not affected by dynamin inhibition.

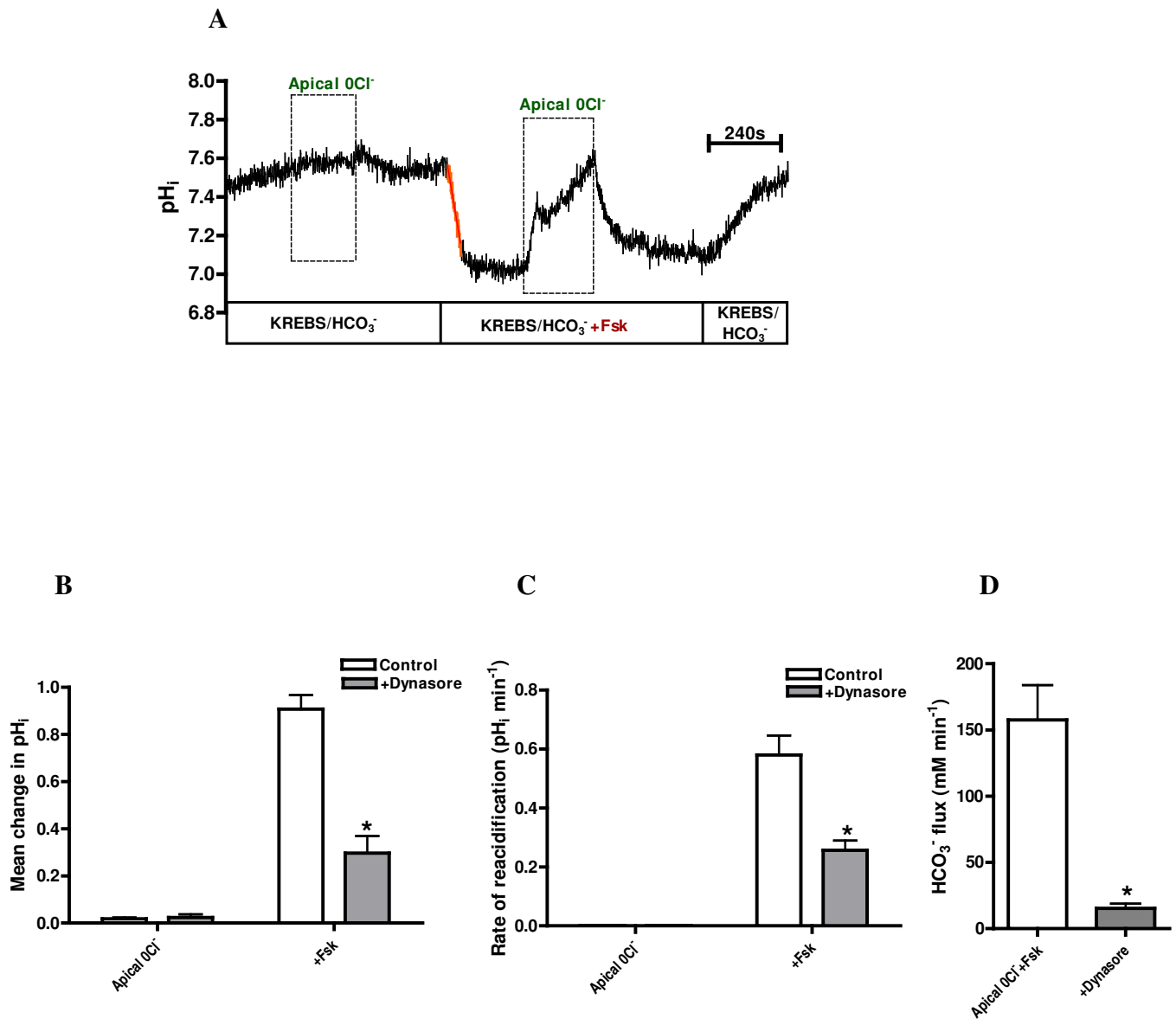


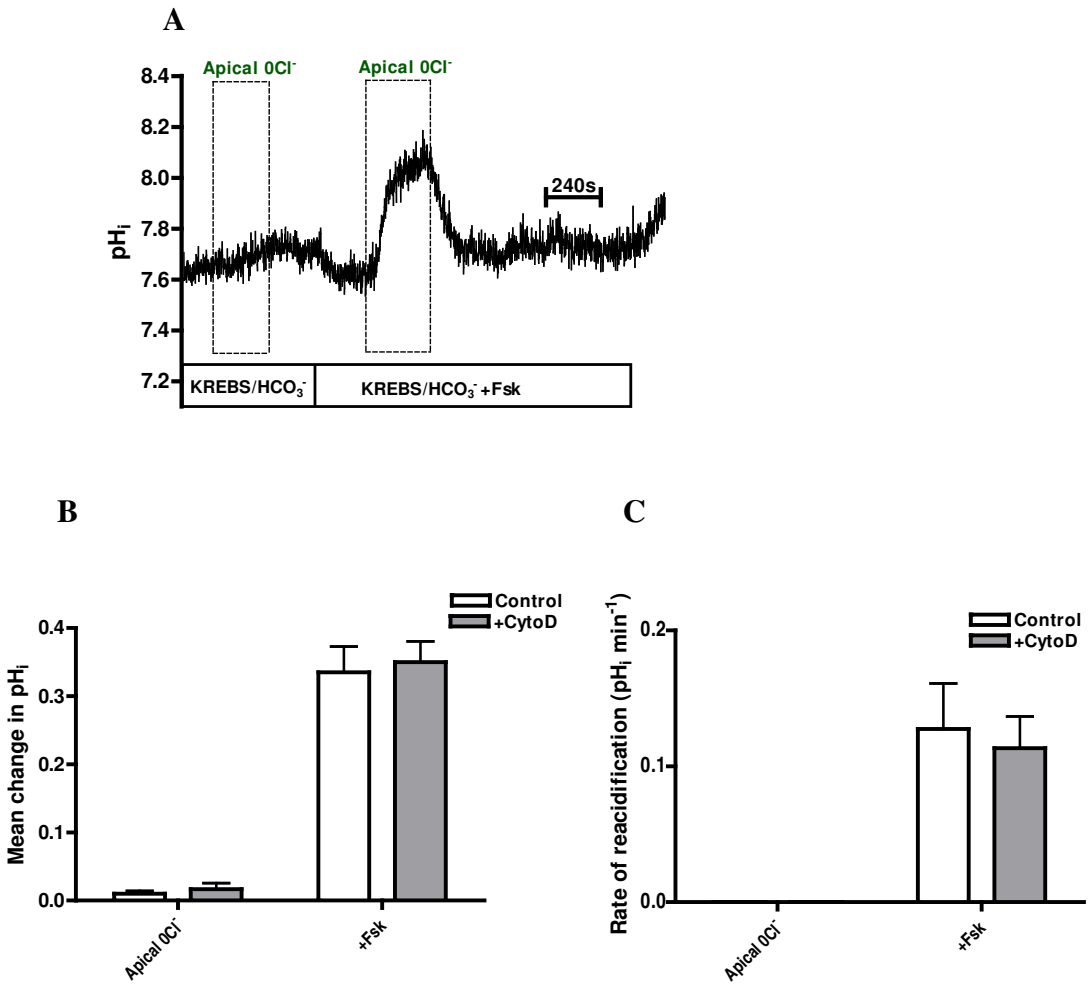
Figure 3. 17: **Dynamin inhibition reduces apical Cl^-/HCO_3^- AE activity in Calu-3.** (A) Representative pH_i trace showing the effect of dynamin disruption on apical Cl^-/HCO_3^- AE activity in dynasore preincubated Calu-3 cells (for 60) min under basal and Fsk stimulated condition. Summary of the effect of dynamin disruption on mean alkalinisation in pH_i in response to apical Cl^- removal (B), and the rate of reacidification upon Cl^- readdition(C), under resting and stimulated condition. (D) Summary of the rate of HCO_3^- flux produced by apical Fsk stimulation compared to control. *P<0.001 compared to control. Data are shown as mean ±SEM, n=4 for control, and n=3 for +Dynasore. Control cells run in parallel.

3.7 Role of actin-cytoskeleton disruption on apical Cl^-/HCO_3^- exchange activity

Since dynamin has an actin binding domain that binds directly to actin filaments which has been shown to play a critical role in the regulation of the actin cytoskeleton in podocytes (Gu *et al.*, 2010), I also assessed the role of the actin cytoskeleton in the regulation of apical Cl^-/HCO_3^- AE activity under basal and cAMP-stimulated conditions in Calu-3 cells. It has been shown that apical CFTR is confined within a macromolecular complex which forms a microdomain to enable efficient cAMP signalling transduction to CFTR (Guggino and Stanton, 2006). There is a physical interaction between CFTR and the protein scaffold NHERF1, which also helps place PKA close to CFTR via the protein kinase A anchoring protein known as AKAP ezrin (Sun *et al.*, 2000), and this complex plays an essential role in the cAMP-induced activation of CFTR (Huang *et al.*, 2001). Recently, the importance of actin-cytoskeleton integrity for cAMP compartmentalization and PKA activity in the regulation of CFTR activity has been shown in human bronchial epithelial cells (Monterisi *et al.*, 2012). They found that pharmacological disruption of the actin cytoskeleton caused a defective accumulation of cAMP in the subcortical compartment, but also a high concentration of cytosolic cAMP, which was accompanied by reduced subcortical PKA activity and CFTR dependent Cl^- efflux. Pharmacologically, the actin cytoskeleton can be disrupted by cytochalasin-D (CytoD) which is a widely used as an inhibitor of actin dynamics. CytoD disrupts the interaction between cofilin, which is a key regulator of actin filament dynamics, with G- and F-actin and causes a reduction in the rate of both actin polymerization and depolymerisation, in intact cells (Shoji *et al.*, 2012). To assess the effect of actin cytoskeleton disruption on the apical Cl^-/HCO_3^- AE activity, Calu-3 cells were preincubated with 10μM cytochalasin D for one hour and then apical Cl^-/HCO_3^- AE was measured in Cl^- free solutions under basal and cAMP stimulated conditions. Figure 3.18 shows that actin disruption had no significant effect on apical Cl^-/HCO_3^- AE activity, under both basal and Fsk stimulated conditions compared to untreated cells (Figure 3.18B and C). I also tested if actin disruption led to changes in G-protein coupled receptor stimulation of apical Cl^-/HCO_3^- AE activity by using ADO. As seen for Fsk, the ability of ADO to activate apical Cl^-/HCO_3^- AE activity was also not

impaired (Fig 3.18. D and E). Taken together these results suggest that the actin cytoskeleton/cAMP compartmentalization is not essential for activation of apical anion exchange activity by cAMP in Calu-3 cells.

To ensure that CytoD treatment had disrupted actin cytoskeleton integrity, confocal images of phalloidin-stained actin cytoskeleton (Figure 3.19A), were made. These images clearly show that CytoD caused a marked disruption of the cytoskeleton, which was more punctate and less organised (Figure 3.19A, Right panel), compared to the untreated cells (Figure 3.19A, left panel). In addition, CytoD caused a significant decrease in TEER in Calu-3 cells within the first 20 mins of treatment that was maintained over a two hour period (Figure 3.19B).



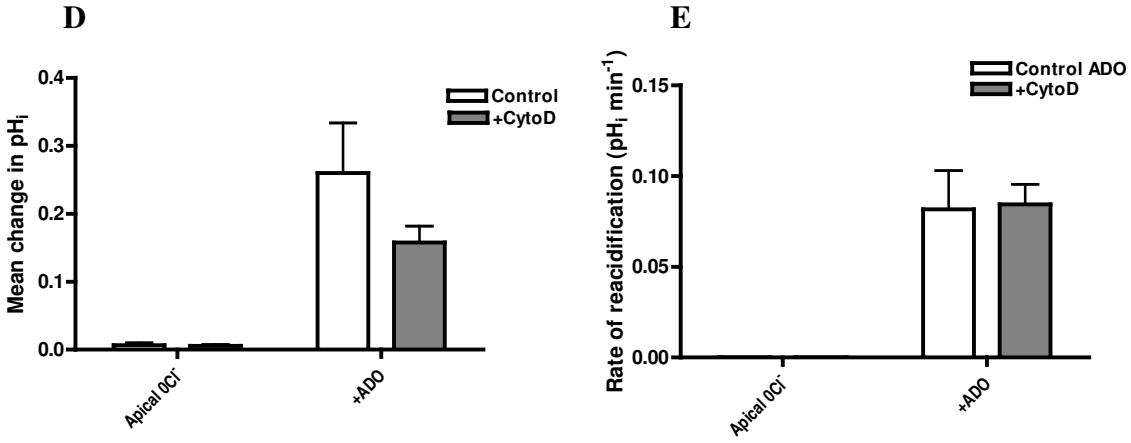


Figure 3. 18: Cytochalasin-D did not affect apical Cl^-/HCO_3^- AE activity in Calu-3. (A) Representative pH_i trace showing the effect of Cl^- removal in the apical perfusate on pH_i under basal and stimulated conditions in cytochalalsin-D preincubated Calu-3 cells. Summary of the impact of actin-cytoskeleton disruption on apical Cl^-/HCO_3^- AE activity by preincubation of Calu-3 cells with cytochalasin-D for 60 min. on mean alkalinisation in pH_i in response to apical Cl^- free solution (B), and the rate of reacidification upon Cl^- readdition (C) under basal and Fsk stimulated condition, n=4 for control, and n=3 for cytochalasin-D preincubated. (D) Mean alkalinisation in pH_i in response to Cl^- free solution, (E) the rate of reacidification under basal and adenosine stimulated condition, n=6 for control, and n=9 for cytochalasin-D preincubated. Data are shown as mean ±SEM, no significant difference (P>0.05) between cytochalasin-D treated and untreated cells.

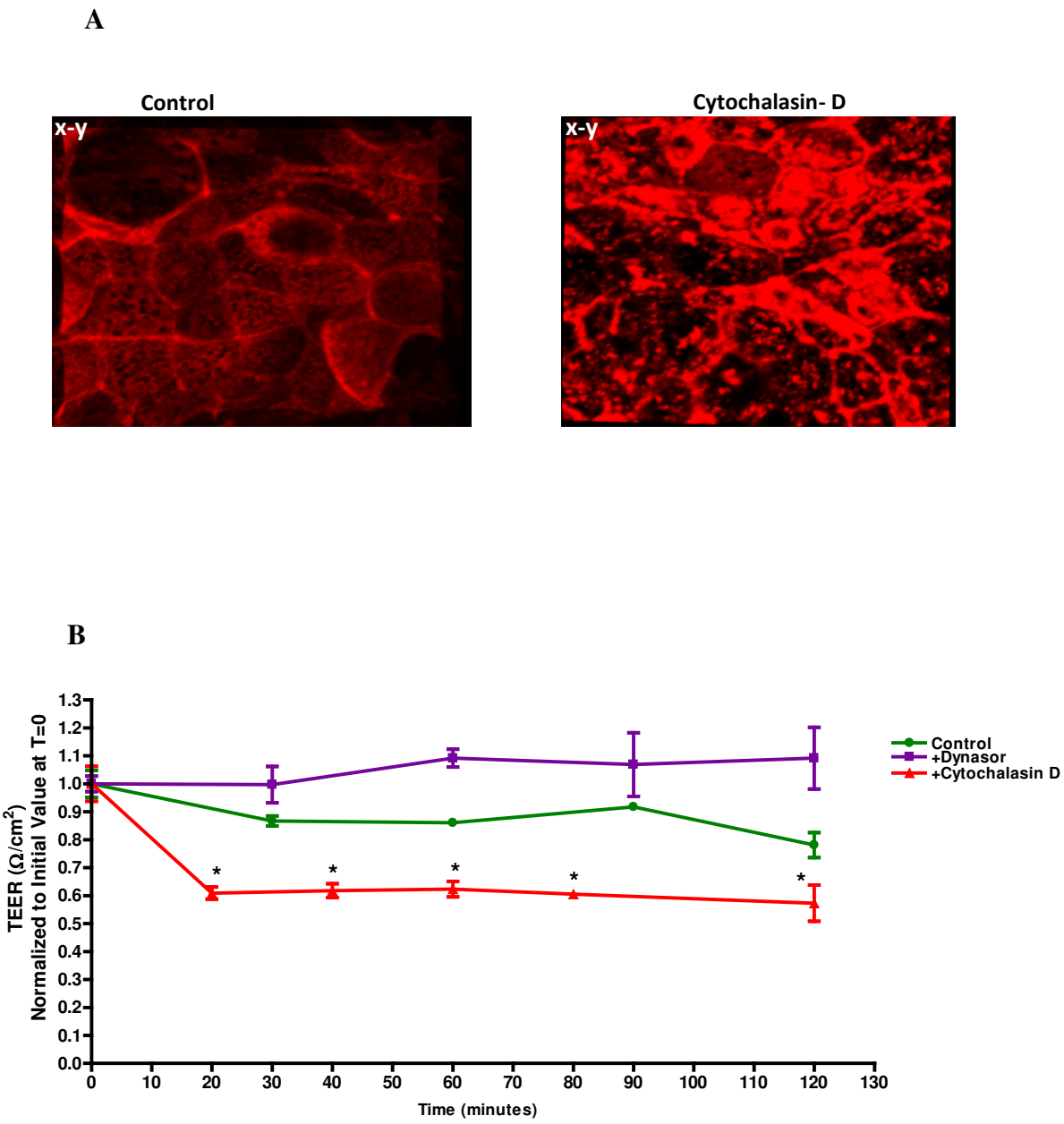


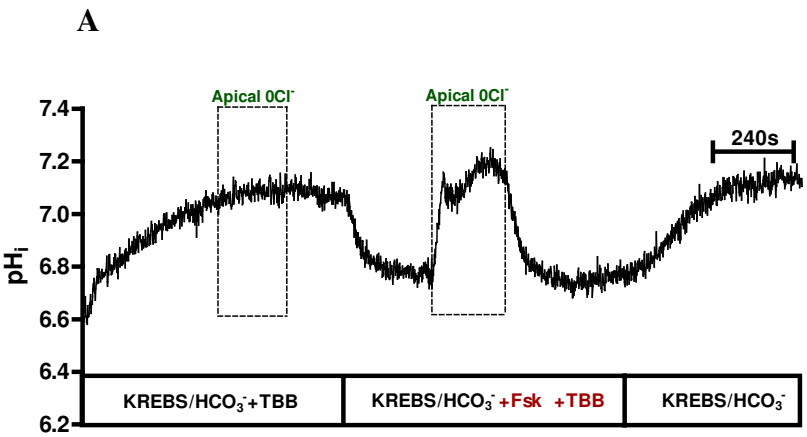
Figure 3. 19: The effect of dynamin inhibition and actin cytoskeleton disruption on transepithelial electrical resistance in control and treated Calu-3 cells. (A) shows Calu-3 cells that were untreated (left panel) compared to cells incubated for one hour with 10µM CytoD (Right panel) and stained with 0.25% Texas-Red Phalloidin to visualize F-actin using confocal microscopy. I performed the actin staining of Calu-3 cells with my colleague Mark Turner. (B) TEER was measured in Calu-3 cells before adding the inhibitors (time 0), and then cells were preincubated for two hours with 10µM cytochalasin D or dynasore in 5% CO_2. TEER measurements were made over the 2 hour period every 20 or 30 mins, to see whether the effect of dynamin or actin skeleton disruption was time dependent. *P<0.05 compared to control and +Dynasore, n=3 for each condition. The actual values for TEER at time 0 (prior to adding inhibitors) were; 563.3±27.2 for control, 580±16 for Dynasore, and 925±57.9 for CytoD treated cells.

3.8 Regulation of apical Cl^-/HCO_3^- exchange activity by CK2

A previous study demonstrated that CK2 (formerly known as casein kinase 2)is a very interesting kinase that plays an essential role in the regulation of CFTR biogenesis, trafficking and activity (Luz, 2008). It has also been shown that CK2 colocalized with CFTR in the apical membrane of airway epithelial cells, and inhibition of CK2 decreased CFTR-dependent Cl^- transport in CFTR over expressing, as well as in native pancreatic duct epithelial cells, which endogenously express CFTR. Moreover, coimmunoprecipitation studies suggested that there is a direct interaction between CK2 and CFTR, but not with F508del CFTR (Treharne *et al.*, 2009). Furthermore, it has been found that application of the CK2 inhibitor TBB (4,5,6,7-tetrabromo-benzotriazole; 10μM) inhibited CFTR activity and significantly reduced the short-circuit current under cAMP stimulation, in both distal colonic and airway epithelial cell monolayers. The onset of the inhibition with TBB occurred at about 1μM which is highly specific for CK2 (Luz *et al.*, 2011). TBB is a specific pharmacological agent and highly selective for CK2 as coexpression of a TBB-insensitive form of CK2 eliminated the ability of TBB to inhibit cAMP/PKA-dependent CFTR activity (Treharne *et al.*, 2009).

To assess the role of CK2 in the regulation of apical Cl^-/HCO_3^- AE activity, Calu-3 cells were preincubated with 10μM TBB for 60 min and then apical Cl^-/HCO_3^- AE activity was measured in Cl^- free solutions, but in the continued presence of TBB (Figure 3.20A). Note that at the start of the experiment the resting pH_i was significantly decreased in TBB preincubated cells (6.7±0.05, n=4, compared to untreated cells, 7.5±0.07, n=4, $P<0.05$), but this did partially recover over the first 5-10 mins of recording (despite the continued presence of TBB) to a new steady state pH_i of (6.88±0.06, n=4). The apical Cl^-/HCO_3^- AE activity was measured when pH_i had partially recovered. Results showed that CK2 inhibition significantly reduced both the mean pH_i change produced by apical Cl^- removal (0.30±0.06, n=4, compared to control of 0.90±0.05, n=4, $P<0.001$), and the rate of reacidification following Cl^- readdition (0.32±0.01, n=4, compared to control of 0.58±0.06, n=4, $P<0.001$), under Fsk stimulated conditions. However, it did not stimulate apical Cl^-/HCO_3^- AE activity under

basal conditions, compared to the control response (Figure 3.20B and C). CK2 inhibition also caused a significant decrease in the rate of HCO_3^- flux produced by apical Cl^- removal under Fsk stimulation (Figure 3.20D). Also, as shown in Figure 3.22A, CK2 inhibition produced a marked decrease in the volume of Fsk-stimulated fluid secretion (229±2.0µl/transwell, n=3, compared to the control response of 245±3.0 µl/transwell, n=3, P<0.05). However, the pH of the secreted fluid was not changed (See figure 3.22B). Therefore, these data imply that CK2 plays an important role in the regulation of apical Cl^-/HCO_3^- AE activity by cAMP, as well as fluid secretion in Calu-3 cells.



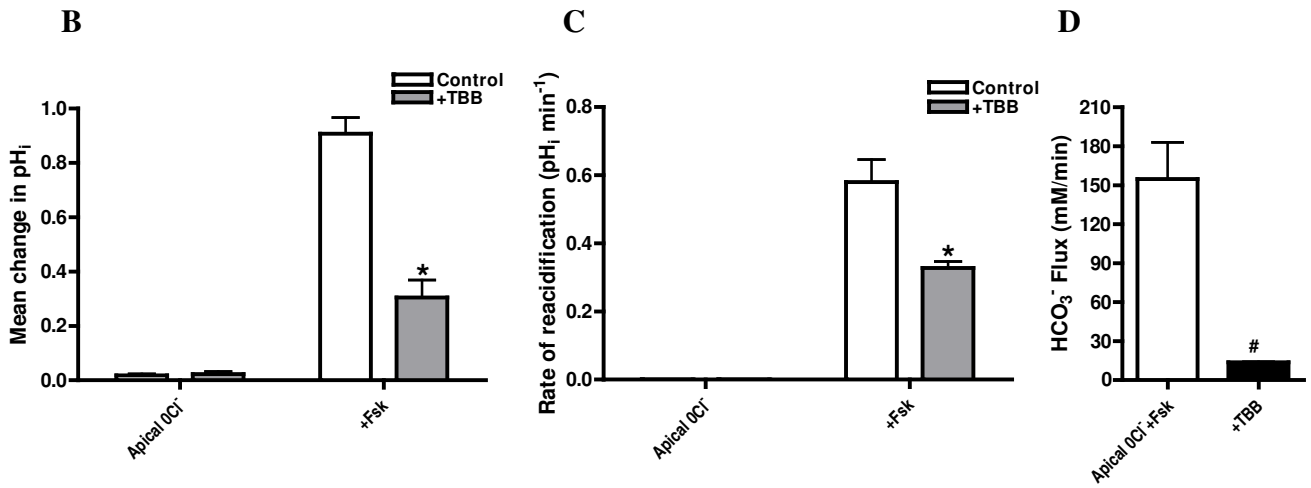


Figure 3. 20: Inhibition of apical Cl^-/HCO_3^- AE activity by TBB in Calu-3 cells. (A) Raw pH_i trace showing the effect of TBB preincubation and perfusion (10μM) on apical Cl^-/HCO_3^- AE activity under basal and Fsk stimulated conditions in Calu-3 cells. Summary of the effect of CK2 inhibition on (B) mean alkalinisation (pH_i) produced by apical Cl^- removal, (C) the rate of reacidification upon Cl^- readdition, (D) the rate of HCO_3^- flux resulting from apical Cl^- removal in the presence of Fsk in control and TBB treated Calu-3 cells. Data are shown as Mean±SEM. *P<0.001, $^{\#}$P<0.05 compared to control, n=4 for each condition. Control experiments were run in parallel.

To further investigate the temporal effects of CK2 inhibition on apical Cl^-/HCO_3^- activity, in a separate series of experiments Calu-3 cells were acutely exposed to 10μM TBB for several mins (both apical and basolateral perfusates) and then AE activity measured (Figure 3.21A). Results showed that in TBB-treated cells, forskolin addition caused a larger intracellular acidification compared to untreated cells (0.53±0.03 compared to 0.25±0.02 pH units, P<0.05). However, TBB had no effect on either the Fsk stimulated apical Cl^-/HCO_3^- AE activity, nor the rate of HCO_3^- flux produced by apical Cl^- removal under Fsk stimulation (Figure 3.21B, C and D). This suggests that TBB needs time to cause inhibition of CK2 and exert its effect on the apical Cl^-/HCO_3^- AE activity in Calu-3 cells.

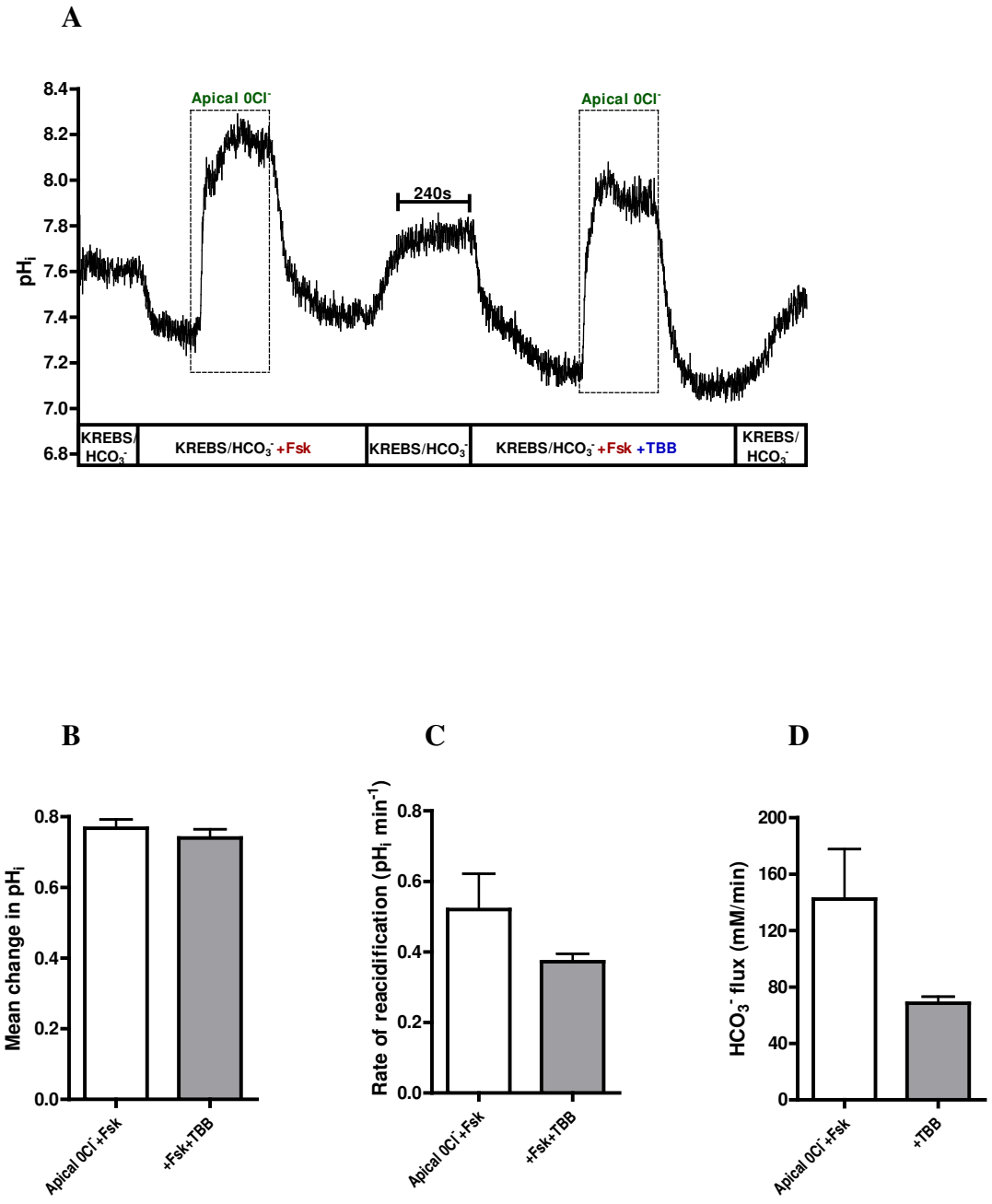


Figure 3. 21: Effect of acute bilateral TBB exposure on the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing the effect of acute bilateral TBB exposure (10μM) on Fsk stimulated apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of acute TBB exposure (10μM) on the mean alkalinisation (pH_i) produced by apical Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C) in control and TBB treated Calu-3 cells. Rate of HCO_3^- flux resulting from forskolin stimulated apical Cl^- removal in control and TBB treated Calu-3 cells (D). No significant difference ($P>0.05$) compared to control response. Data are shown as Mean±SEM. n=4 for each condition, paired observations.

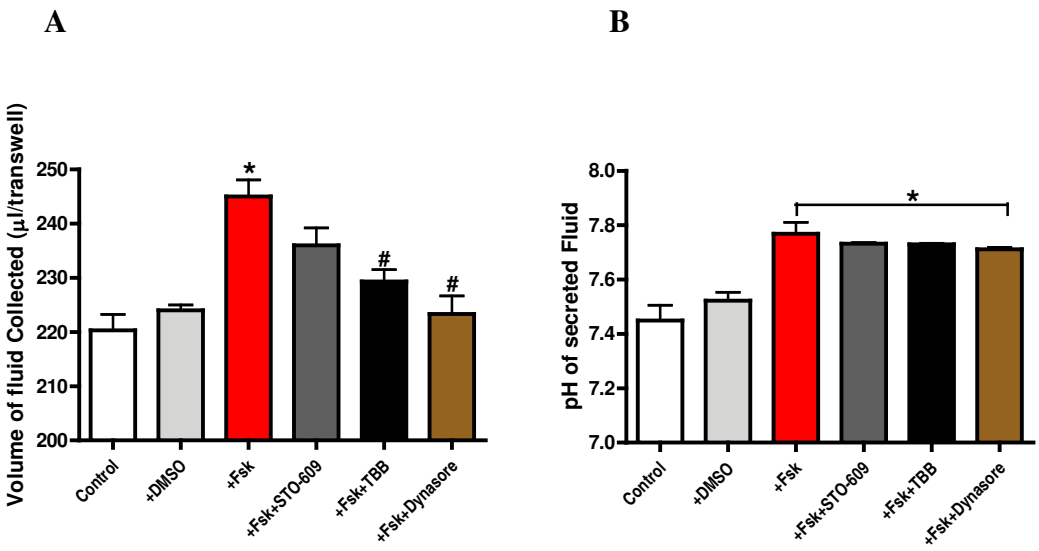


Figure 3. 22: Summary of the effect of STO-609, Dynasore and TBB on rates of Fsk-stimulated fluid secretion and pH in Calu-3 cells. Cells were stimulated with 5µM forskolin and incubated for 24 hours in 5% CO_2 (v/v) in high Cl^- Krebs solution at 37˚C. (A) Shows the effect of Fsk and different inhibitors on the volume of fluid secreted over 24 hours; *= P<0.05 significant effect of forskolin stimulation compared to unstimulated control cells, $^{\#}$=P<0.05 significant effect of forskolin stimulation compared to +Fsk+TBB and Fsk+Dynasore. (B) Shows the pH of Fsk-stimulated secreted fluid compared to unstimulated control cells under different reatments as indicated. Data represents mean ± S.E.M.; n = 3 for each condition.

3.9 Discussion

3.9.1 The effect of cAMP and cGMP in the regulation of apical Cl^-/HCO_3^- AE activity

Using pH_i measurements from polarised cultures of Calu-3 cells my results have shown that in the absence of cAMP stimulation these model human serous airway cells exhibit little Cl^-/HCO_3^- anion exchange activity at the apical membrane. In contrast, stimulation of Calu-3 cells with several different cAMP agonists (ADO, Fsk, IBMX and dbcAMP) reversed this situation, with the cells showing robust anion exchange activity at the apical membrane as evidenced by the magnitude of the alkalinisation following apical Cl^- removal and the subsequent rate of reacidification upon Cl^- readdition. However, stimulation of Calu-3 cells with Fsk caused a significantly larger increase in apical Cl^-/HCO_3^- AE activity, compared to ADO stimulated cells. One potential explanation for this difference could be that ADO might produce a smaller increase in $[cAMP]_i$ compared to Fsk, which is consistent with studies by Huang *et al.* (2001) who demonstrated that stimulation of Calu-3 cells with adenosine produced very little increase in $[cAMP]_i$, compared to forskolin (Huang *et al.*, 2001).

Although a previous study by Illek *et al*., (1997) concluded that CFTR functions as a Cl^- and HCO_3^- channel (Illek *et al.*, 1997), which was supported by studies performed on Calu-3 cells that showed CFTR to possess both an apical Cl^- and HCO_3^- conductance (Shan *et al.*, 2012), my studies are also consistent with the most recent work obtained by Garnett *et al*., 2011 and 2013 (Garnett *et al.*, 2011; Garnett *et al.*, 2013) that apical Cl^-/HCO_3^- AE directly regulates HCO_3^- transport in Calu-3 cells. In the Garnett study the apical Cl^-/HCO_3^- AE was identified as SLC26A4, also known as pendrin, a member of the SLC26A transporter family (Mount and Romero, 2004). The cAMP-induced activation of apical Cl^-/HCO_3^- AE activity that I have described was markedly inhibited by two different PKA inhibitors, H-89 and RpcAMP, as well as by the Epac inhibitor ESI-09, which clearly indicates that stimulation of apical Cl^-/HCO_3^- AE activity is through cAMP/PKA/Epac-dependent mechanisms. Furthermore, Epac inhibition enhanced apical Cl^-/HCO_3^- AE activity under resting conditions, which was potentially CFTR dependent. Either way these results suggest that

Epac plays two distinct roles in Calu-3 cells. Under resting conditions, where cAMP levels are likely to be low, Epac appears to inhibit apical Cl^-/HCO_3^- AE activity. In contrast, when cells are strongly stimulated by Fsk, Epac appears to be required to maintain maximal Cl^-/HCO_3^- AE activity. Exactly how Epac is able to play these distinct roles requires further investigation, but it is possible that under resting conditions Epac could help keep cAMP levels sufficiently low to prevent PKA activation, by acting as a cAMP 'buffer'. In contrast to cAMP, although it has been previously shown in some cells that there is a synergistic interaction between the two intracellular second messengers, cAMP and cGMP, (de Wit *et al.*, 1994), my results showed that cGMP is not involved in the regulation of the apical Cl^-/HCO_3^- AE activity in Calu-3 cells, under both basal and cAMP stimulated conditions.

Furthermore, cAMP stimulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells was significantly reduced by CFTR inhibition, using the CFTR pore blocker GlyH-101, which produced a very fast intracellular acidification, suggesting that anion transport by CFTR modulated the apical Cl^-/HCO_3^- AE activity. However, the GlyH-101 induced acidification was prevented by addition of basolateral DIDS, in a dose-dependent manner, with 100µM DIDS completely blocking the acidification. This result suggests that a DIDS-sensitive basolateral transporter appears to regulate apical Cl^-/HCO_3^- AE activity in Calu-3 cells. This is consistent with the previous finding that 500µM H_2DIDS abolished the CFTR inhibitor-induced acidification under $0Cl^-$ conditions (J Garnett PhD thesis, 2010). My experiments have not uncovered the exact identity of the basolateral HCO_3^- transporter so future experiments will be required to do this. However, because I have shown that cAMP stimulation inhibits the basolateral anion exchanger in Calu-3 cells, it is unlikely that this protein is involved, although at this stage it cannot be ruled out. Another possibility is that the basolateral NBC could be involved as GlyH-101 prevents anion efflux through CFTR and this could therefore lead to a hyperpolarisation of the membrane potential, which could be large enough to reverse the electrogenic basolateral NBC, thereby causing HCO_3^- efflux rather than entry, and so $[HCO_3^-]_i$ decreases. However, previous results showed that GlyH-101 induced acidification was independent of membrane potential (J Garnett PhD thesis, 2010).

3.9.2 Intracellular Ca^{2+} signaling plays a significant role in the regulation of apical Cl^-/HCO_3^- AE activity

The mechanism by which cAMP induced activation of apical Cl^-/HCO_3^- AE was assessed by changing the concentration of intracellular and extracellular Ca^{2+}, because it has been shown that there is a synergistic interaction between cAMP and Ca^{2+} in stimulation of HCO_3^- secretion in Calu-3 cells (Krouse *et al.*, 2004). Consistent with this, my results showed that decreasing intracellular Ca^{2+} concentration, by BAPTA-AM, significantly reduced the cAMP-induced intracellular alkalinisation and rate of reacidification produced by apical Cl^- removal. These data imply that normal (resting) concentration of $[Ca^{2+}]_i$ is required for cAMP to stimulate apical Cl^-/HCO_3^- AE activity in Calu-3 cells. However, an increase in $[Ca^{2+}]_i$ concentration, using thapsigargin, significantly reduced the cAMP stimulated apical Cl^-/HCO_3^- AE activity which was not blocked by treating the cells with BAPTA-AM; instead this caused a further significant reduction in apical Cl^-/HCO_3^- AE activity. This indicates that the effect of an increase $[Ca^{2+}]_i$ is independent of BAPTA-AM; which potentially could be through a PKA-dependent mechanism because it has been shown that an increase in $[Ca^{2+}]_i$ enhances calcineurin, a serine-threonine protein phosphatase, which inhibits PKA activity via dephosphorylation of PKA effectors (Santana *et al.*, 2002) or even direct inhibition of PKA itself (Orie *et al.*, 2009). Thus, even in the presence of BAPTA-AM, thapsigargin might produce some increase in $[Ca^{2+}]_i$ to affect the PKA-dependent apical Cl^-/HCO_3^- AE activity. Consistent with this, my colleague, Waseema Patel, has measured intracellular Ca^{2+} in HEK293T cells (using the Ca^{2+} sensitive dye, Fura2-AM), in the presence of BAPTA-AM and thapsigargin, and her results showed that there is still a small increase in $[Ca^{2+}]_i$. On the other hand, removing extracellular Ca^{2+} did not affect the cAMP-induced activation of apical Cl^-/HCO_3^- AE activity. These data collectively suggest that BAPTA-AM might disrupt the synergistic cross-talk between cAMP and Ca^{2+} to maintain apical Cl^-/HCO_3^- AE activity under cAMP stimulation, while an increase in $[Ca^{2+}]_i$ exerts its effect via PKA inhibition, independently of intracellular Ca^{2+} chelation by BAPTA-AM. Also extracellular Ca^{2+} is not involved in intracellular cAMP signalling cascades in the regulation of Fsk stimulated apical Cl^-/HCO_3^- AE activity. Future experiment would be of interest to measure the PKA activity in

the presence of BAPTA-AM and thapsigargin to establish whether a decrease or an increase in $[Ca^{2+}]_i$ affects PKA activity in forskolin-stimulated Calu-3 cells, which could involve performing ELISA assays.

Based on the fact that cAMP stimulated apical Cl^-/HCO_3^- AE activity needs normal (resting) $[Ca^{2+}]_i$ to maintain the apical Cl^-/HCO_3^- AE activity, I investigated further the relationship between cAMP and Ca^{2+}/calmodulin dependent protein kinase (CaMK) in the regulation of cAMP stimulated apical Cl^-/HCO_3^- AE activity, because extensive cross-talk has been found between CaMK and some cAMP-dependent protein kinases, that could be involved in the regulation of signalling cascades inside the cells (Soderling, 1999). Results showed that preincubation of cells with the CaMKK inhibitor STO-609 (which inhibits CaMKI and CaMKIV), did not stimulate basal AE activity, but did produce a significant reduction in intracellular alkalinisation upon apical Cl^- removal, following Fsk stimulation, in STO-609 treated cells, but did not affect the rate of reacidification compared to control cells. A possible mechanism for this might be that inhibition of CaMK interferes with the ability of AC1 to generate cAMP, in a Ca^{2+}-dependent manner, as AC1, which colocalizes with CFTR, is a Ca^{2+}/calmodulin stimulated enzyme in the apical membrane of human bronchial epithelial cells (Namkung *et al.*, 2010). However, CaMKII inhibition had no effect on the apical Cl^-/HCO_3^- AE activity, suggesting that this Ca^{2+}/calmodulin dependent kinase is not involved in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Furthermore, in order to obtain an insight into the role of CaM in the regulation of apical Cl^-/HCO_3^- AE activity, Calu-3 cells were treated with a CaM inhibitor and results revealed that CaM did not contribute to the regulation of apical Cl^-/HCO_3^- AE activity under basal and cAMP stimulated condition. This is consistent with the lack of a role for CaMKK and CaMK in the regulation of the apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Also, another Ca^{2+}-dependent protein kinase, PKC, has also been shown not to be involved in the regulation of the apical Cl^-/HCO_3^- AE activity under basal and stimulated conditions (J Garnett, PhD thesis, 2010).

3.9.3 The effect of dynamin on apical Cl^-/HCO_3^- AE activity and fluid secretion

There is a strong relationship between intracellular Ca^{2+} and dynamin as it has been shown that relocation of dynamin from the cytoplasm to the plasma membrane is enhanced by an elevation of intracellular Ca^{2+} (Liu *et al.*, 1994) which could be via direct binding of Ca^{2+} to dynamin, or indirectly, by dephosphorylation of dynamin through its binding with a Ca^{2+}-sensitive phosphatase, known as calcineurin (Bauerfeind *et al.*, 1997; Hens *et al.*, 1998; Marks and McMahon, 1998). Dynamin also plays an important role in the formation of a transport vesicle derived from the trans Golgi network, which travels to the plasma membrane (Nabi and Le, 2003; Abazeed *et al.*, 2005; Cao *et al.*, 2005). It has also been shown that dynamin plays a key role in the expansion of the fusion pore within milliseconds of granule fusion, and also regulates the topological fate of the released granules after fusion and spreading of membrane proteins into the plasma membrane (Anantharam *et al.*, 2011). Thus, I also investigated the role of dynamin in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells since increasing $[Ca^{2+}]_i$ markedly reduced the cAMP stimulated apical Cl^-/HCO_3^- AE activity, which could be potentially by enhancing dynamin mediated endocytosis of the AE.

Dynamin was inhibited via treating cells with dynasore, and this caused a significant decrease in cAMP stimulated apical Cl^-/HCO_3^- AE activity, but had no effect on the apical Cl^-/HCO_3^- AE activity under basal conditions. However, dynamin inhibition did not affect the pH of secreted fluid, but it did reduce the amount of fluid secreted over a 24 h period, compared to control responses. One possible explanation for these apparent discrepant results could be that dynamin inhibition may have reduced surface levels of CFTR, which would decrease Cl^- efflux, and thereby reduce the driving force for fluid secretion by Calu-3 cells. However, the residual CFTR activity may have been enough to maintain sufficient apical Cl^-/HCO_3^- AE activity to ensure that the pH of secreted fluid was alkalinised over a 24 h period. This would be consistent with previous results from our group which showed that both CFTR KD, or treatment of cells with CFTR blockers, also reduced the amount of fluid secreted over 24 h but not the final pH of the secreted fluid (Garnett *et al.*, 2011). Another possible explanation could be that apical AE activity in dynamin treated cells for 24

hours may not reflect the acute exposure of dynamin in pH_i experiments. Thus, it would be worth to measure apical AE activity in dynasore treated Calu-3 cells following 24 hour preincubation, under both resting and Fsk-stimulated conditions. However, how dynamin inhibition could reduce levels of CFTR is not clear and needs further investigation. Note that dynamin inhibition could also reduce the expression level of the apical Cl^-/HCO_3^- AE and subsequently reduce the Cl^-/HCO_3^- exchange activity. However, previous work from our lab (Garnett *et al.*, 2011), showed that while pendrin KD in Calu-3 cells caused a reduction in apical anion exchange activity, it had modest effects on fluid secretion, but markedly reduced the pH of the secreted fluid. Thus the results from dynamin inhibition do not match those from pendrin KD cells. Overall, my results imply that dynamin plays a role in the regulation of apical Cl^-/HCO_3^- AE activity, and this may be via a change in surface levels of CFTR in Calu-3 cells.

3.9.4 Role of the actin-cytoskeleton in the regulation of apical Cl^-/HCO_3^- AE activity

I also have assessed the dependency of apical Cl^-/HCO_3^- AE activity on an intact cytoskeleton and cAMP compartmentalization. The pharmacological agent, CytoD, was used to disrupt F-actin polymerization and thus actin cytoskeleton organization, which was appeared much more punctuate and disorganised by phalloidin staining. Treatment was found to significantly reduce TEER in Calu-3 cells suggesting that CytoD affected the organization of the actin cytoskeleton to modulate tight junction properties. However, CytoD did not affect either the basal or the cAMP stimulated apical Cl^-/HCO_3^- AE activity under Fsk and ADO stimulated conditions. Given that forskolin or ADO still provoked apical Cl^-/HCO_3^- AE activity suggested that cAMP compartmentalization wasn't necessary in order to produce this response. This is somewhat surprising because Monterisi *et al.* (2012) has demonstrated that actin-cytoskeleton disruption in human bronchial epithelial cells (HBE) significantly decreased cAMP-stimulated CFTR activity. This potentially suggests that (i) there is an important difference between Calu-3 and HBE cells in actin cytoskeleton organization or (ii) forskolin or ADO were still able to increase $[cAMP]_i$ to some extent, that

even a reduction in a specific cAMP microdomain, was not sufficient to reduce the activity of cAMP-stimulated apical Cl^-/HCO_3^- AE in Calu-3 cells. Further investigations into the effects of cytoskeleton disruption on cAMP compartmentalization and $[cAMP]_i$ at specific microdomains in cAMP stimulated Calu-3 cells are required, which could involve performing FRET analysis with cAMP or PKA sensors, which is an accurate method for the analysis and interpretation of changes in $[cAMP]_i$ detected at different subcellular regions (Salonikidis *et al.*, 2008).

3.9.5 CK2 exhibits a significant role in regulating apical Cl^-/HCO_3^- AE activity

To further understand the mechanism of apical Cl^-/HCO_3^- AE activation by cAMP signalling, Calu-3 cells were treated with two different CK2 inhibitors (TBB or CX4945), because CK2 inhibition has been shown to reduce CFTR channel activity and reduce the Cl^- conductance of airway cells. CK2 co-immunoprecipitated and colocalized with WT-CFTR in the apical membrane of human airway epithelial cells (Treharne *et al.*, 2009). Here, researchers also demonstrated that CK2 is absent in the apical membrane of CF nasal epithelia which makes CFTR unable to traffic to the apical membrane. It has also been shown that CK2 inhibition blocked the cAMP-dependent PKA activation of CFTR (Mehta, 2008). My results demonstrated for the first time that CK2 inhibition caused a significant decrease in cAMP stimulated apical Cl^-/HCO_3^- AE activity in Calu-3 cells, but it did not affect the basal AE activity. Also, CK2 inhibition caused a significant reduction in the cAMP stimulated HCO_3^- flux by the apical Cl^-/HCO_3^- exchanger. This is consistent with the previous finding that CK2 can modulate cAMP-dependent HCO_3^- secretion in pancreatic duct epithelial cells (Treharne *et al.*, 2009). My findings also provide further support for an interaction between CK2 and CFTR, as CK2 inhibition would potentially reduce CFTR anion transport function, and subsequently reduce cAMP-stimulated apical Cl^-/HCO_3^- AE activity. This is consistent with previous finding from our laboratory, which showed that knocking down CFTR in Calu-3 cells decreased the rate of cAMP-stimulated apical Cl^-/HCO_3^- AE activity (Garnett *et al.*, 2011). However, I found that CK2 inhibition produced a significant decrease

in the amount of secreted fluid collected after 24 h preincubation of Calu-3 cells with TBB, but it did not change the pH of secreted fluid. This paradoxical effect of CK2 inhibition could be explained by a similar mechanism described for dynamin inhibition, namely a selective reduction in CFTR expression/activity (see section 3.83). Further investigation could involve testing the effect of CK2 inhibition on the apical Cl^-/HCO_3^- AE activity in CFTR-KD Calu-3 cells.

The exact mechanisms involved in the regulation of apical Cl^-/HCO_3^- AE activity by CK2 under cAMP stimulated condition are not clear. However, CK2 is known to be the main, if not the only, protein kinase that can phosphorylate CaM in living cells (Arrigoni *et al.*, 2004). Since I have shown that CaM is not involved in the regulation of apical Cl^-/HCO_3^- AE activity, this suggests that CK2 exerts its effect on the apical Cl^-/HCO_3^- AE activity through a CaM-independent mechanism, possibly via direct phosphorylation of CFTR and/or the apical AE itself.

The main findings of this chapter are summarized below (see Figure 3.23):

- cAMP agonists stimulate CFTR dependent apical Cl^-/HCO_3^- AE activity in Calu-3 cells through PKA and Epac-dependent mechanisms.

- cGMP is not involved in the regulation of the apical Cl^-/HCO_3^- AE activity in Calu-3 cells.

- Epac inhibition stimulates CFTR-dependent apical Cl^-/HCO_3^- AE activity under basal conditions.

- A decrease in $[Ca^{2+}]_i$ significantly attenuated cAMP stimulated apical Cl^-/HCO_3^- AE activity in Calu-3 cells potentially via inhibiting the synergistic interaction between Ca^{2+} and cAMP.

- cAMP stimulated apical Cl^-/HCO_3^- AE activity was significantly reduced by intracellular elevation of Ca^{2+}, perhaps through PKA inhibition.

  CaMKI, CaMKII and CaMKIV were not significantly involved in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells.
- Dynamin inhibition caused a significant reduction in cAMP stimulated apical Cl^-/HCO_3^- AE activity, as well as the amount, but not the pH, of the secreted fluid.

- An intact actin-cytoskeleton was not required for cAMP-induced apical Cl^-/HCO_3^- AE activity in Calu-3 cells.

- CK2 plays an essential role in the regulation of cAMP stimulated apical Cl^-/HCO_3^- AE activity through a CaM-independent mechanism in Calu-3 cells.

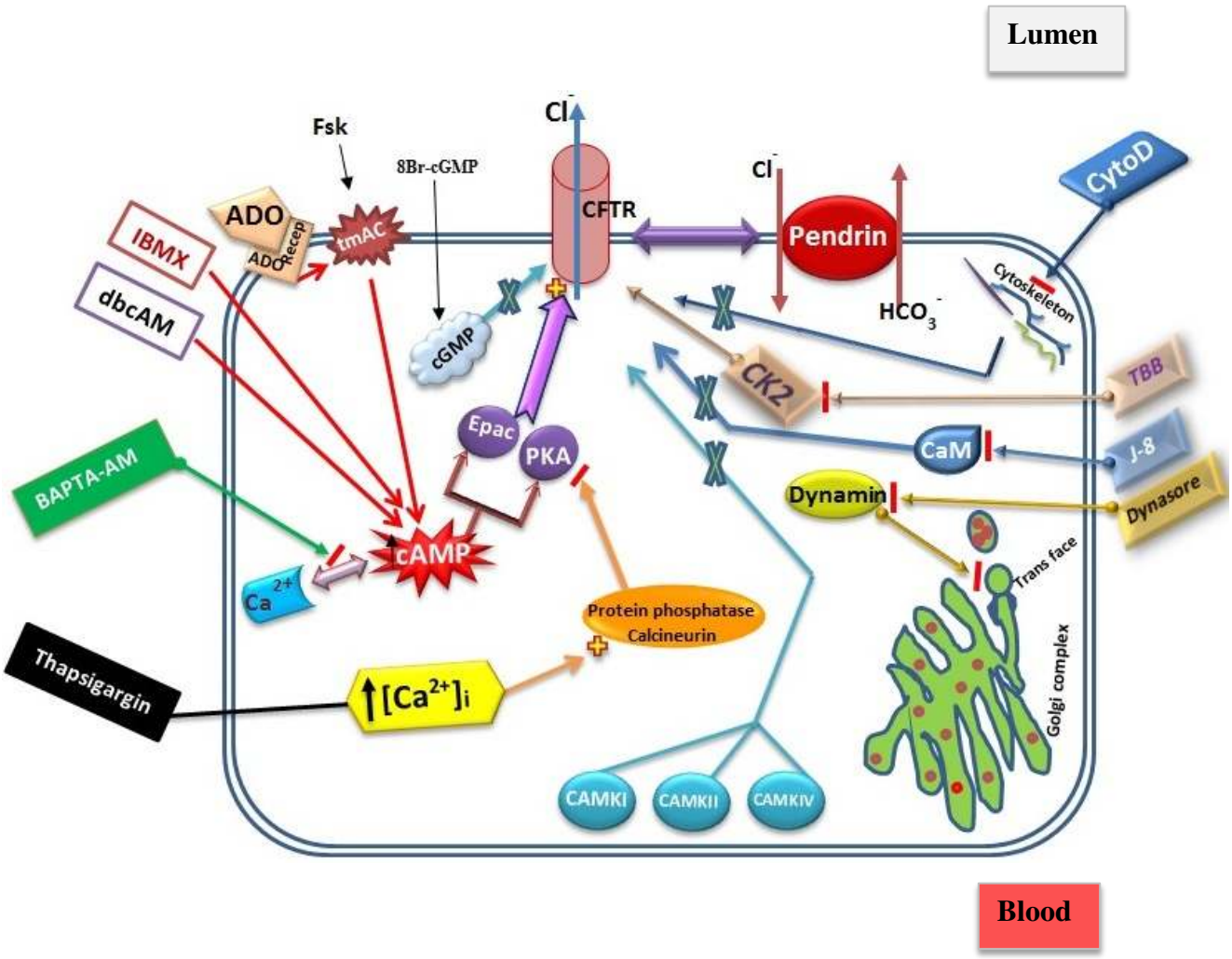


Figure 3. 23: Schematic illustration of intracellular signaling pathways that regulate CFTR and apical Cl^-/HCO_3^- AE activity in Calu-3 cells. Adenylyl cyclase (tmAC) activators (forskolin or adenosine), and the membrane permeable cAMP analogue ,dibutyryl cAMP, or the phosphodiesterase inhibitor, IBMX, cause a rise in cAMP, which is a key event in PKA and Epac-dependent stimulation of CFTR and apical Cl^-/HCO_3^- AE activity. Chelation of intracellular Ca^{2+} by BAPTA-AM, or elevation of $[Ca^{2+}]_i$ by thapsigargin, reduced cAMP stimulated CFTR and pendrin activity, through a different mechanism. CK2 and dynamin play a critical role in the regulation of cAMP stimulated CFTR and pendrin activity. (-) denotes inhibition, (+) denotes stimulation, and (X) denotes not contribute.

Chapter 4 Basolateral Cl^-/HCO_3^- anion exchange activity in Calu-3 cells

4.1 Introduction

A previous immunofluorescence study (Loffing *et al.*, 2000), and recent results by Garnett *et al*, have demonstrated that there is a functional Cl^-/HCO_3^- anion exchanger at the basolateral membrane of Calu-3 cells which is active under resting conditions (Garnett *et al.*, 2011; Garnett *et al.*, 2013) and which was originally proposed to be a housekeeping Cl^-/HCO_3^- exchanger (Alper *et al.*, 1999). This transporter is most likely AE2 or SLC4A2, which is one of the eight members of the SLC4 family of solute transporters that encode proteins that are involved in transmembrane HCO_3^- transport (Romero *et al.*, 2004). Immunohistochemical analysis has shown that AE2 is present in airway epithelial cells with a similar level of mRNA expression from the trachea down to the small bronchi (Al-Bazzaz *et al.*, 2001). The basolateral Cl^-/HCO_3^- AE is thought to play an important role in the regulation of intracellular pH (pH_i) by participating in base efflux, and it can also work in concert with the sodium-potassium chloride cotransporter, NKCC1, to facilitate the inward movement, and accumulation, of Cl^- across the basolateral membrane (Loffing *et al.*, 2000; Inglis *et al.*, 2002). In most epithelial cells the basolateral Cl^-/HCO_3^- AE plays a critical role in the regulation of intracellular pH during intracellular alkalinisation, as well as in cell volume control, by regulating the Cl^- concentration inside the cells (Stewart *et al.*, 2002). However, it has recently been proposed that the basolateral Cl^-/HCO_3^- AE also plays an essential role in cAMP-stimulated transepithelial Cl^- secretion in Calu-3 cells, through coupling of its transport activity with that of the electrogenic sodium-bicarbonate cotransporter (NBC). In this novel 'tertiary' active process, Cl^- is effectively accumulated within Calu-3 cells through the combined activity of the two basolateral transporters (driven by the inward directed Na^+ gradient maintained by the Na^+-K^+ ATPase), with HCO_3^- cycling across the basolateral membrane (Huang *et al.*, 2012; Shan *et al.*, 2012). The study by Shan *et al*, 2012 also demonstrated that Fsk stimulation of Calu-3 cells led to an increase in

basolateral Cl^- loading and HCO_3^- influx by stimulation of the basolateral Cl^-/HCO_3^- AE and NBC activity, respectively, thereby enhancing fluid secretion across the apical membrane (Shan *et al.*, 2012). Furthermore, a recent pH_i study by Kim *et al*., (2014) revealed that although the basolateral Cl^-/HCO_3^- AE appeared to be almost completely inhibited in Fsk stimulated Calu-3 cells, it could in fact be shown to be active (unmasked) if CFTR was inhibited (in the presence of Fsk), suggesting that cAMP stimulation was not inhibiting the basolateral anion exchanger, it was simply being 'masked' by the more dominant apical anion exchanger activity (Kim *et al.*, 2014). This result is consistent with our own studies where it was found that knocking down CFTR expression, or blocking its activity with GlyH-101, led to incomplete inhibition of the basolateral Cl^-/HCO_3^- AE by cAMP in Calu-3 cells (Garnett *et al.*, 2013). However, we also found that the basolateral anion exchanger was still inhibited in cells treated with both Fsk as well as the PKA inhibitor, H-89, which reduced apical anion exchanger activity by ~ 85%. This latter result is not compatible with the observations of Kim *et al*., (2014), and furthermore suggested that an increase in intracellular cAMP inhibited basolateral Cl^-/HCO_3^- AE activity through a novel PKA-independent mechanism (Garnett *et al.*, 2013). The reason for these differing results/interpretations is not resolved.

However, since human airway serous cells from SMGs play an important role in regulating the magnitude and pH of the ASL that lines the surface of the conducting airways (Ballard and Inglis, 2004; Tarran *et al.*, 2006; Garnett *et al.*, 2011), which is essential for the innate defence mechanisms of the lung to function adequately (Pezzulo *et al.*, 2012b) and inhibition of the basolateral Cl^-/HCO_3^- anion exchanger could theoretically provide additional HCO_3^- to be transported by the apical AE in Calu-3 cells, the molecular mechanism that regulates the basolateral Cl^-/HCO_3^- AE under resting, as well as cAMP stimulated conditions, is an important area of research. In this chapter I have characterised in more detail the cellular signalling mechanisms that regulate the basolateral anion exchanger, and provide additional support that a rise in cytosolic cAMP inhibits AE2 activity, through a PKA-independent mechanism.

4.2 Cl^- and HCO_3^- dependence of the basolateral Cl^-/HCO_3^- anion exchanger

As shown in Figure 4.1, and previously reported by Garnett *et al*., (2011); Garnett *et al*., (2013), Calu-3 cells exhibit a basolateral Cl^-/HCO_3^- AE activity under resting situations. In HCO_3^-/KREBS condition, basolateral Cl^- removal produced an alkalinisation in pH_i of 0.36±0.02 pH units (n=6). It was noticeable that the alkalinisation caused by basolateral Cl^- removal was monophasic, with a fast initial increase in pH_i to a new plateau level (Figure 4.1A), in contrast to the alkalinisation produced by apical Cl^- removal under cAMP-stimulated conditions, which was biphasic (see Chapter 3). To investigate the anion transport properties of this basolateral anion exchanger in more detail, the Cl^- and HCO_3^--dependence was assessed by removing Cl^- and HCO_3^- from the perfusate and replacing with HEPES buffer, under resting conditions. Acute exposure of the basolateral membrane to a HCO_3^- free (but Cl^- rich) HEPES buffered solution failed to produce any intracellular alkalinisation, but instead produced a large, and significant, intracellular acidification of 0.40±0.01 pH units (n=3, $P<0.001$; Figure 4.1A & B), which may be due to HCO_3^- efflux via the basolateral Cl^-/HCO_3^- AE activity in exchange with extracellular Cl^- or more likely due to inhibition of the NBC. This suggests that the basolateral Cl^-/HCO_3^- AE cannot transport significant amounts of OH^- anions. Addition of a HCO_3^- and Cl^- free HEPES solution to the basolateral compartment did not produce any change in pH_i, which was significantly different to the control response and the response to HCO_3^--free, Cl^--rich HEPES conditions (n=6, $P<0.001$; Figure 4.1), suggesting that Cl^--dependent HCO_3^- transportation was responsible for the obvious intracellular pH changes.

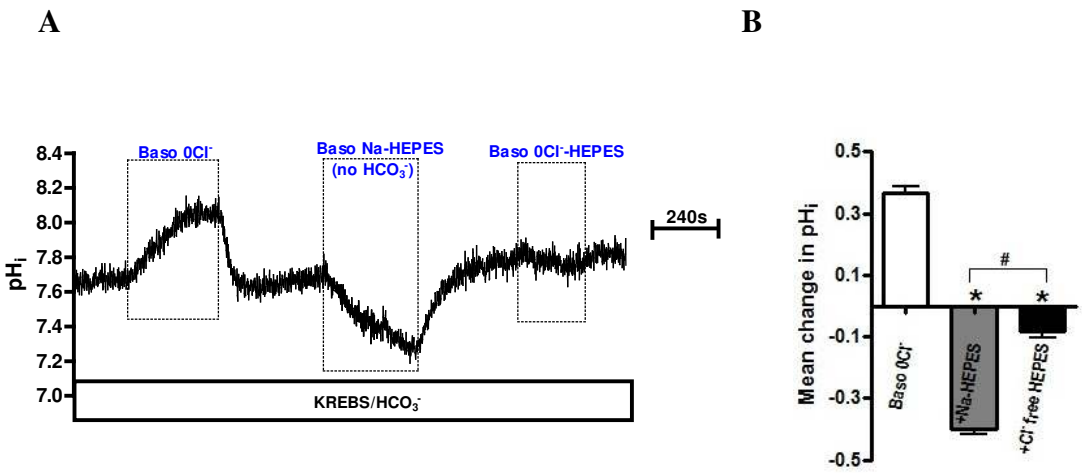


Figure 4. 1: Cl^- and HCO_3^- dependence of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells. (A) Representative pH_i trace showing the basolateral Cl^-/HCO_3^- AE activity in HCO_3^- and Cl^- free HEPES buffer solution in Calu-3 cells. (B) mean change in pH_i produced by basolateral Cl^-/HCO_3^- AE activity in HCO_3^-/KREBS solution compared to HCO_3^- free and HCO_3^-+Cl^- free HEPES solution respectively. *P<0.001 compared to control response, #P<0.001 compared to Na-HEPES. Data are shown as mean ±SEM, n=6 for control, n=3 for +Na-HEPES, and n=6 for Cl^- free HEPES.

4.3 DIDS sensitivity of the basolateral Cl^-/HCO_3^- anion exchanger

To investigate the pharmacological properties of the basolateral anion exchanger the response to a basolateral Cl^- free solution was evaluated in the presence of the anion exchange inhibitor 4-4-Diisothiocyanatostilbene-2,2′-Disulfonic Acid (DIDS). Figure 4.2A shows that basolateral Cl^- removal produced an alkalinisation in pH_i of 0.33±0.05 pH unit, which recovered following Cl^- readdition at a rate of 0.57±0.07 pH_i min^{-1} (n=8). This response was not affected by 0.01µM DIDS, (0.34±0.01 pH units, and the rate of reacidification following Cl^- readdition of 0.57±0.01 pH_i min^{-1}, n=4); however, the response to a Cl^- free solution was completely abolished by 500µM DIDS (P<0.05, paired observation; n=3). There was a concentration-dependent inhibition of basolateral Cl^-/HCO_3^- AE activity by DIDS. Results showed that there was a significant reduction in the mean pH_i when perfused with Cl^- free solution containing 30µM (0.10 ± 0.01 pH units, n=5, P<0.05) and 100 µM DIDS (0.05 ± 0.02 pH units, n=3, P<0.05) and a marked change in the rate of reacidification upon readdition

of high Cl^- solution. The percent inhibition of the basolateral AE activity is shown in Figure 4.2B and C, and from these data an IC_{50} value 16.5 ± 1.3 µM was obtained for DIDS inhibition of the mean change in pH_i. The IC_{50} value for inhibition of the rate of reacidification was 7.5 ± 1.2 µM. These results are consistent with the presence of a basolateral DIDS-sensitive Cl^-/HCO_3^- anion exchanger (SLC4A2), as previously been reported in Calu-3 cells (Loffing *et al.*, 2000). Taken together, these data imply that the basolateral Cl^-/HCO_3^- AE activity is clearly a Cl^- and HCO_3^--dependent, DIDS-sensitive, anion exchanger in Calu-3 cells.

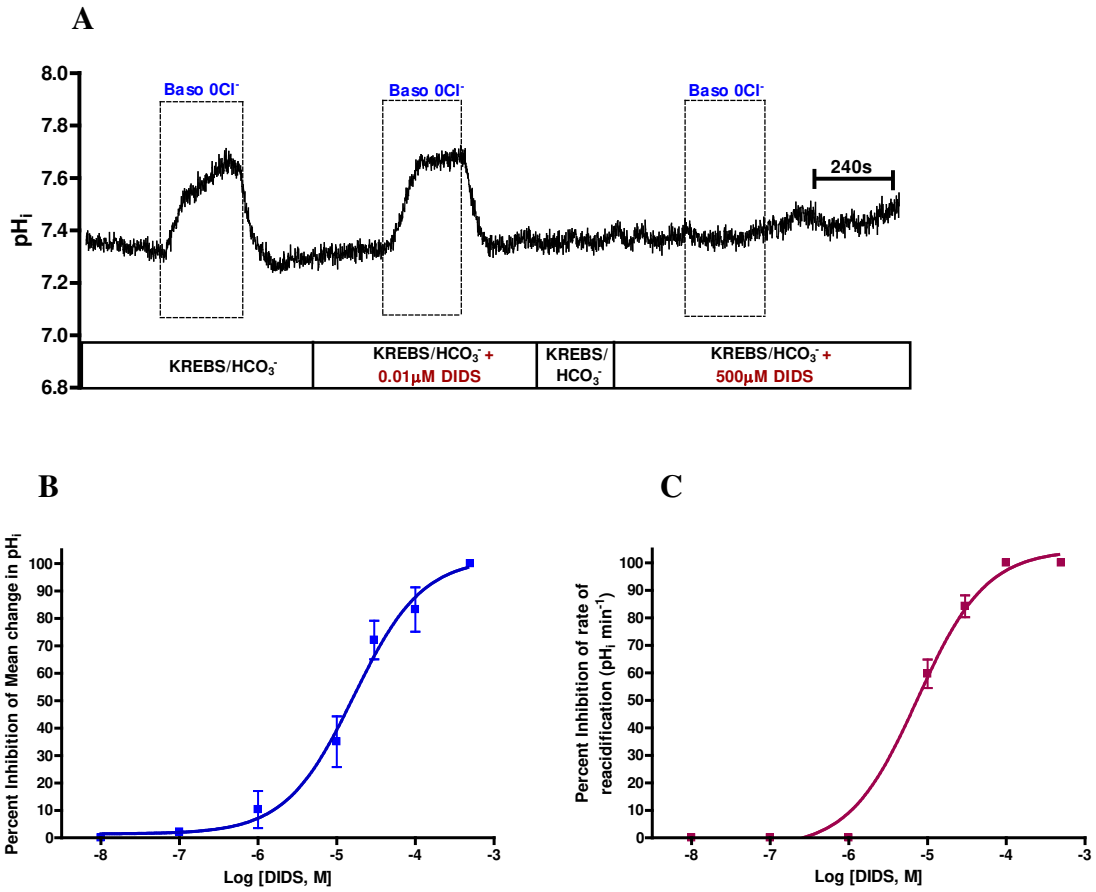


Figure 4. 2: Inhibitory effect of DIDS on basolateral Cl^-/HCO_3^- AE activity. (A) Representative pH_i traces showing the effect of DIDS (0.01 µM and 500µM) on pH_i changes after perfusion of basolateral Cl^- free solution in Calu-3 cells. DIDS dose response curve for inhibition of the basolateral Cl^-/HCO_3^- anion exchanger. Plot of percent inhibition of the mean change in pH_i caused by basolateral Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C) at different DIDS concentrations. Non-linear regression fit to the data, n=8 for control; n=4 for 0.01µM and 500µM DIDS; n=5 for 10µM, 30µM, and n=3 for 0.1µM; 1µM; 100µM DIDS.

Note that because the inhibitory effect of DIDS on the basolateral AE was not fully reversible for concentrations above 10 µM, (as shown in Figure 4.3), dose-response experiments had to be done on separate cultured monolayers.

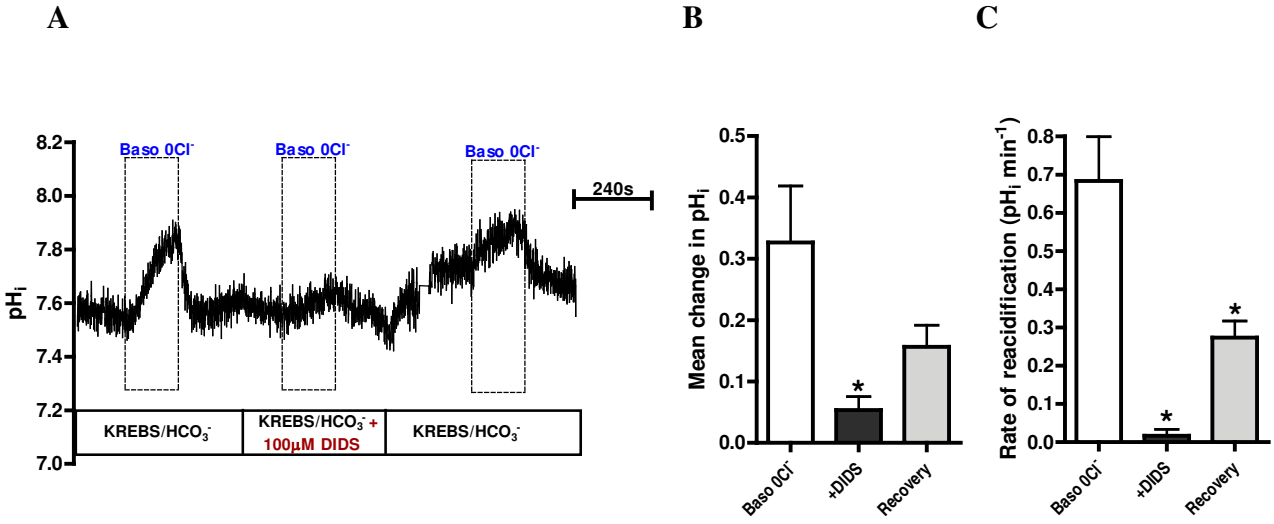


Figure 4. 3: **Recovery of basolateral Cl^-/HCO_3^- AE activity after DIDS inhibition.** (A) Representative pH_i trace showing the effect of DIDS (100µM) on pH_i changes after perfusion of basolateral Cl^- free solution in Calu-3 cells. Summary of the effect of 100µM DIDS on mean pH_i changes (B), and the rate of reacidification after Cl^- readdition (C). DIDS inhibition of the basolateral Cl^-/HCO_3^- exchanger was partially reversible after 8 min of wash off in high Cl^- solution. Data are shown as mean± SEM *P<0.05 compared to control, paired observation, n=3 for each condition.

In the DIDS experiments, DMSO was used as the vehicle to dissolve the inhibitor. In order to test if DMSO alone had any effect on basolateral Cl^-/HCO_3^- AE activity, Calu-3 cells were acutely exposed to 0.25% DMSO (~5min) and then basolateral Cl^-/HCO_3^- AE activity was measured in the presence of DMSO. Results showed that basolateral Cl^- removal in the presence of 0.25% DMSO did not affect the basolateral Cl^-/HCO_3^- AE activity compared to control responses, for both the mean change in pH_i produced by basolateral Cl^- removal (0.30±0.01, for controls and 0.34±0.02 for DMSO treated responses, n=5, P>0.05) and the rate of reacidification following Cl^- readdition (0.77±0.09, n=5

for controls and 0.88±0.08 for DMSO treated responses, n=5, P>0.05). This suggests that DMSO is not involved in producing any changes in the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

Previous work from our group has shown that in the absence of cAMP stimulation, Calu-3 cells secrete an isotonic solution with a pH of ~ 7.4 (Garnett et al., 2011). I therefore investigated the effect of blocking the basolateral anion exchanger with DIDS on transepithelial fluid and HCO_3^- secretion. Although complete inhibition of the basolateral Cl^-/HCO_3^-AE by 500µM DIDS did not affect the amount of secreted fluid, the pH of the secreted fluid was significantly increased after 24 h incubation of cells with DIDS, in a high Cl^- Krebs solution at 37˚C in 5% CO_2 (Figure 4.4A and B). These results provide support that changes in basolateral Cl^-/HCO_3^-AE activity can modulate transepithelial HCO_3^- secretion, as inhibition of the basolateral Cl^-/HCO_3^- AE activity should cause accumulation of HCO_3^- inside the cells, and thereby increase the driving force for HCO_3^- secretion, by apical AE and/or CFTR activity.

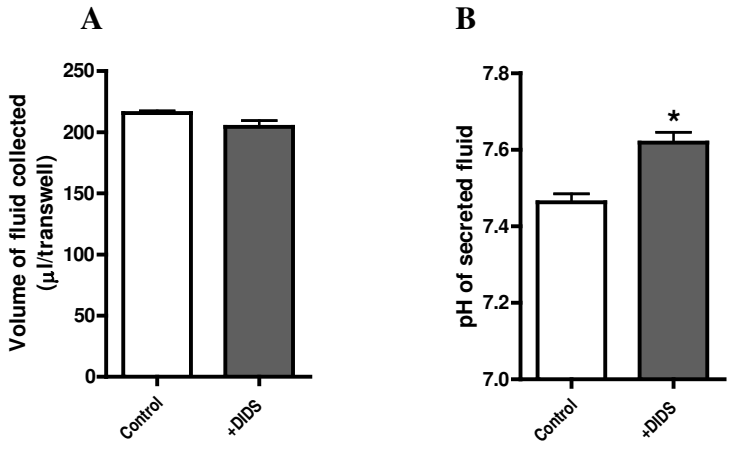
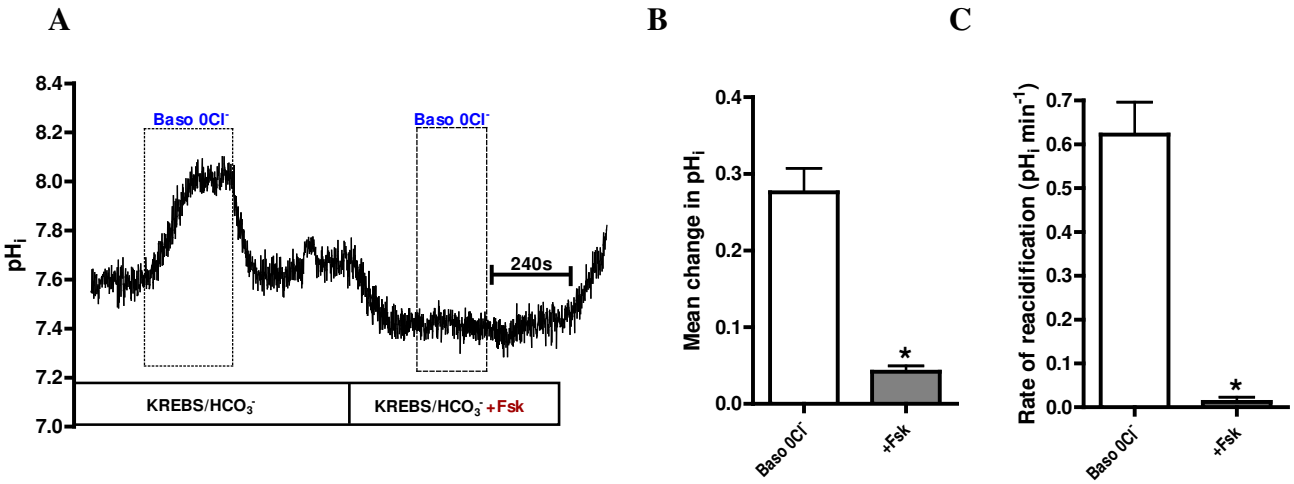


Figure 4. 4: **Summary of the effect of DIDS (500µM) on fluid secretion in Calu-3 cells.** Cells were incubated with 500µM DIDS for 24 hours in 5% CO_2 (v/v) in air in high Cl^- Krebs solution at 37˚C. Summary shows the effect of DIDS on the volume (A) and pH (B) of the secreted fluid after 24 h; *P<0.05 significant effect compared to control cells, n=3.

4.4 Role of cyclic nucleotides (cAMP and cGMP) in the regulation of basolateral Cl^-/HCO_3^- anion exchange activity

4.4.1 cAMP agonists:

Adenosine 3′,5′ cyclic monophosphate (cAMP) is one of the ubiquitous intracellular secondary messengers that plays an important role in the regulation of various cellular functions, and its intracellular concentration is controlled by enzymes such as adenylyl cyclase and phosphodiesterases (Fantidis, 2010). As shown in Figure 4.5A, the basolateral Cl^-/HCO_3^- anion exchange activity was nearly completely abolished by the addition of the cAMP agonist Fsk, which reduced the magnitude of alkalinisation induced by basolateral Cl^- removal by 85.2±2.6% and the rate of reacidification by 98.4±1.6%. The basolateral Cl^-/HCO_3^- exchange activity was also inhibited by bilateral addition of 10µM adenosine (ADO), which was as effective as Fsk in reducing the basolateral AE activity. ADO reduced the magnitude of alkalinisation by 73.9±4.8% and the rate of reacidification by 81.8± 5.1% (Figure 4.5D and E). The results for ADO inhibition are also of interest since it was shown in Chapter 3 (section 3.2.1), that ADO was much less effective at activating apical AE activity compared to Fsk. Overall, these results suggest that intracellular increases of cAMP were responsible for the inhibition of the basolateral Cl^-/HCO_3^- exchange activity in Calu-3 cells.



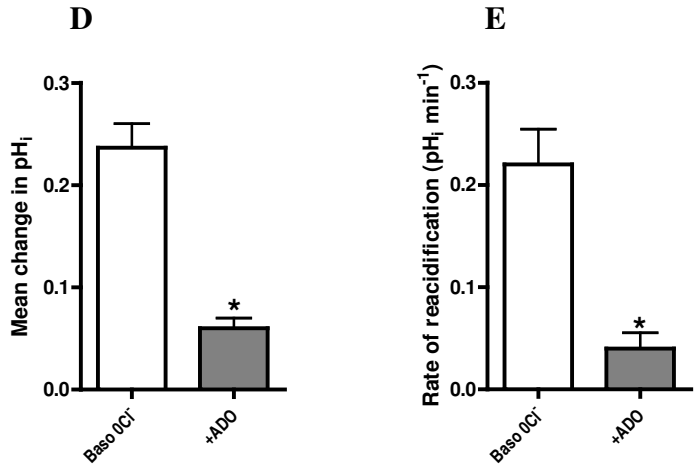


Figure 4. 5: **Forskolin and adenosine reduced basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.** (A) Representative pH_i trace showing the effect of Fsk (5µM) on pH_i changes after perfusion of basolateral Cl^- free solution in Calu-3 cells. Summary of the impact of cAMP agonist forskolin on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, (B) mean pH_i change (alkalinisation) caused by Cl^- removal, and the rate of reacidification (C) following Cl^- readdition, under resting and forskolin (5µM) stimulated condition. *P<0.05 compared to Baso $0Cl^-$. Data are shown as mean ±SEM, n=10 for each condition. (D) Impact of ADO on mean pH_i change (alkalinisation) caused by Cl^- removal, and the rate of reacidification following Cl^- readdition (E), under resting and adenosine (10µM) stimulated conditions. *P<0.05 compared to Baso $0Cl^-$. Data are shown as mean ±SEM, n=3 for each condition, paired observations.

Another way to increase cAMP inside cells, aside from Fsk and ADO, and downstream of tmAC was to use the phosphodiesterase (PDE) inhibitor, IBMX (1mM added apically only), or to treat cells with a membrane permeable analogue of cAMP, db-cAMP, (added bilaterally at a concentration of 800µM) to further clarify the role of cAMP in mediating the inhibition of basolateral AE activity. It has been shown that inhibitions of PDEs, which are responsible for cAMP breakdown, are accompanied by an increase of intracellular cAMP, and CFTR activation in Calu-3 cells (Cobb *et al.*, 2003). As shown in Figure 4.6A, stimulation of Calu-3 cells with IBMX completely abolished the basolateral AE activity compared to the control response, and which was fully reversed after 5 min washing off the IBMX. The PDE inhibitor caused a significant decrease in both mean change in pH_i, produced by basolateral Cl^- removal, and the rate of reacidification, upon Cl^- readdition, compared to control response (Figure 4.6B and C). Moreover, db-cAMP significantly reduced the activity of the basolateral Cl^-/HCO_3^- anion exchanger, as both the magnitude of alkalinisation produced

by basolateral Cl^- removal, as well as the rate of reacidification following Cl^- readdition were significantly decreased (Figure 4.6D and E). Note that the effect of dbcAMP was not readily reversible. Therefore, these data provide further strong support that an increase of intracellular cAMP is clearly responsible for abolishing basolateral Cl^-/HCO_3^- AE activity.

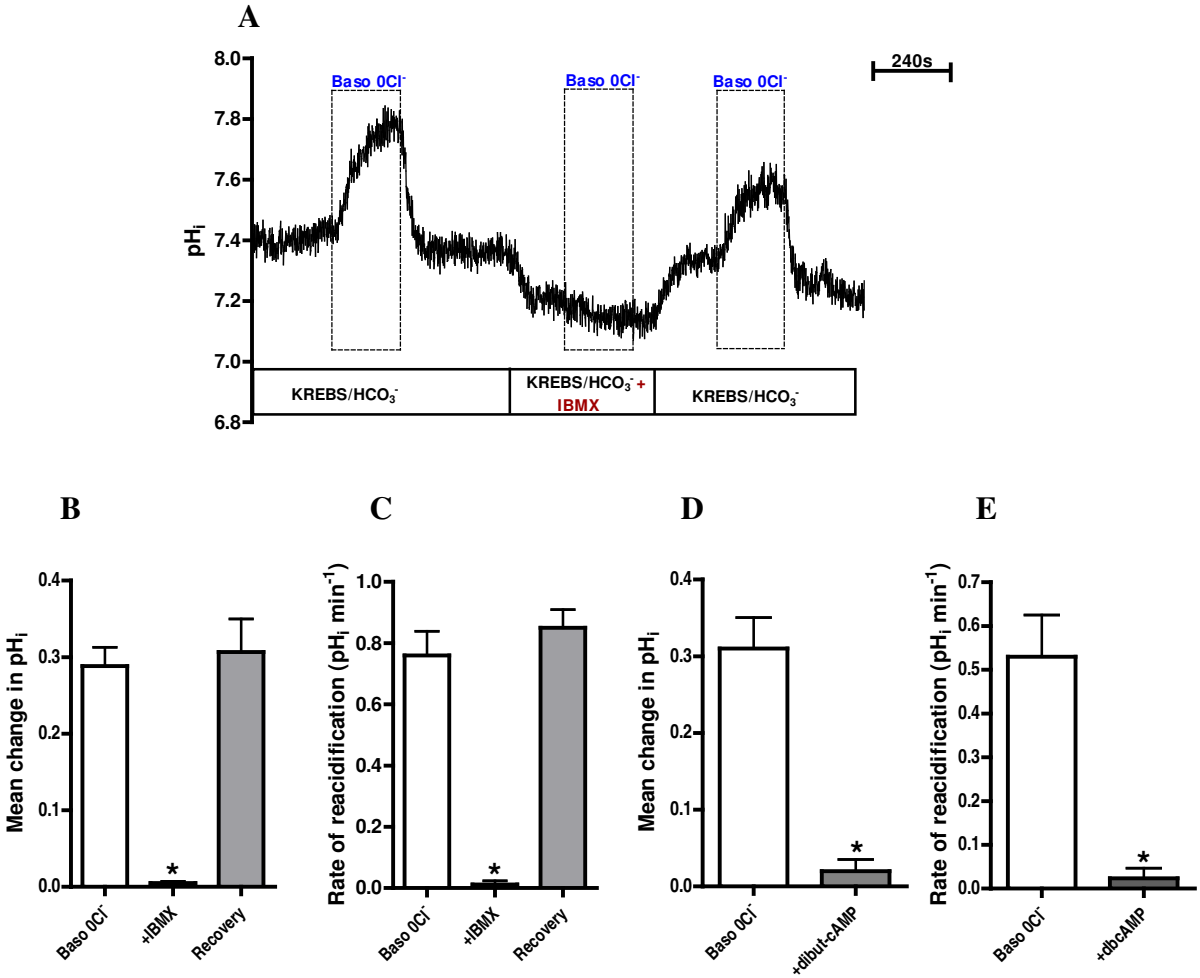


Figure 4. 6: IBMX and dbcAMP inhibit basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing that IBMX inhibits the activity of the basolateral AE activity in Calu-3 cells. Summary of the impact of IBMX on the basolateral Cl^-/HCO_3^- AE activity for both mean pH_i change (alkalinisation) caused by Cl^- removal (B), and the rate of reacidification after Cl^- readdition (C) under resting and stimulated condition with cAMP agonists (IBMX), .*P<0.05, n=6 for each condition. (D) Mean pH_i change (alkalinisation) caused by Cl^- removal, (E) the rate of reacidification after Cl^- readdition under resting and stimulated condition with dbcAMP (800µM) in Calu-3 cells. Data are shown as Mean±SEM. *P<0.05, n=3 for each condition, paired observations.

4.4.2 The role of Multidrug Resistance Proteins

The multidrug resistance proteins (MRP) are also members of the ABC transporter family, and have been shown to transport cyclic nucleotides from inside to outside of cells (Dean *et al.*, 2001). It has been demonstrated that a functional MRP1 is expressed on the basolateral membrane of Calu-3 cells (Hamilton *et al.*, 2001). MRP4 has been shown to be expressed in epithelial cells lining lung, kidney, intestine, etc., on both the apical (van Aubel *et al.*, 2002) and basolateral membranes (Lai and Tan, 2002) of polarized cells. MRP4 plays an essential role as a high affinity cAMP efflux pump (Chen *et al.*, 2001; van Aubel *et al.*, 2002). Moreover, it has been reported that MRP4 is physically and functionally linked to CFTR in gut epithelial cells, and that MK571 an inhibitor of MRP4, potentiates the CFTR mediated Cl^- conductance at the apical membrane of gut epithelial cells (Li *et al.*, 2007). Our hypothesis was that inhibition of these transporters should cause cAMP accumulation inside the cells, and reduce the basolateral Cl^-/HCO_3^- AE activity. Therefore, in order to investigate the role of these MRP transporters in the regulation of basolateral Cl^-/HCO_3^- AE activity, Calu-3 cell cells were preincubated with the MRP4 inhibitor, MK-571, (10µM apically) for 60 min and then basolateral AE activity was measured in Cl^- free solutions under resting (i.e. non-cAMP stimulated) conditions. Results showed that MRP inhibition caused a significant reduction in basolateral Cl^-/HCO_3^- AE activity, and both the mean change in pH_i produced by basolateral Cl^- removal, and the rate of reacidification following Cl^- readdition, were significantly reduced compared to control responses (Figure 4.7A and B). This implies that inhibition of the cAMP efflux pump caused an increase in intracellular cAMP and inhibition of the basolateral Cl^-/HCO_3^- activity in Calu-3 cells, which further reinforces the fact that cAMP is responsible for inhibition of the basolateral AE activity. These results also indicate that under 'resting 'conditions, Calu-3 cells must have a basal turnover of cAMP in the absence of an external cAMP agonist, and the transport activity of the MRP transporters helps maintain a low intracellular level of cAMP, which has been found in other epithelial cells (van Aubel *et al.*, 2002).

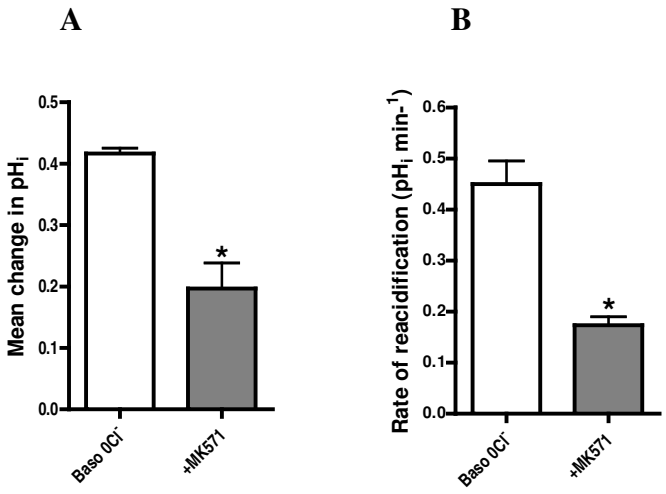


Figure 4. 7: Inhibition of the MRP transporter reduced the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of the MRP inhibitor, MK571 (10µM), on mean pH_i change (alkalinisation) caused by Cl^- removal (A), and the rate of reacidification (B) following Cl^- readdition under resting condition in Cau-3 cells. Data are shown as Mean±SEM.*P<0.05, n=3 for each condition. Control cells run in parallel.

4.4.3 cGMP

As mentioned in chapter 3 (section 3.2.2) cGMP plays an important role in the activation of CFTR and anion secretion in many epithelial cells. Although my results suggested it did not regulate CFTR-dependent pendrin activity in Calu-3 cells, it was still of interest to gain an insight into the role of cGMP in the regulation of the basolateral anion exchanger in Calu-3 cells, and to see whether cGMP influences the cAMP-dependent inhibition of this exchanger. Thus, Calu-3 cells were preincubated with the cGMP agonist, 8Br-cGMP, for 60 min and then basolateral Cl^-/HCO_3^- AE activity measured in response to Cl^- free solutions. My results showed that, similar to the lack of effect on apical anion exchange activity, cGMP stimulation had no effect on either the magnitude of alkalinisation produced by basolateral Cl^- removal, or the rate of reacidification, under resting conditions, nor did it alter the subsequent Fsk-induced inhibition of the basolateral Cl^-/HCO_3^- anion exchanger (Figure 4.8A and B). This suggests

that that cGMP dependent protein kinase is not involved in the regulation of basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

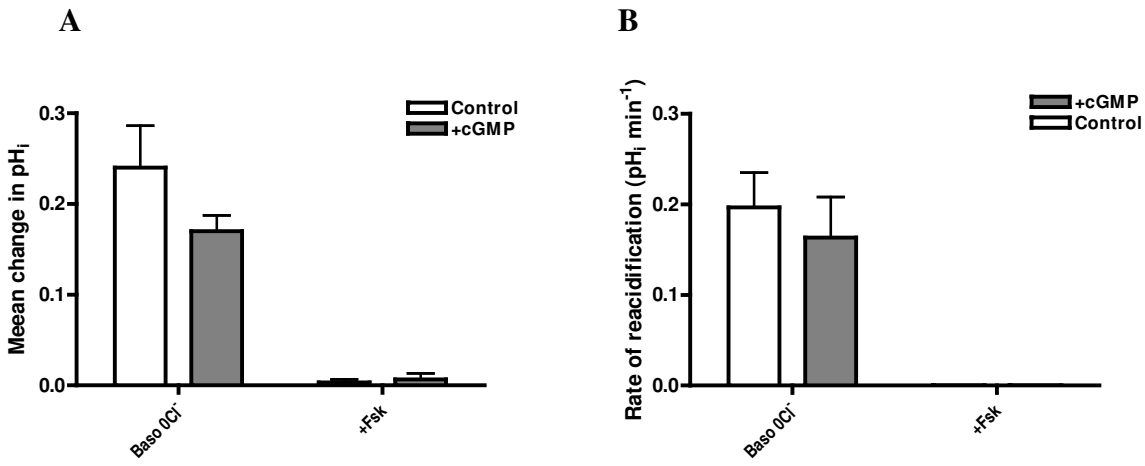


Figure 4. 8: cGMP agonist, 8Br-cGMP, did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of the cGMP agonist on basolateral Cl^-/HCO_3^- AE activity. Calu-3 cells were preincubated apically with 1mM 8Br-cGMP for 60 min. (A) mean alkalinisation in pH_i in response to Cl^- free solution, (B) the rate of reacidification upon Cl^- readdition. Data are shown as Mean±SEM, no significant difference ($P>0.05$) compared to control, n=3 for each condition, control cells run in parallel.

Overall, my results demonstrate that raising intracellular levels of cAMP, using cAMP agonists or inhibiting cAMP efflux, was clearly responsible for the marked reduction in basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. However, it was not clear whether cAMP directly or indirectly inhibited the basolateral exchanger. Therefore, my next experiments focused on identifying the role of downstream targets of cAMP in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

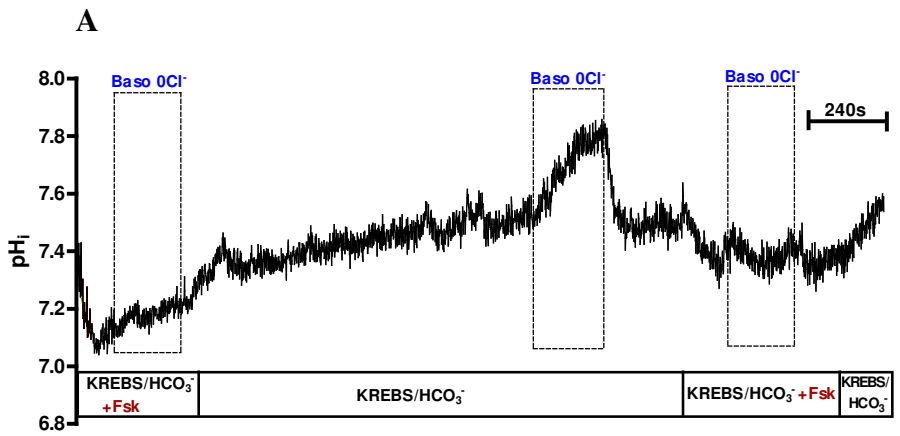
4.5 Regulation of basolateral Cl^-/HCO_3^- anion exchanger activity by downstream targets of cAMP

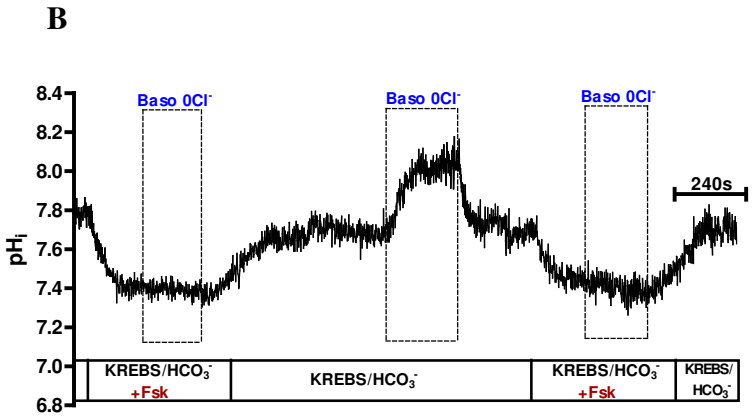
To investigate the mechanisms behind the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in more detail, I next assessed the potential downstream targets of cAMP. There are three major intracellular targets for cAMP which include PKA, Epac and cyclic nucleotide-gated ion channels (CNGCs), which are non-selective cation channels that are particularly important in the olfactory and visual system (Craven and Zagotta, 2006). Moreover, it has been reported by Kim *et al*., (2010) that intracellular elevation of cAMP can lead to the activation of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway through a PKA-independent mechanism (Kim *et al.*, 2010).

4.5.1 Role of PKA

H-89, which is a competitive inhibitor of PKA, was tested on the basolateral response to Cl^- free KREBS solution, in Calu-3 cells pre-incubated with H-89 for 60 min, and then exposed to Fsk. Note that previous data from our group showed that H-89 had no effect on the resting basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells using similar conditions (J Garnett PhD thesis, 2010). As shown in Figure 4.9, the inhibitory effect of Fsk on basolateral AE activity was not changed in the presence of H-89. Furthermore, normal basolateral AE activity returned after ~ 15 mins of washing off the H-89 (with high Cl^- KREBS solution), which was completely inhibited by subsequent addition of Fsk. This is despite the fact that H-89 (50μM) significantly decreased the Fsk stimulated apical AE activity in Calu-3 cells (see chapter 3, figure 3.3). As H-89 is a non-specific inhibitor of PKA, an alternative PKA inhibitor, Rp-adenosine-3',5'-cyclic monophosphorothioate (RpcAMP) was used. RpcAMP has a different structure to H-89, and acts as a specific competitive antagonist of the cyclic nucleotide–binding domains on PKA (de Wit *et al.*, 1984). Calu-3 cells were preincubated with 1mM RpcAMP on the apical side for one hour, and then the

basolateral response to Cl^- removal in the presence of Fsk assessed. Results showed that the basolateral AE activity was completely abolished in RpcAMP pretreated cells, which was completely recovered after 10 min washing off of the inhibitor. A representative pH_i trace from RpcAMP treated cells is shown in Figure 4.9B. The effect of both H-89 and RpcAMP are summarized in Figure 4.9C and D. As can be seen, the PKA inhibitor had no effect on the inhibitory effect of Fsk on the basolateral AE activity, suggesting that PKA is not involved in the cAMP-dependent inhibition of the basolateral AE activity in Calu-3 cells.





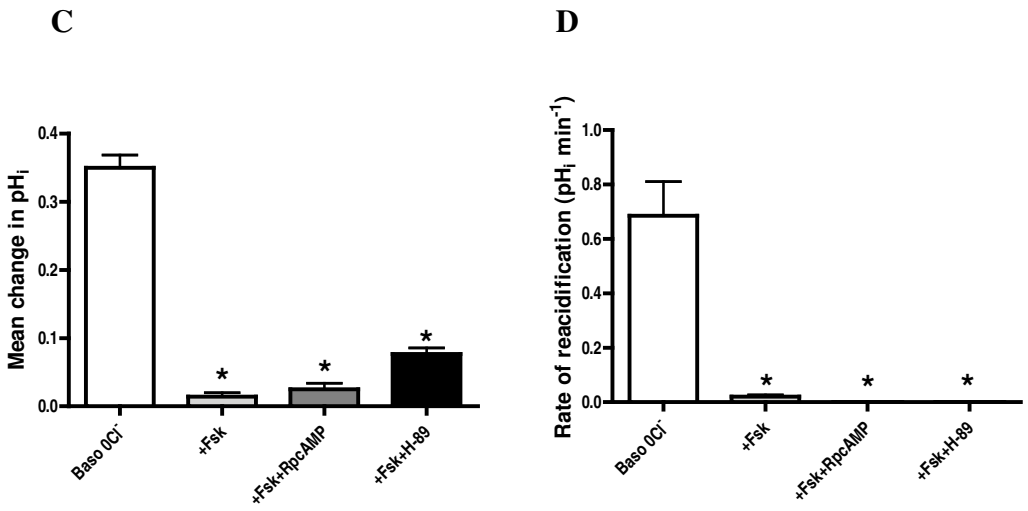


Figure 4. 9: PKA inhibitors, H-89 and RpcAMP, had no effect on the cAMP induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) and (B) pH_i traces showing that inhibition of PKA by either 50µM H-89 , or 1mM RpcAMP (both inhibitors preincubated with cells for 60 min), did not affect the Fsk induced inhibition of the basolateral AE in Calu-3 cells. Summary of the effect of both PKA inhibitors, H-89 and RpcAMP, on mean pH_i change (alkalinisation) caused by Cl^- removal (C), and the rate of reacidification after Cl readdition (D) in Calu-3 cells. Data are shown as Mean±SEM.*P<0.05 compared to control, n=3 for each, except for control n=6, which were run in parallel.

4.5.2 Exchange protein directly activated by cAMP (Epac)

It has been demonstrated that Epac is a novel cAMP target which has properties independent of PKA (Schmidt et al., 2013). Therefore, Calu-3 cells were preincubated with the Epac agonist (8CPT-2Me-cAMP-AM) for 60 min, and basolateral Cl^-/HCO_3^- AE activity measured in response to Cl^- free solutions under both resting and cAMP stimulated conditions. Results showed that Epac stimulation did not alter basolateral Cl^-/HCO_3^- AE activity (Figure 4.10A and B), nor did it alter Fsk-induced inhibition of the basolateral anion exchanger.

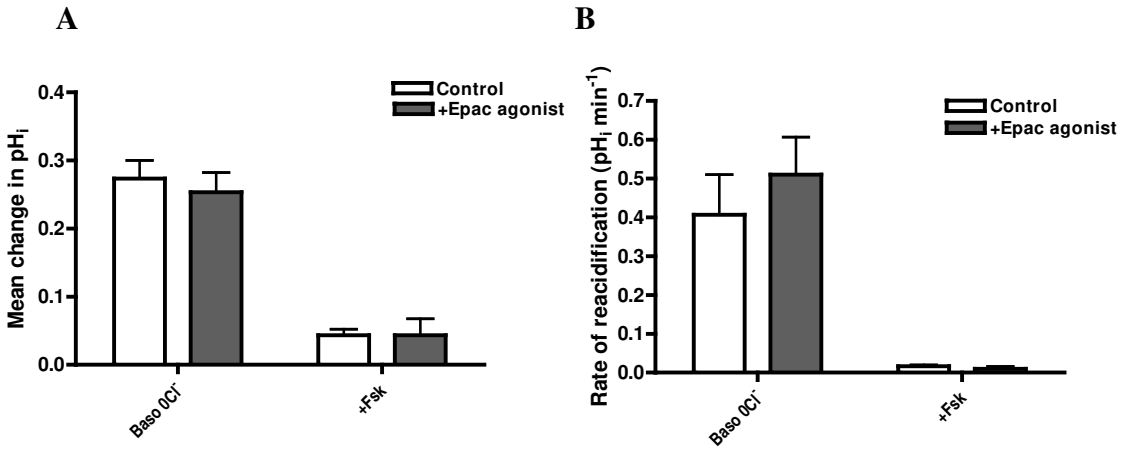


Figure 4. 10: Epac agonist, 8CPT-2Me-cAMP-AM, did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of the Epac agonist (8CPT-2Me-cAMP-AM) 10µM preincubation for 60 min on the mean alkalinisation in pH_i in response to Cl^- free solution (A) and the rate of reacidification upon Cl^- readdition (B) in the presence and absence of Fsk in Calu-3 cells. Data are shown as Mean±SEM. No significant difference (P>0.05) compared to control, n=3 for each condition. Control cells run in parallel.

Although the experiments using the Epac agonist showed that Epac appeared not to be involved in regulating basolateral AE activity, inhibition of Epac by preincubation of Calu-3 cells with the Epac inhibitor (ESI-09), which is a novel and specific Epac inhibitor (Almahariq *et al.*, 2013), resulted in a marked reduction in the basolateral Cl^-/HCO_3^- AE activity under resting conditions, both in the mean change in pH_i (alkalinisation) produced by basolateral Cl^- removal, and the rate of reacidification upon Cl^- readdition, compared to control responses. However, the residual basolateral AE activity could still be inhibited by Fsk (Figure 4.11A, B and C). The rate of HCO_3^- flux was also significantly reduced in ESI-09 treated cells compared to control cells (Figure 4.11F). The effect of Epac inhibition on the basolateral Cl^-/HCO_3^- AE activity was significantly recovered after washing off the inhibitor (Figure 4.11D and E). This result clearly implies that (1) Epac appears to be required to maintain the basolateral Cl^-/HCO_3^- AE activity under resting conditions, and (2) the Fsk-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity is through an Epac-independent mechanism.

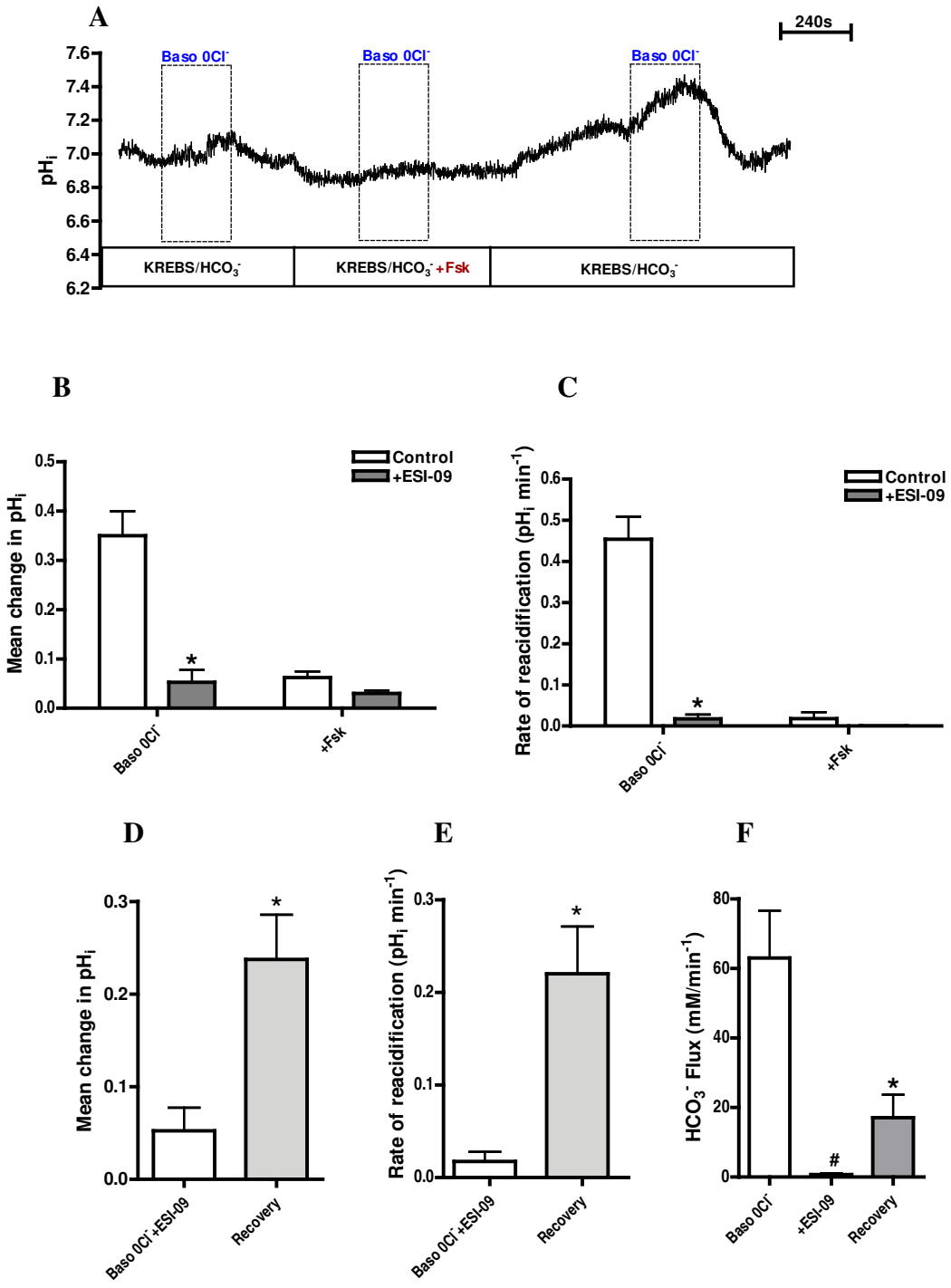


Figure 4. 11: The Epac inhibitor, ESI-09, reduced the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting conditions. (A) pH_i trace showing that inhibition of Epac by preincubation of Calu-3 cells with 10µM ESI-09 for 60 min reduced the basolateral Cl^-/HCO_3^- AE activity which was recovered after washing off the inhibitor. Summary of the effect of ESI-09 on the mean alkalinisation in pH_i in response to Cl^- free solution (B) and the rate of reacidification after Cl^- readdition (C) under resting and Fsk stimulated condition. The basolateral AE activity was recovered after washing off the Epac inhibitor for both mean change in pH_i (D) and the rate of reacidification (E). (F) The rate of HCO_3^- flux in control and ESI-09 treated cells. Data are shown as Mean±SEM, *P<0.05, #P<0.01 compared to control, paired observation, n=4 for each condition.

4.5.3 Role of cyclic nucleotide-gated cation (CNG) channels

It has been shown that CNG channels are stimulated by cAMP and increase transepithelial sodium and calcium absorption in rat colon (Qiu *et al.*, 2000b). It has also been demonstrated that mRNA for CNG channels are expressed in human bronchial airway cells, which contribute to both sodium and calcium absorption in the adult lung (Qiu *et al.*, 2000a). Since CNG channels are voltage gated ion channels (Kaupp and Seifert, 2002), and mediate membrane depolarization in neurons (Zufall *et al.*, 1994; Finn *et al.*, 1996), and there is no specific CNG channel inhibitor, I investigated the effect of depolarising the membrane potential by perfusing Calu-3 cells with a high K^+ HCO_3^-/KREBS solution, which should inhibit the CNG channels (Figure 4.12A). Results showed that depolarising cell did not prevent the inhibitory effect of cAMP on the basolateral Cl^-/HCO_3^- AE activity, although the mean pH_i change and rate of reacidification were affected under resting conditions (Figure 4.12B and C). This suggested that CNG channels are not involved in the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. The enhanced alkalinisation seen under zero Cl^- conditions in the presence of high K^+ (Figure 2.12B), might be due to stimulation of the electrogenic basolateral NBC by membrane depolarisation, which would provide more HCO_3^- influx across the basolateral membrane into the cells. However, under Fsk-stimulated conditions, in depolarized cells, where the basolateral anion exchanger is inhibited, there was a marked acidification observed upon zero Cl^- perfusion (Figure 4.12A). This pH_i data could be explained by the presence of an electrogenic anion exchanger, or alternatively an anion channel on the basolateral membrane, which is active under cAMP stimulated conditions (see Chapter 3).

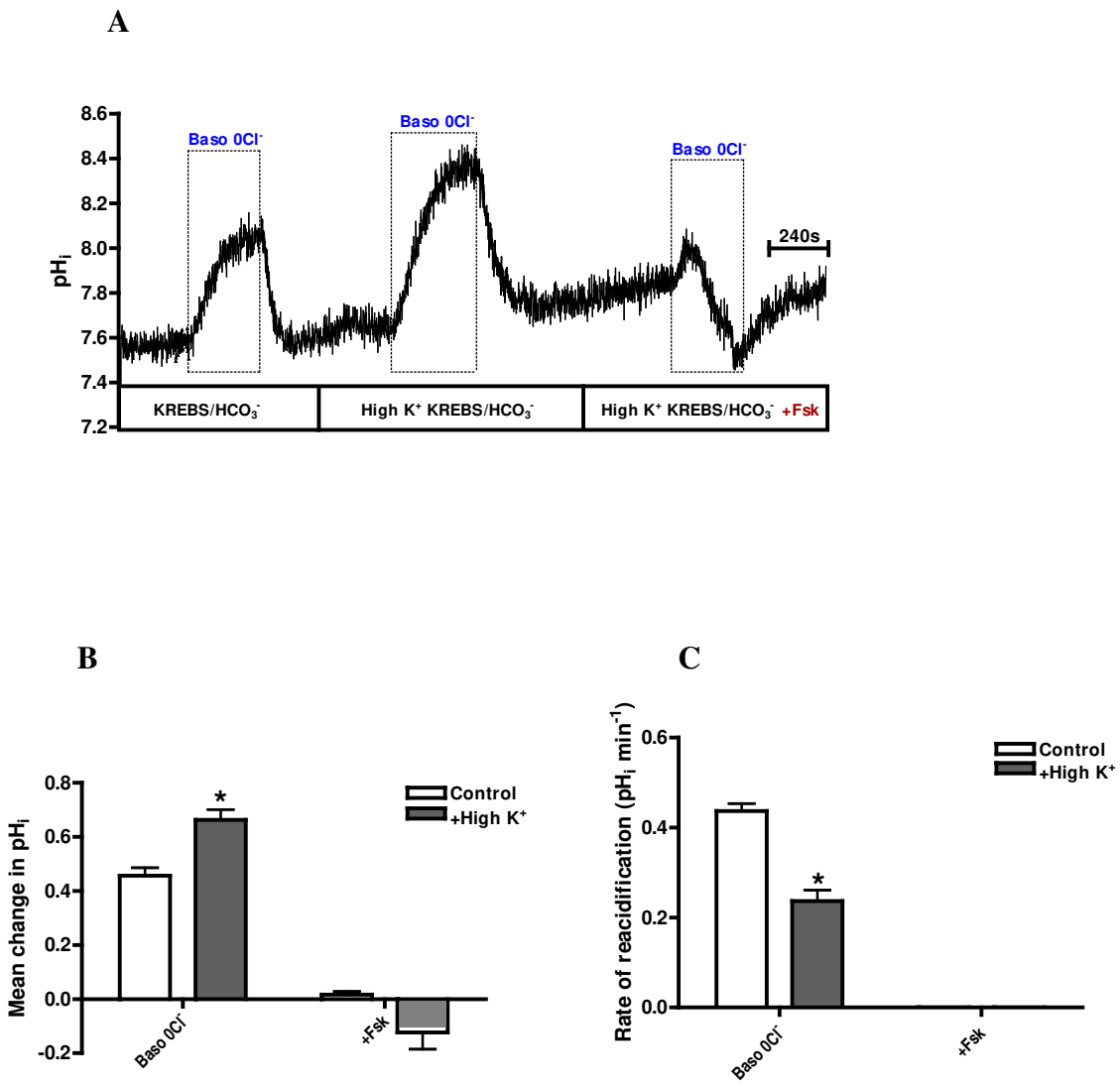


Figure 4. 12: Depolarization of Calu-3 cells did not affect the cAMP induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. pH_i trace showing the effect of high K^+ KREBS solution on the basolateral AE activity. Calu-3 cells were perfused with bilateral high K^+ Krebs solution to depolarize cell membrane potential, and inhibit CNG channel. Summary of mean pH_i change (alkalinisation) caused by Cl^- removal (A) and the rate of reacidification upon Cl^- readdition (B) under resting and Fsk stimulated condition in depolarized Calu-3 cells. Data are shown as Mean±SEM.*P<0.05 compared to control, paired observation, n=3 for each condition.

4.5.4 Mammalian target of rapamycin (mTOR) kinase

In order to assess the role of the cAMP-dependent protein kinase, mTOR, in the regulation of the basolateral Cl^-/HCO_3^- AE activity, Calu-3 cells were preincubated for 60 mins with rapamycin, which is selective blocker of mTOR protein kinase (Ballou and Lin, 2008). Results showed that inhibition of mTOR kinase did not produce any changes in either the magnitude of alkalinisation produced by basolateral Cl^- removal or the rate of reacidification following Cl^- readdition, and did not affect the cAMP induced inhibition of the basolateral Cl^-/HCO_3^- AE activity (Figure 4.13A and B). This suggests that mTOR kinase is not involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity under both resting and cAMP stimulated conditions.

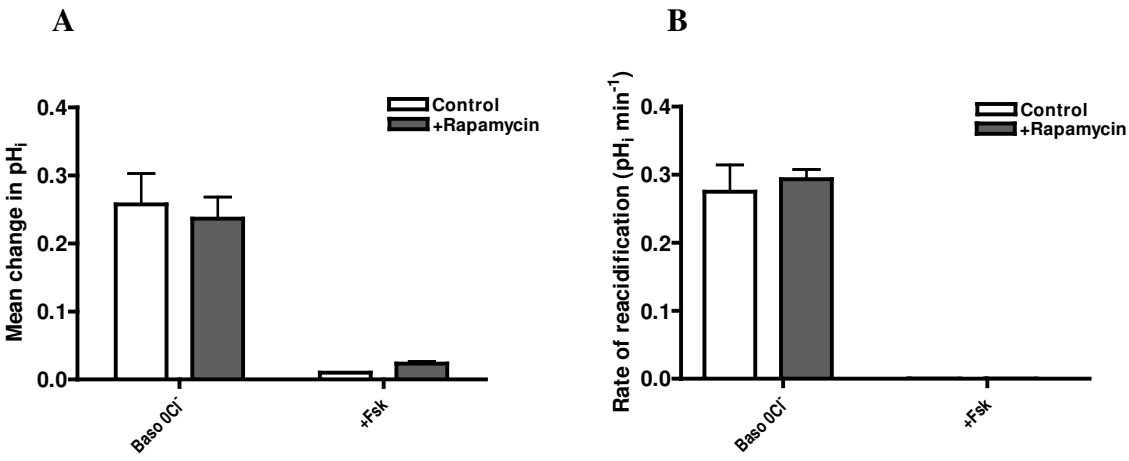


Figure 4. 13: mTOR inhibition did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of mean pH_i change (alkalinisation) caused by Cl^- removal (A) and the rate of reacidification upon Cl^- readdition (B) in Calu-3 cells, preincubated for 60 min and perfused with 100nM rapamycin, under resting and Fsk stimulated condition. Data are shown as Mean±SEM. No significant difference (P>0.05) compared to control response, n=4 for control, and n=3 for +Rapamycin. Controls run in parallel.

4.6 Role of Ca^{2+} in the regulation of basolateral Cl^-/HCO_3^- anion exchanger activity

It has been found that changes in $[Ca^{2+}]_i$ can alter cAMP levels (either by stimulation or inhibition of cAMP production), through a number of Ca^{2+}-sensitive isoforms of AC (Willoughby and Cooper, 2007). Evidence has also shown that there is a direct link between the ER-Ca^{2+}-store operated signaling pathway and cAMP production by ACs, which is independent of changes in cytosolic Ca^{2+} concentration (see Chapter 3). This response requires the translocation of the transmembrane ER Ca^{2+}-sensor protein, STIM1, into a large immobile aggregate under the plasma membrane and activation of AC (Lefkimmiatis *et al.*, 2009). Therefore, I investigated the role of Ca^{2+} in the regulation of the basolateral Cl^-/HCO_3^- AE activity under both resting and cAMP stimulated conditions.

4.6.1 Intracellular Ca^{2+}

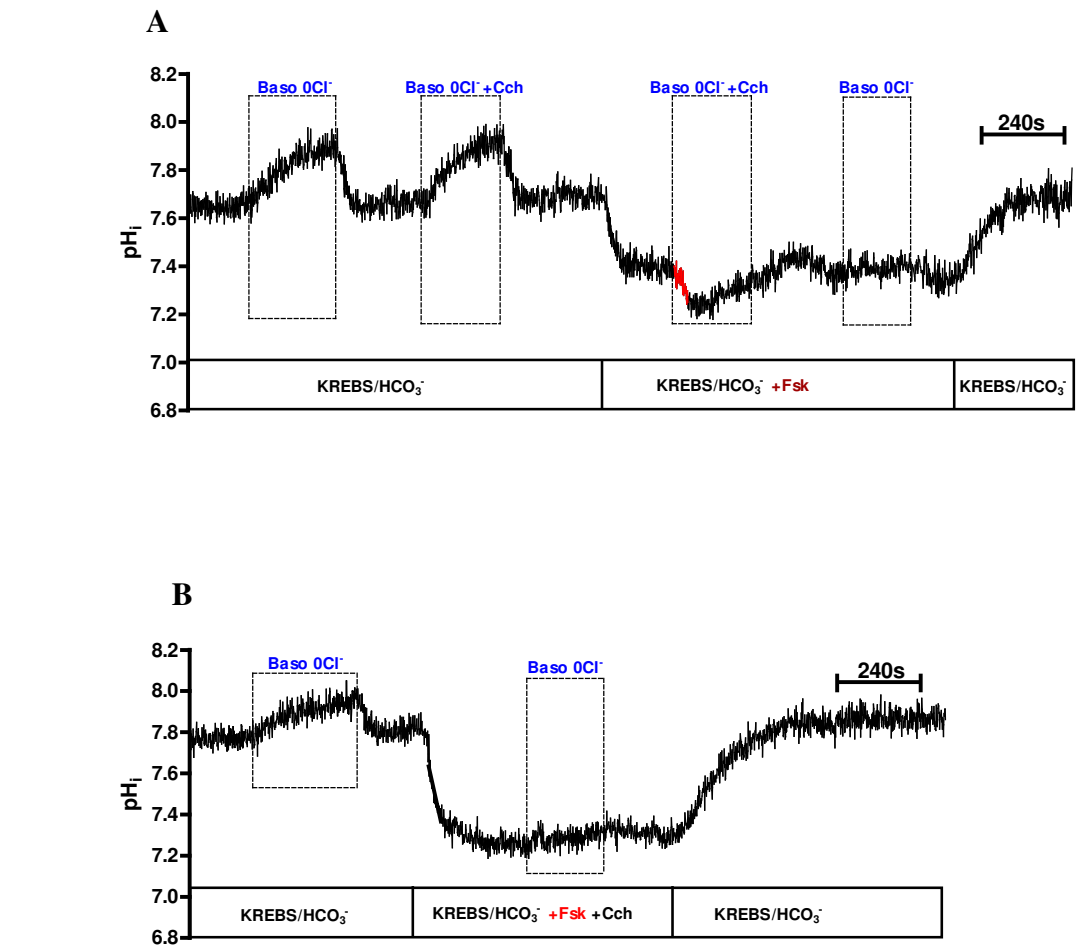
4.6.1.1 Effect of increases in $[Ca^{2+}]_i$ on anion exchange activity

It has been found that elevation of $[Ca^{2+}]_i$ by thapsigargin and muscarinic receptor (M3) stimulation, markedly enhanced basolateral Cl^-/HCO_3^- AE activity in mouse salivary acinar cells (Nguyen *et al.*, 2004). Moreover, it has been shown that intracellular elevation of Ca^{2+}, using Ca^{2+} agonists, plays an important role in the stimulation of adenylyl cyclase and elevation of cAMP (Namkung *et al.*, 2010). A well-known synergistic interaction between cAMP and Ca^{2+} signals has also been reported in human airway epithelial cells, which plays a critical role in HCO_3^- and fluid secretion, in response to carbachol and *vasoactive intestinal peptide* (VIP), which is absent in CF cells (Choi *et al.*, 2007). Therefore it was important to test whether increasing $[Ca^{2+}]_i$ could affect the activity of the basolateral Cl^-/HCO_3^- anion exchanger. I used two different methods to investigate this which involved studying the effects of Ca^{2+} release

from IP_3-sensitive ER stores by the muscarinic agonist carbachol (Cch) (Mayerhofer *et al.*, 1992), and by using the selective SERCA pump inhibitor thapsigargin (Thastrup *et al.*, 1990).

4.6.1.1.1 Effect of carbachol on basolateral AE activity

It has been shown that Cch induces intracellular cAMP elevation in a dose dependent manner in rat pancreatic islets (Tian and Laychock, 2001). However, it has not been investigated whether Cch application could influence basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Thus, Calu-3 cells were exposed to either unilateral (Figure 4.14A, C, D, E & F) or bilateral Cch (Figure 4.14G & H), and results showed that Cch did not affect the basolateral AE activity under resting or Fsk stimulated conditions, compared to the control response (Figure 4.14A-C-F). However, a transient acidification was produced by basolateral Cl^- removal in Fsk stimulated cells compared to the control response, when Cch was applied only basolaterally, (Figure 4.14A, red trace). This transient acidification might be due to a change in membrane potential, as an increase in $[Ca^{2+}]_i$ could stimulate Ca^{2+}-sensitive K^+ channels in the basolateral membrane. This would cause hyperpolarisation, which would inhibit NBC activity, reduce HCO_3^- influx into the cells, and thereby produce a transient acidification. In order to investigate whether this acidification was Ca^{2+}-dependent, cells were preincubated with the Ca^{2+} chelator BAPTA-AM (50μM) for 1hr. Results showed that BAPTA-AM abolished the carbachol-induced transient acidification (Figure 4.14B, n=3), suggesting that this acidification was Ca^{2+} dependent. Note that BAPTA-AM loading significantly reduced basolateral AE activity, which is discussed in more detail in section 4.6.1.2.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Baso 0Cl-
Baso 0Cl- +Cch
Baso 0Cl- +Cch
Baso 0Cl-
240s
pHi
8.2
8.0
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7.4
7.2
7.0
6.8
KREBS/HCO3-
KREBS/HCO3- +Fsk
KREBS/HCO3-
B
Baso 0Cl-
Baso 0Cl-
240s
pHi
KREBS/HCO3-
KREBS/HCO3- +Fsk +Cch
KREBS/HCO3-
C
Mean change in pHi
0.4
0.3
0.2
0.1
0.0
-0.1
Control
+Basolateral Cch
Baso 0Cl-
+Fsk
D
Rate of reacidification (pHi min-1)
0.4
0.3
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0.1
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Control
+Basolateral Cch
Baso 0Cl-
+Fsk

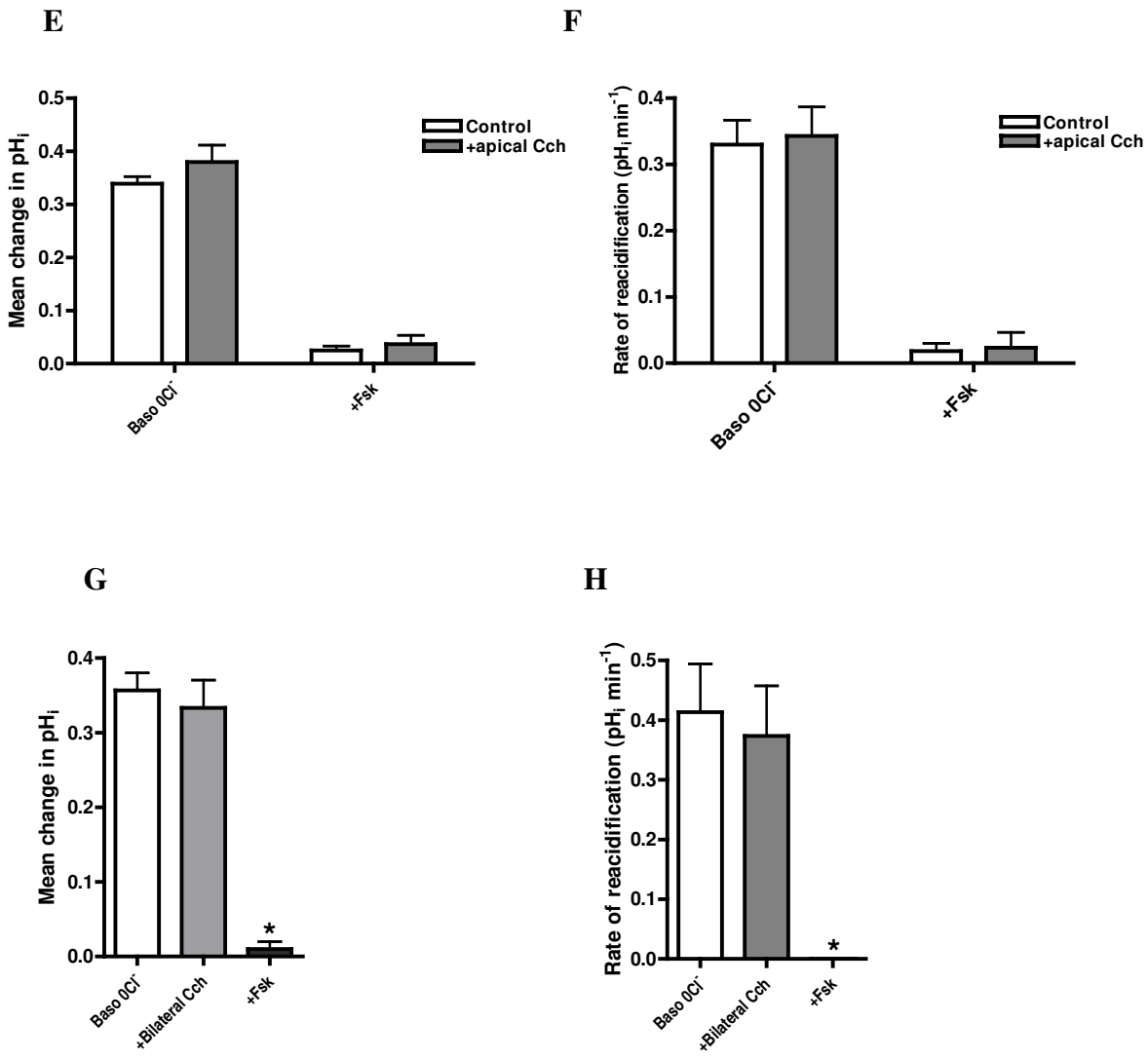

Figure 4. 14: Carbachol (Cch) did not affect the activity of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing lack of effect of Cch (20µM), applied basolaterally, on basolateral AE activity under resting condition, while carbachol induced a transient acidification in pH_i when applied with Fsk stimulation in Calu-3 cells. (B) Representative pH_i trace showing that BAPTA-AM loading abolished the transient acidification caused by Cch. Summary of the effect of basolateral carbachol on the basolateral AE activity on mean pH_i changes after basolateral Cl^- removal (C), and the rate of reacidification upon Cl^- readdition (D), paired observation, n=3 for each condition. Perfusion of apical carbachol did not affect mean pH_i change (alkalinisation) following Cl^- removal (E) and rate of reacidification after readdition of Cl^- (F) in forskolin stimulated and non-stimulated condition in Calu-3 cells, n=10 for control, n=7 +apical carbachol, n=6 +Fsk, and n=3 +Fsk+apical carbachol. Bilateral carbachol did not affect mean pH_i change following Cl^- removal (G) and the rate of reacidification after readdition of Cl^- (H) in Calu-3 cells, n=3 for each condition. Data are shown as Mean±SEM.*P<0.05 compared to control**.**

Furthermore, in order to test whether the effect of Cch, under Fsk stimulation, was produced via muscarinic or nicotinic receptor stimulation, Calu-3 cell were perfused simultaneously with atropine and Cch, and then basolateral AE activity was measured under resting and Fsk stimulated conditions (Figure 4.15A). Application of atropine and Cch did not change the basolateral Cl^-/HCO_3^- AE activity under resting conditions, and did not abolish the Fsk induced inhibition of the basolateral AE activity (Figure 4.15B and C). However, the Cch induced transient acidification, under Fsk stimulation, was completely eliminated by atropine, verifying that Cch signaling pathway was through muscarinic cholinergic receptors. These results suggest that intracellular elevation of Ca^{2+}, by stimulation of muscarinic receptors, does not regulate basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under both resting and Fsk stimulated conditions.

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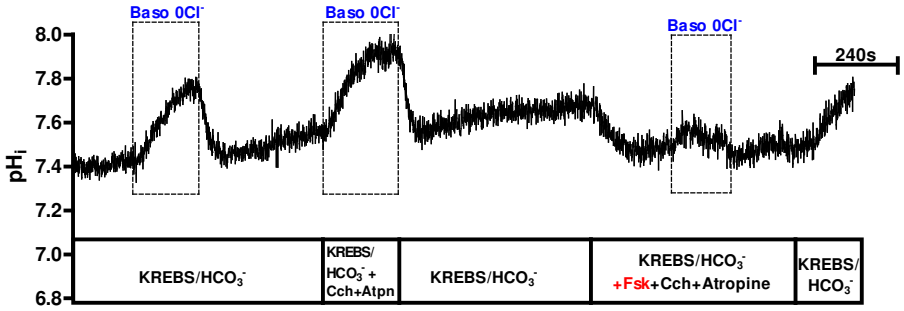


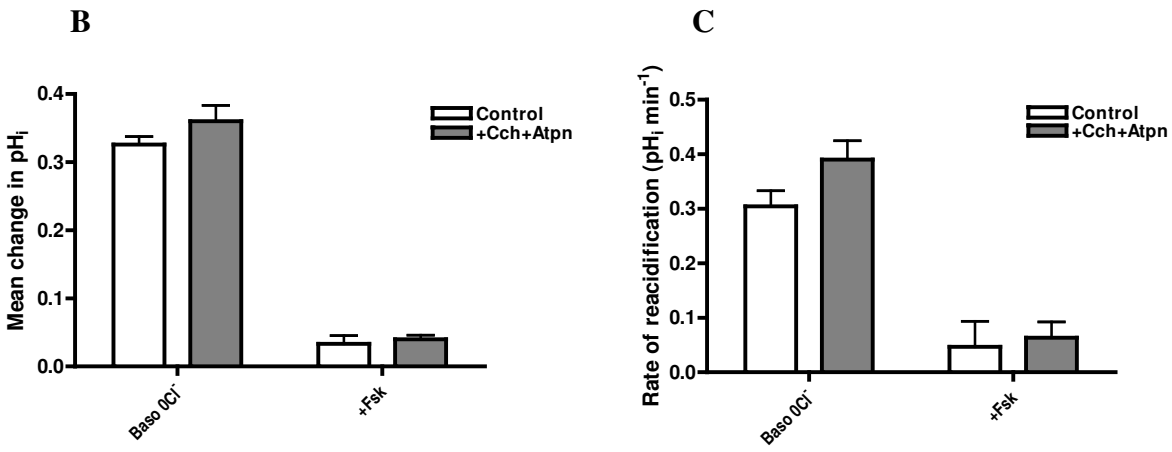


Figure 4. 15: Atropine blocked the transient acidification induced by carbachol in forskolin stimulated Calu-3 cells. (A) Representative pH_i trace showing that apical atropine (10µM), blocked the acidification induced by carbachol, but did not affect the basolateral Cl^-/HCO_3^- AE activity, under resting and Fsk stimulated conditions in Calu-3 cells. Summary of the effect of carbachol and atropine on mean pH_i changes after basolateral Cl^- removal (C), and the rate of reacidification upon Cl^- readdition (D) under resting and Fsk stimulation condition in Calu-3 cells. Data are shown as Mean±SEM, No significant difference (P>0.05) compared to control response, n=7 for control, n=4 for +carbachol+atropine, n=3 for +Fsk and Fsk+carbachol+atropine.

4.6.1.1.2 Thapsigargin

A recent study showed that ER [Ca^{2+}] depletion caused adenylyl cyclase-dependent cAMP production through a Ca^{2+} sensor, STIM-dependent pathway (Maiellaro *et al.*, 2012). In order to assess the effects of Ca^{2+} release from the ER on the basolateral Cl^-/HCO_3^- AE activity, Calu-3 cells were first exposed to 200nM thapsigargin for 5 mins (Figure 4.16A), which previous work from our group has shown that leads to calcium elevation in Calu-3 cells (Garnett *et al.*, 2011). The basolateral Cl^-/HCO_3^- AE response to Cl^- free KREBS solution revealed no significant effect of thapsigargin on mean pH_i change produced by basolateral Cl^- removal, or rate of reacidification upon Cl^- readdition, compared to control Calu-3 cells. In addition, the inhibitory effect of Fsk on basolateral Cl^-/HCO_3^- AE activity was also not changed (Figure 4.16B and C). This suggests

that ER Ca^{2+}-store depletion does not generate sufficient cAMP in Calu-3 cells to inhibit the basolateral anion exchanger.

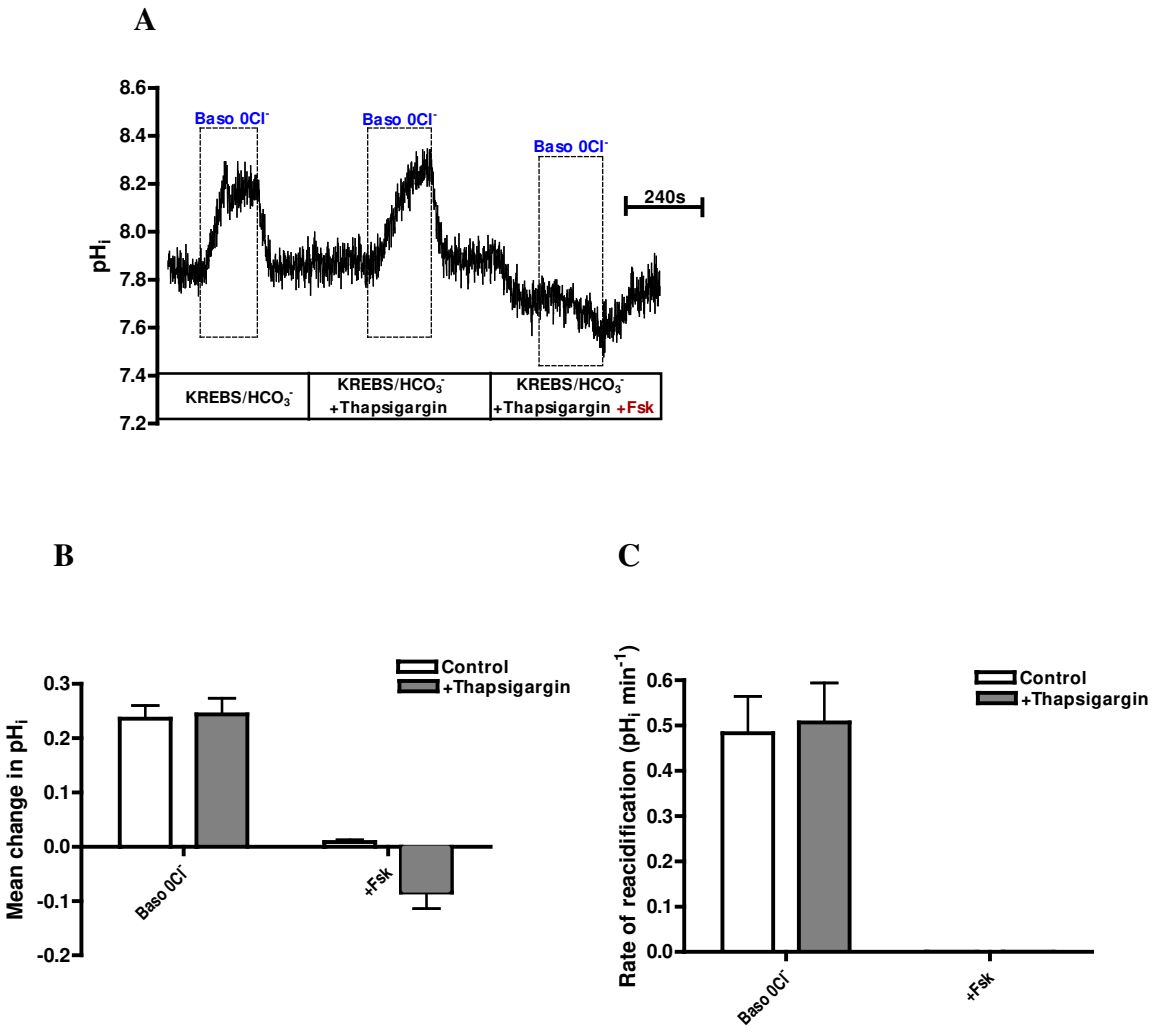


Figure 4. 16: Thapsigargin did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i traces showing the effect of thapsigargin on the basolateral Cl^-/HCO_3^- AE activity under resting and stimulated condition. Summary of the effect of thapsigargin (200nM) on the mean change in pH_i following basolateral Cl^- removal (B) and the rate of reacidification upon readdition of Cl^- (C) under resting and Fsk stimulated condition in Calu-3 cells. Data are shown as Mean±SEM, no significant difference (P>0.05) compared to control, n=10 for Baso 0 Cl^-, and n=7 for +Fsk.

4.6.1.2 Effect of a decrease in $[Ca^{2+}]_i$ on basolateral anion exchange activity, using BAPTA-AM

As mentioned in chapter 3 (section 3.6) since there is a synergistic interaction between Ca^{2+} and cAMP for controlling fluid and electrolyte secretion by epithelial cells, another series of experiments were performed in order to assess the effect of intracellular Ca^{2+} depletion on the resting and the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. For these experiments, Calu-3 cells were preincubated with 50µM BAPTA-AM (both apical and basolateral sides) for one hour and then the basolateral Cl^-/HCO_3^- AE response to Cl^- free solution were measured under resting and cAMP stimulated conditions (Figure 4.17B) and compared to control responses (Figure 4.17A). As shown in Figure 4.18C and D, BAPTA-AM significantly reduced resting basolateral AE activity by 65.1±2.8% (n=8) in the mean pH_i change in response to basolateral Cl^- free solution. It was also noticeable that BAPTA-AM significantly reduced the rate of reacidification, caused by readdition of Cl^- to the basolateral side, by 57.1±8.1% (n=8) compared to control cells, not preincubated with BAPTA-AM. However, BAPTA-AM loaded cells still showed normal forskolin-induced inhibition of the basolateral AE. These results suggest that decreasing intracellular Ca^{2+} plays an important role in regulating the resting level of basolateral Cl^-/HCO_3^- AE activity, but it is not involved in the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

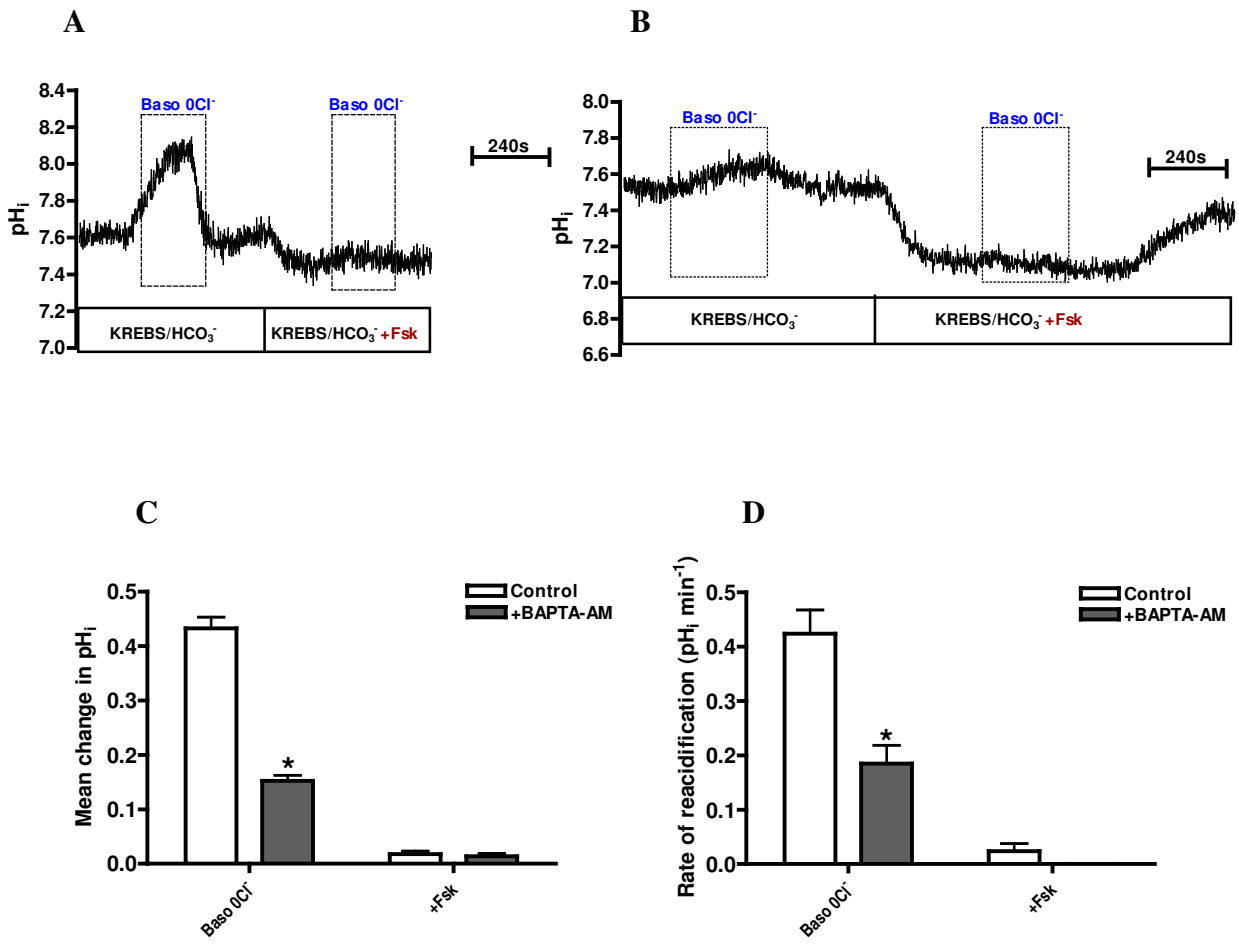


Figure 4. 17: BAPTA-AM induced changes in the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. pH_i experimental trace showing the effect of BAPTA-AM (50µM) preincubation on changes in pH_i following the removal of basolateral Cl^- in Calu-3 cells in the absence and presence of forskolin (B), compared to control response (A). Summary of the effect of BAPTA-AM on mean alkalinisation produced by Cl^- removal (C), and the rate of reacidification upon chloride readdition (D) under resting and stimulated conditions. Data are shown as Mean±SEM, *P<0.05 compared to control. n= 8 for each condition. Control cells run in parallel.

Interestingly, in another set of experiments, perfusion of cells with 200nM thapsigargin in BAPTA-AM treated Calu-3 cells (Figure 4.19A), partially reversed the inhibitory effect of BAPTA-AM on the mean pH_i change in response to Cl^- free perfusion under resting conditions, although the rate of reacidification was still significantly reduced (Figure 4.19B and C). This implies that thapsigargin may be capable of causing a small increase in $[Ca^{2+}]_i$ from a thapsigargin-sensitive store in BAPTA-AM treated cells that was capable of

partially reversing the BAPTA-AM-induced inhibition of the basolateral AE activity. This result reinforces the fact that the normal resting $[Ca^{2+}]_i$ plays an important role in the regulation of the basolateral AE activity in Calu-3 cells.

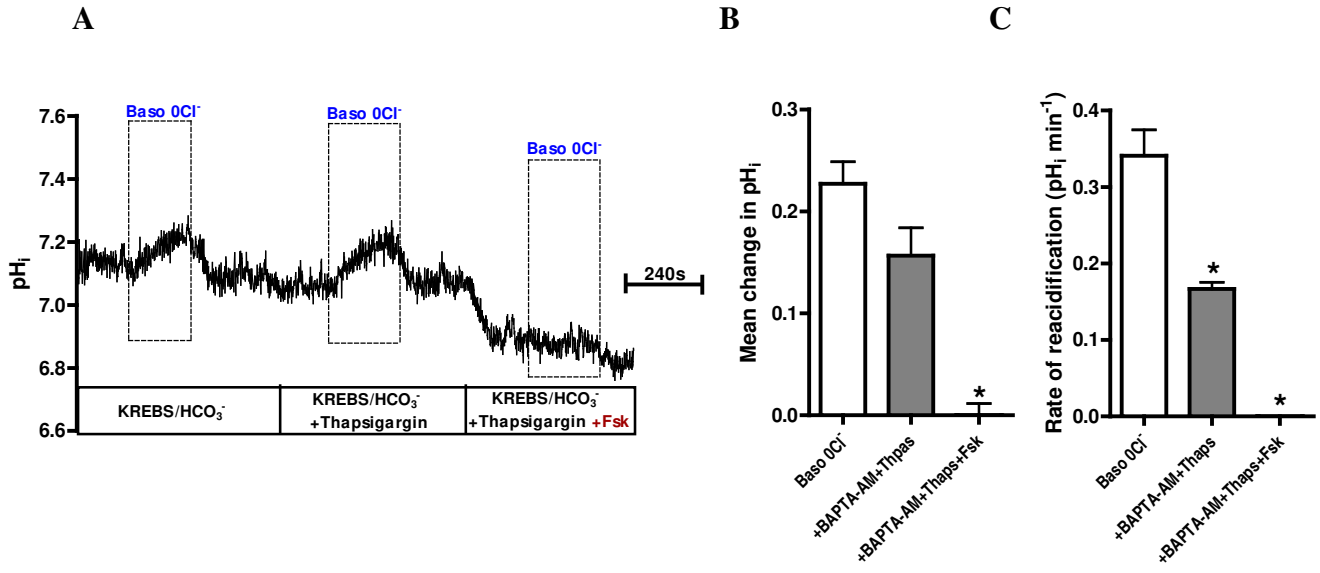
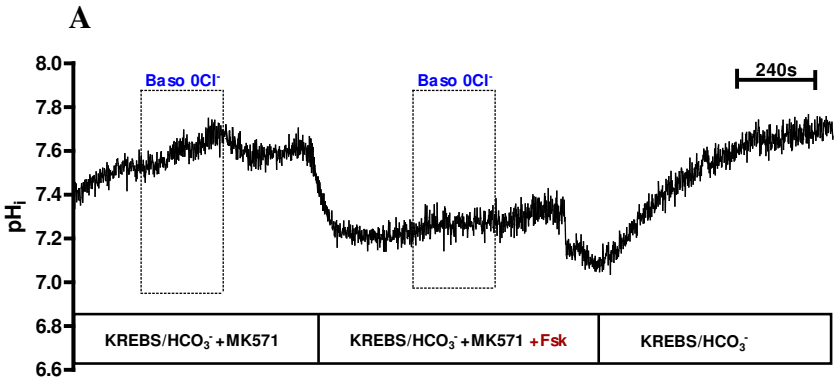


Figure 4. 18: The Effect of thapsigargin and BAPTA-AM on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i traces showing the effect of thapsigargin and BAPTA-AM on the basolateral Cl^-/HCO_3^- AE activity under resting and Fsk stimulated condition in Calu-3 cells. Summary of the effect of BAPTA-AM (50μM) and thapsigargin (200nM) on mean pH_i change (alkalinisation) following basolateral Cl^- removal (B) and the rate of reacidification after readdition of Cl^- (B), under resting and forskolin stimulated conditions in Calu-3 cells. Data are shown as Mean±SEM.*P<0.05 compared to control. n=3 for each, except for control n=10. Control cells run in parallel.

In order to test whether cAMP and Ca^{2+} exert their effects on the basolateral Cl^-/HCO_3^- AE activity through the same pathway, Calu-3 cells were pretreated with the cAMP efflux pump inhibitor, MK571, simultaneously with BAPTA-AM, for 60 min, and then basolateral AE activity measured under both resting and cAMP

stimulated conditions (Figure 4.19A). Results showed that there was a further reduction in basolateral Cl^-/HCO_3^- AE activity under resting conditions, both in the magnitude of alkalinisation produced by basolateral Cl^- removal, and the rate of reacidification upon Cl^- readdition (Figure 4.19B and C) compared to BAPTA-AM on its own (4.17C and D). However, the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity was not changed. As shown in Figure 4.20A and B, preincubation of Calu-3 cells with BAPTA-AM and MRP inhibitor, MK571, caused a marked reduction in the rate of reacidification produced by basolateral Cl^- readdition compared to BAPTA-AM treated only, or MK-571 treated only cells, although the mean pH_i changes showed no-significant change. These data imply that both intracellular elevation of cAMP and decreasing $[Ca^{2+}]_i$ produced their effects on the basolateral Cl^-/HCO_3^- AE activity via distinct mechanisms.



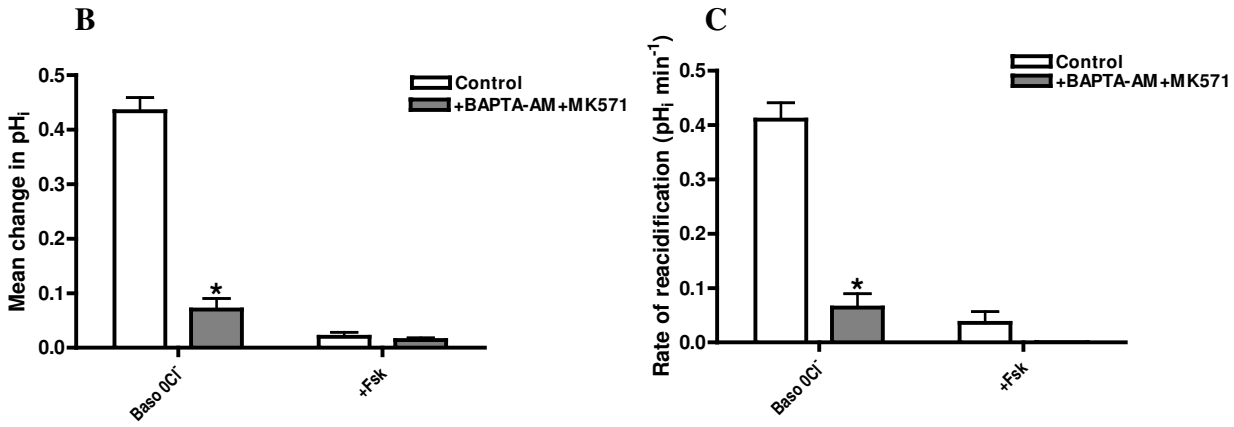


Figure 4. 19: BAPTA-AM+MK571 almost completely inhibited the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Experimental pH_i trace showing the effect of BAPTA-AM (50µM) preincubation and perfusion of MK571 on changes in pH_i following the removal of basolateral Cl^- in Calu-3 cells in the absence and presence of forskolin. Summary of the effect of BAPTA-AM+MK571 on mean alkalinisation produced by chloride removal (B), and the rate of reacidification upon chloride readdition (C). Data are shown as Mean±SEM, *P<0.001 compared to control, n=5 for each condition. Control cells run in parallel.

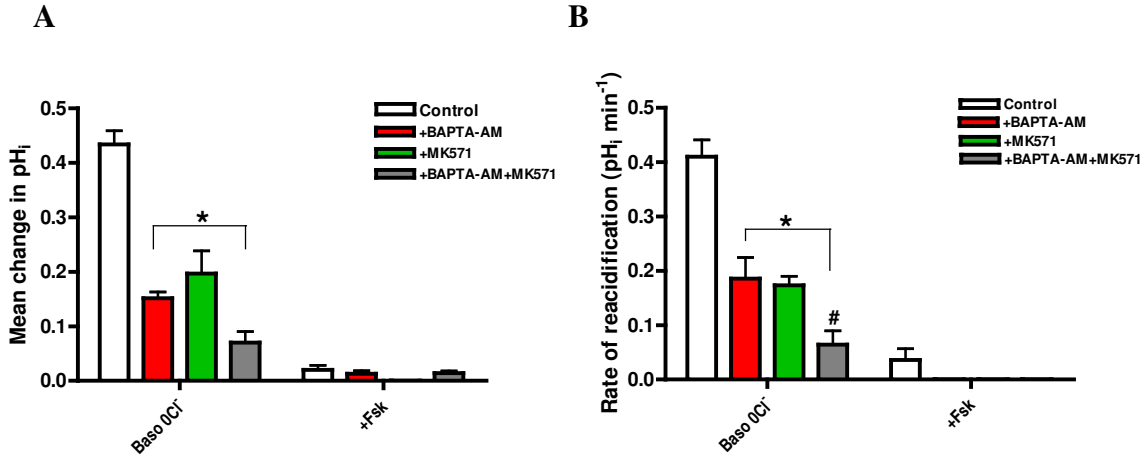


Figure 4. 20: Summary of the effect of BAPTA-AM and MK571 on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Mean alkalinisation (pH_i) produced by chloride removal (A), and the rate of reacidification upon chloride readdition (B). *P<0.001 compared to control, # =Significant difference (P<0.001) compared to MK571, and BAPTA-AM. Data are shown as mean ±SEM. n= 5 for control, n=7 for BAPTA-AM, n=3 for +MK571 and n=5 for BAPTA-AM+MK571.

4.6.2 Effect of changing extracellular Ca^{2+} concentration

The extracellular Ca^{2+} sensing receptor (CaSR) is a G-protein coupled receptor whose main physiological ligand is extracellular Ca^{2+} (Magno *et al.*, 2011). Since it has been found that CaSR plays an important role in the regulation of anion and fluid secretion in human pancreatic ducts (Racz *et al.*, 2002), it was of interest to assess whether extracellular Ca^{2+} mediated signaling pathway regulates the basolateral Cl^-/HCO_3^- AE activity under resting and cAMP stimulated conditions. It has been shown that a decrease in extracellular Ca^{2+} concentration reduces the activity of the CaSR (Brown, 2007). Thus, Calu-3 cells were exposed to Ca^{2+}-free extracellular solution and anion exchange activity measured.

4.6.2.1 Effect of unilateral Ca^{2+} free solutions:

Apical or basolateral perfusion of extracellular Ca^{2+} free HCO_3^-/KREBS solution, in which $CaCl_2$ was replaced with $MgCl_2$ and 0.5mM EGTA added to chelate any remaining Ca^{2+}, did not produce any changes in basolateral AE activity. Also the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- exchanger was not affected (Figure 4.21A-D). This suggest that neither apical or basolateral CaSR mediated signaling pathways were involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

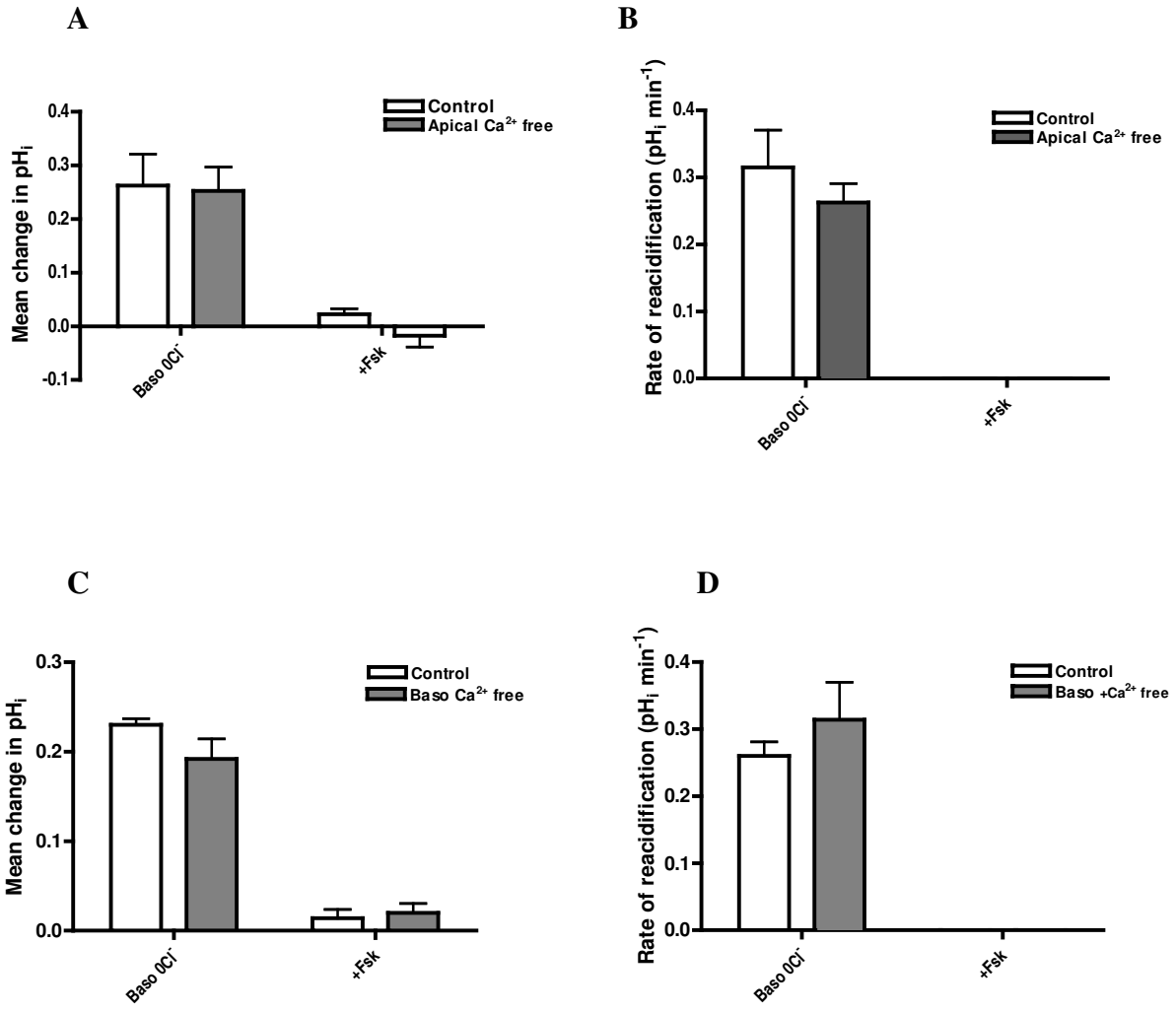


Figure 4. 21: Unilateral Ca^{2+} free solutions did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of apical Ca^{2+} free solution on the basolateral Cl^-/HCO_3^- AE activity on mean pH_i changes produced by basolateral Cl^- removal (A), and rate of reacidification upon Cl^- readdition (B). Data are shown as Mean ±SEM.*P<0.05 compared to Baso $0Cl^-$, n=4, paired observation. Basolateral Ca^{2+} free solution on the mean pH_i change caused by basolateral Cl^- free solution (C), and the rate of re acidification (D) in Calu-3 cells. Data are shown as Mean±SEM. No significant difference (P>0.05) compared to control, n=5, paired observations.

4.6.2.2 Effect of bilateral Ca^{2+} free solutions:

To further investigate the possibility of cross-talk between apical and basolateral CaSR mediated signaling that might affect the activity of the basolateral Cl^-/HCO_3^- AE, Calu-3 cells were perfused bilaterally with a Ca^{2+} free HCO_3^-/KREBS solution (Figure 4.22A). Although resting AE activity was not altered under these conditions there was a significant relief in the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^-AE activity, when mean pH_i changes were compared to the 'control' Fsk stimulated response. However, although the rate of reacidification was partially recovered under cAMP stimulated conditions in treated cells, it was not significant compared to control responses (Figure 4.22B and C) in Ca^{2+} free conditions. This indicates that, to some extent, there is a cross-talk between apical and basolateral CaSR-mediated signaling pathways that regulate the basolateral Cl^-/HCO_3^- AE activity under cAMP stimulated condition in Calu-3 cells. Note that the effect of bilateral Ca^{2+} free conditions on basolateral AE activity did not mirror the response seen in BAPTA-AM treated cells (Figure 4.17), suggesting that the bilateral removal of extracellular Ca^{2+} was not exerting an effect on basolateral AE activity simply by reducing intracellular Ca^{2+} levels.

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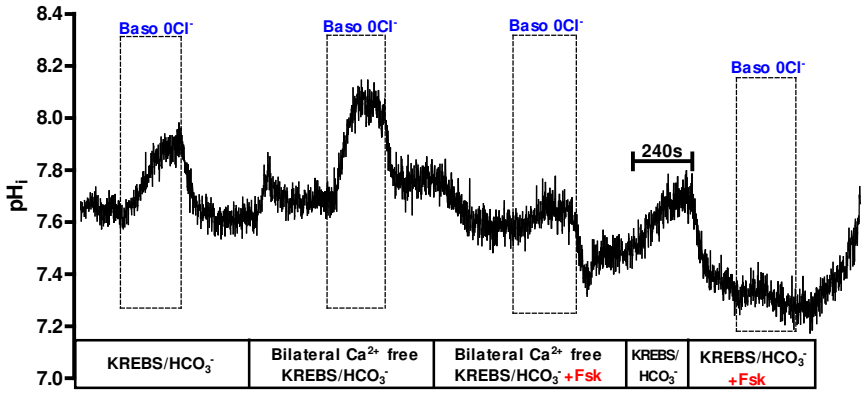


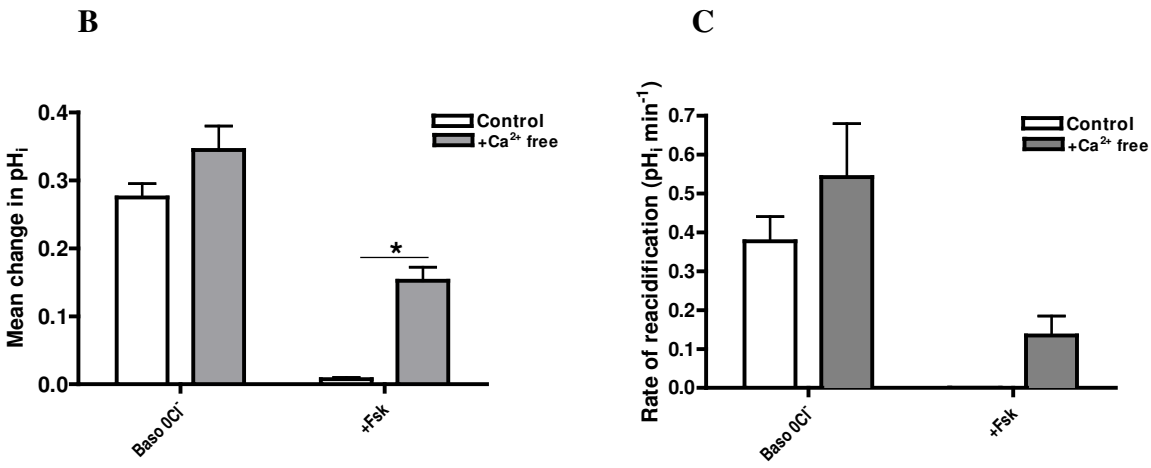
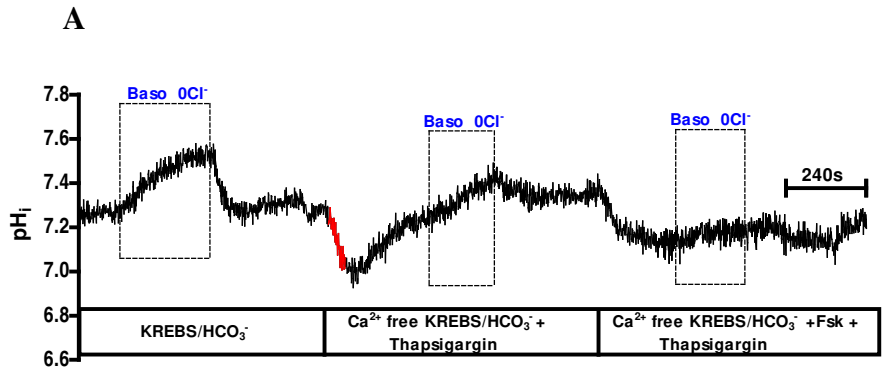


Figure 4. 22: Impact of bilateral Ca^{2+} free solutions on the activity of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i experiment showing the effect of bilateral perfusion of Ca^{2+} free Krebs solution on the basolateral Cl^-/HCO_3^- AE activity. Summary of the effect of bilateral Ca^{2+} free Krebs solution on mean pH_i changes produced by basolateral Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C), in non-stimulated and Fsk stimulated condition in Calu-3 cells. Data are shown as Mean ±SEM.*P<0.05 compared to +Fsk, n=4 for each condition, paired observation.

Although the resting basolateral Cl^-/HCO_3^- AE activity was not significantly changed in the absence of extracellular Ca^{2+}, it has been found that there is intracellular cAMP production, through stimulation of AC (independent of PDEs), by ER Ca^{2+}-store depletion in the absence of extracellular Ca^{2+}, via the ER potential Ca^{2+}-sensor STIMI, that couples ER-Ca^{2+} to cAMP production (Lefkimmiatis *et al.*, 2009). Thus, I performed a set of experiments in Calu-3 cells to assess the activity of the basolateral Cl^-/HCO_3^- AE in the absence of extracellular Ca^{2+-}, but after ER Ca^{2+}-store depletion. ER stores were depleted using thapsigargin and basolateral AE activity was measured, under resting and cAMP stimulated conditions. As shown in Figure 4.23A, basolateral Cl^-/HCO_3^- AE was significantly decreased, both the magnitude of alkalinisation produced by basolateral Cl^- removal, and the rate of reacidification following Cl^- readdition, compared to the control response (Figure 4.23B and C). This result is consistent with my previous results which clearly showed that an increase in

[cAMP]$_i$ is responsible for inhibition of the basolateral AE activity in Calu-3 cells. This suggest that ER Ca^{2+}-store depletion and removal of extracellular Ca^{2+} leads to an increase in [cAMP]$_i$. Interestingly, a transient intracellular acidification was produced following removal of extracellular Ca^{2+} and ER Ca^{2+}-store depletion (Figure 4.23A, red trace), which might be due to an elevation of intracellular cAMP and stimulation of CFTR/Pendrin activity, which would lead to HCO_3^- efflux across the apical membrane. Although removal of extracellular Ca^{2+} partially reduced the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE (see Figure 4.22A and B), the cAMP-induced inhibition was not changed when ER Ca^{2+}-store depletion occurred in the absence of extracellular Ca^{2+} (Figure 4.23). This might be due to the absence of extracellular Ca^{2+} alone, which could affect the rate of intracellular cAMP production by AC compared to complete inhibition of the basolateral Cl^-/HCO_3^- AE by cAMP under both ER Ca^{2+}-store depleted and extracellular Ca^{2+} free conditions.



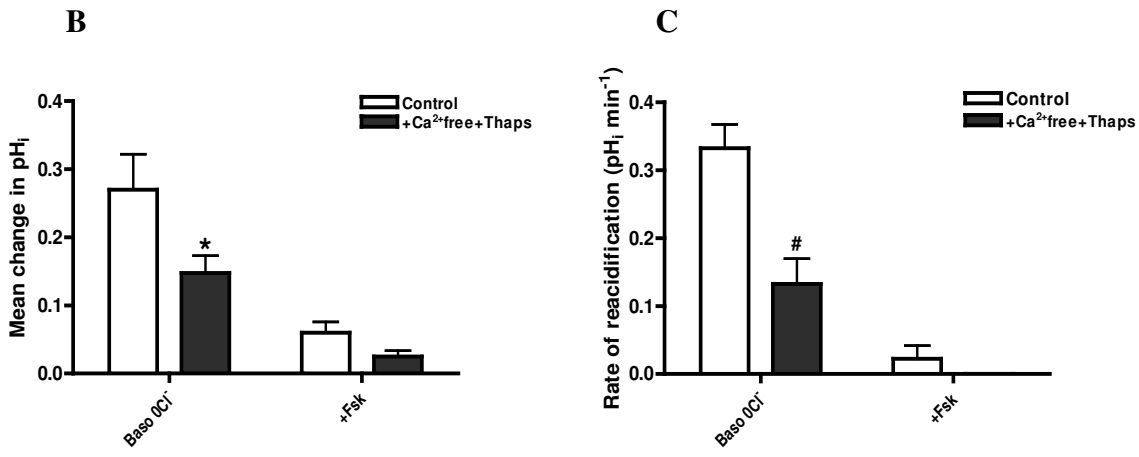


Figure 4. 23: Bilateral Ca^{2+} free solution and thapsigargin reduced the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i experiment shows impact of bilateral perfusion of Ca^{2+} free Krebs solution on the basolateral Cl^-/HCO_3^- AE activity in the presence of 200nM thapsigargin. Summary of the effect of bilateral Ca^{2+} free Krebs solution and 200nM thapsigargin on mean pH_i changes produced by basolateral Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C), under resting and Fsk stimulated Calu-3 cells. Data are shown as Mean ±SEM.*P<0.05, #P<0.001 compared to Baso $0Cl^-$, n=4 for each condition, paired observations.

4.7 Role of CaM and Ca^{2+}/CaM-dependent protein kinases in the regulation of basolateral Cl^-/HCO_3^- AE activity

Intracellular changes in Ca^{2+} are often decoded by the cell through Ca^{2+} binding proteins such as calmodulin (CaM), which play an important role in signal transduction and regulation of a wide range of cellular responses. Ca^{2+} and CaM modulate the activity of several intracellular serine/threonine protein kinase cascades, including CaM-kinase kinase (CaMKK), CaMKI and CaMKIV. Moreover, it has been shown that binding of Ca^{2+}/CaM complex enhances membrane adenylyl cyclase activity, which is accompanied by elevation of intracellular cAMP (Ferguson and Storm, 2004). According to sequence analysis of SLC4A2, there are a number of putative Ca^{2+}/calmodulin-dependent protein kinase (CaMKI, CaMKII and CaMKIV) phosphorylation sites present. Since it is unknown whether these kinases could be involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity under resting and cAMP stimulated conditions, another set of experiments were done to test the impact of these kinases on basolateral AE activity.

4.7.1.1 Role of calmodulin (CaM) in the regulation of basolateral Cl^-/HCO_3^- AE activity

To focus on the mechanism of regulation of the basolateral AE activity by a decrease in intracellular Ca^{2+}, the role of CaM was tested. For these experiments Calu-3 cells were preincubated apically for 60 min with N-(8-aminooctyl)-5-iodonaphthalene-1-sulfonamide (J-8, 50μM), which is a highly specific CaM inhibitor (Tian *et al.*, 2011), and then the basolateral Cl^-/HCO_3^- AE activity was assessed using Cl^- free solutions under resting conditions. Interestingly, CaM inhibition caused a marked reduction in both the mean pH_i alkalinisation produced by basolateral Cl^- removal and the rate of reacidification following Cl^- readdition, compared to control cells, while the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity was not affected (Figure 4.24A and B). CaM inhibition also significantly decreased the rate of HCO_3^- flux produced by basolateral Cl^- removal, compared to untreated cells (Figure 4.24C). This implies that CaM plays an important role in the regulation of the basolateral Cl^-/HCO_3^- AE activity under resting conditions in Calu-3 cells.

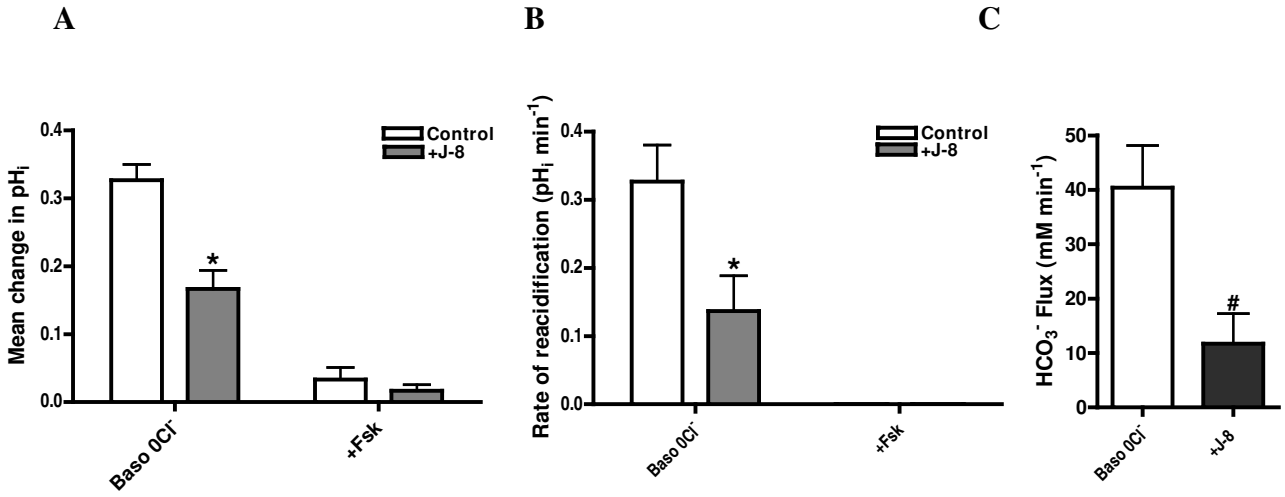


Figure 4. 24: Calmodulin inhibitor, J-8, inhibited basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting conditions. Summary of the effect of apical preincubation of 50μM J-8 for 60 min on mean alkalinisation in pH_i in response to basolateral Cl^- removal (A) and the rate of reacidification upon Cl^- readdition (B) under resting and Fsk stimulated conditions. (C) The rate of HCO_3^- flux during Cl^- readdition in Calu-3 cells. Data are shown as Mean±SEM.*P<0.01, #P<0.05 compared to control, n=3 for each condition, experiments run in parallel.

To further investigate whether the inhibitory impact of CaM inhibition on basolateral AE activity could be changed under conditions where intracellular cAMP was elevated, Calu-3 cells were preincubated with J-8 and then perfused with the cAMP efflux inhibitor, MK571, and basolateral Cl^-/HCO_3^- AE activity was measured with and without cAMP stimulation (Figure 4.25A). Compared to untreated cells (Figure 4.25B), results showed that J-8 and MK571 caused a very significant inhibition of the basolateral Cl^-/HCO_3^- AE activity under resting conditions, but did not change the subsequent Fsk-induced inhibition of the remaining basolateral Cl^-/HCO_3^- AE activity (Figure 4.25A, C and D). As shown in Figure 4.26B, treatment of Calu-3 cells with J-8 and MK572 simultaneously caused a marked decrease in the rate of reacidification produced by basolateral Cl^-/HCO_3^- AE activity, which was nearly abolished, compared to Calu-3 cells treated with MK571 or J-8 alone. However, the mean pH_i change produced by basolateral Cl^- removal, and cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity was not changed (Figure 4.26A and B). This suggests that CaM and cAMP control the basolateral Cl^-/HCO_3^- AE activity via separate regulatory mechanisms.

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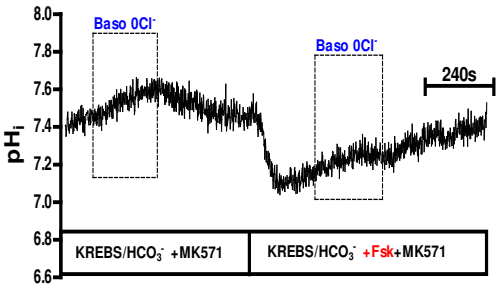

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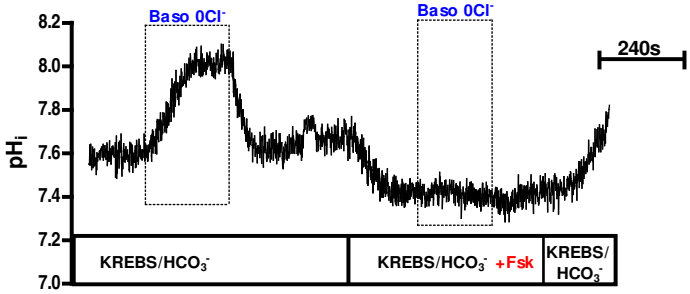


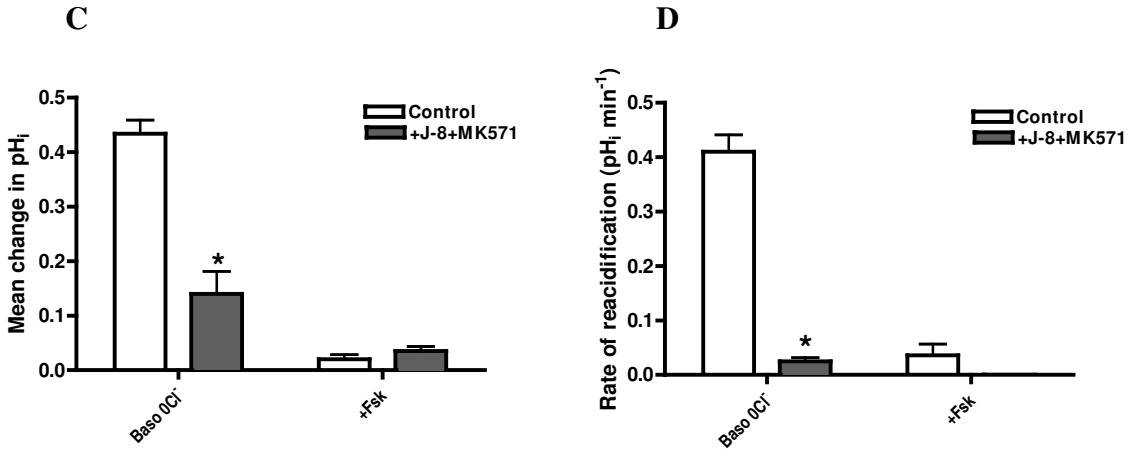


Figure 4. 25: Impact of J-8+MK571 on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Representative pH_i trace showing the effect of J-8 and MK571 on the basolateral Cl^-/HCO_3^- AE activity compared to control response (B) under resting and Fsk stimulated condition. Summary of the effect of apical perfusion of MRP inhibitor, MK572 in Calu-3 cells preincubated with 50µM J-8 for 60 min on mean change in pH_i in response to basolateral Cl^- removal (C) and the rate of reacidification upon Cl^- readdition (D) under resting and Fsk stimulated conditions. Data are shown as Mean±SEM.*P<0.001 compared to control, n=5 for control, and n=4 for J-8+MK571.

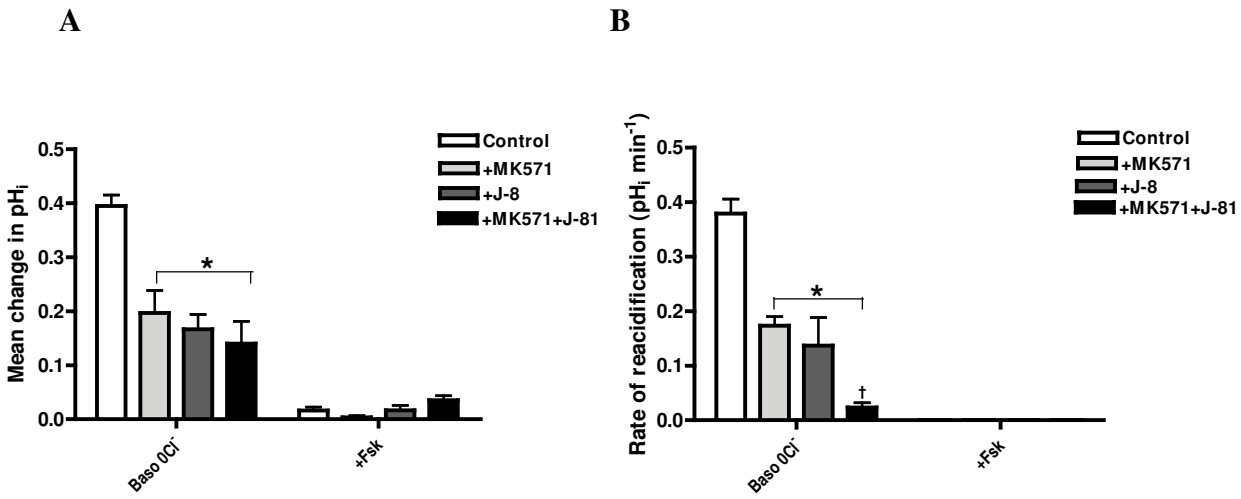


Figure 4. 26: Impact of MK571, J-8 and J-8+MK571 on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. (A) Mean alkalinisation in pH_i in response to basolateral Cl^- free solution, (B) the rate of reacidification upon Cl^- readdition under resting and Fsk stimulated conditions. Data are shown as Mean±SEM.*P<0.001, †P<0.01 compared to MK571 and †P<0.05 compared to +J-8, n=10 for control, n=3 +K571, n=4 for +J-8, and n= 4 J-8+K571.

4.7.1.2 Inhibition of CaMKK by STO-609

It has been shown that the activity of calcium–activated Cl^- channels was abolished by inhibition of the CAMK pathway (using the CAMKK inhibitor STO-609) in *Xenopus oocytes*, while it did not affect CFTR activity (Faria D., 2012 PhD thesis, University of Lisbon). In order to test the role of CaMKK in the regulation of the basolateral Cl^-/HCO_3^- AE activity, Calu-3 cells were preincubated with 20μM STO-609 for 60 min, and then basolateral Cl^-/HCO_3^- AE activity was measured in response to basolateral Cl^- removal under resting and cAMP stimulated conditions. As shown in Figure 4.27A and B, inhibition of the CaMKK mediated signaling pathway did not affect the resting basolateral Cl^-/HCO_3^- AE activity, and did not abolish the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. This suggests that CaMKI and CaMKIV are not involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

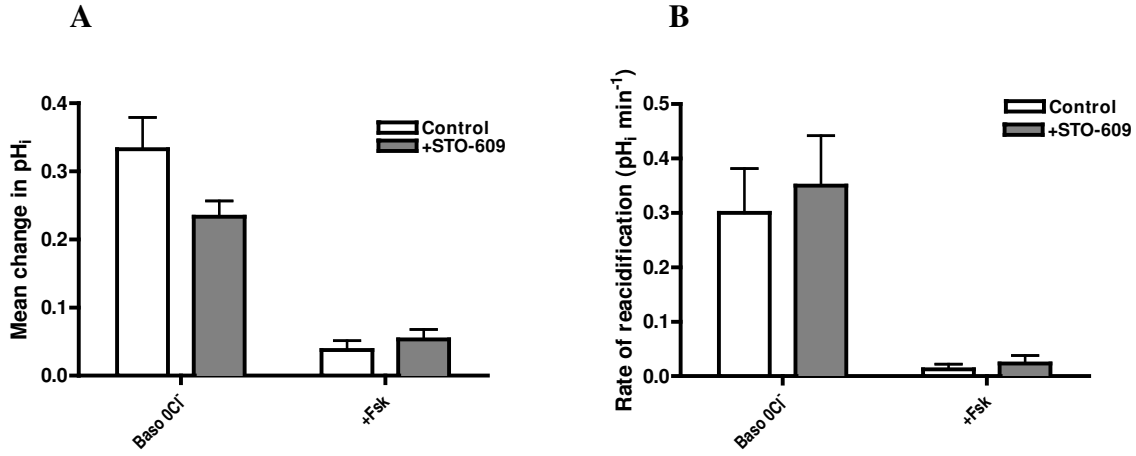


Figure 4. 27: CAMKK inhibitor, STO-609, did not affect the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of apical preincubation of 20μM STO-609 for 60 min on mean alkalinisation in pH_i in response to basolateral Cl^- removal (A) and the rate of reacidification upon Cl^- readdition under resting and Fsk stimulated conditions (B). Data are shown as Mean±SEM. No significant difference ($P>0.05$) compared to Baso $0Cl^-$, n=3 for each, experiments run in parallel.

4.7.1.3 Inhibition of CaMKII by KN-93

It has also been found that CAMKII plays an important role in the regulation of membrane Cl^- permeability through Cl^- channels in a variety of epithelial cells (Hartzell *et al.*, 2005). To further investigate the mechanism of CaM-dependent regulation of the basolateral Cl^-/HCO_3^- AE, Calu-3 cells were preincubated with KN-93 (5μM), a CaMKII-specific inhibitor (Namkung *et al.*, 2010). Results showed that CaMKII inhibition did not affect the resting basolateral Cl^-/HCO_3^- AE activity, nor affect the cAMP-induced inhibition of the basolateral AE, although the rate of reacidification was increased in treated cells, compared to control cells (Figure 4.28A and B). These data indicate that CAMKII was not significantly involved in maintaining resting activity, nor was it involved in the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Together, the above data imply that inhibition of the basolateral Cl^-/HCO_3^- AE activity by decreasing $[Ca^{2+}]_i$ is through a CaM dependent, but CaMK-independent, signaling pathway in Calu-3 cells.

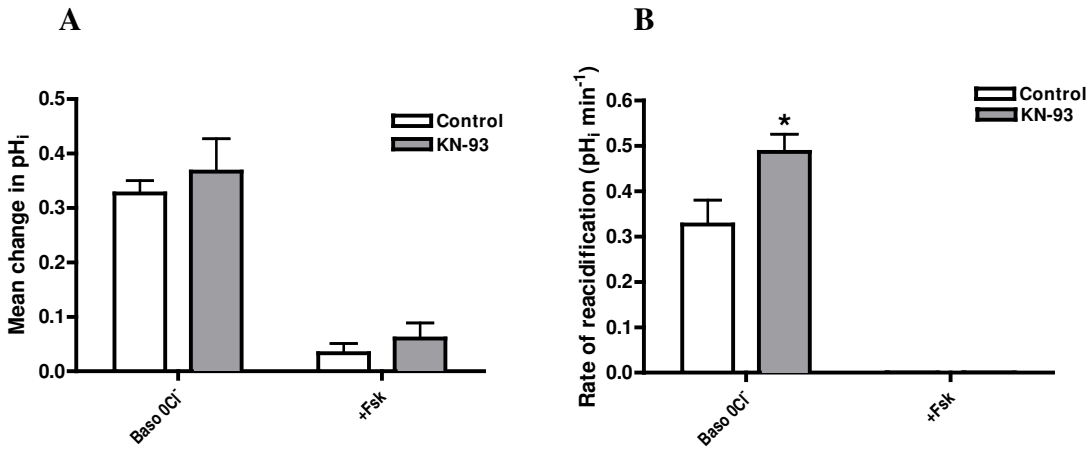


Figure 4. 28: No impact of the CaMKII inhibitor, KN-93, on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of apical preincubation of 5μM KN-93 for 60 min. (A) mean alkalinisation in pH_i in response to basolateral Cl^- removal, and the rate of reacidification following Cl^- readdition (B), under resting and Fsk stimulated condition. Data are shown as Mean±SEM.*P<0.05 compared to control, n=3 for each condition. Control cells run in parallel.

4.8 Regulation of basolateral Cl^-/HCO_3^- anion exchanger activity by dynamin

In order to further investigate the mechanism of Ca^{2+}/CaM-mediated regulation of the basolateral AE activity in Calu-3 cells, the role of dynamin was assessed, because there is a Ca^{2+}-sensing mechanism that regulates dynamin mediated endocytosis (Lai *et al.*, 1999). My previous results showed that inhibition of dynamin, using dynasore, significantly reduced the activity of the apical Cl^-/HCO_3^- AE in Calu-3 cells (see chapter 3, section 3.7). Also, experimental evidence has shown that dynamin plays a critical role in membrane fission of Golgi-derived vesicles in the trans-face of the Golgi network, through an actin-dependent mechanism, and interference with dynamin function inhibits post Golgi protein transportation (Kerkhoff *et al.*, 2001; Carreno *et al.*, 2004; Kessels and Qualmann, 2004; Praefcke and McMahon, 2004; Cao *et al.*, 2005; Kessels *et al.*, 2006). However, it is unknown whether dynamin is involved in the regulation of the basolateral anion exchanger. Thus, Calu-3 cells were preincubated with dynasore for 60 min, and then basolateral Cl^-/HCO_3^- AE activity was measured in Cl^- free solutions. As shown in Figure 4.29A and B, dynamin inhibition caused a marked reduction in the activity of the basolateral Cl^-/HCO_3^- AE activity, both in the magnitude of alkalinisation produced by basolateral Cl^- removal, as well as the rate of reacidification following Cl^- readdition, compared to control cells. However, dynamin inhibition did not alter the Fsk-induced inhibition of the remaining basolateral AE activity. This suggests that dynamin plays an important role in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting conditions, but probably not in the cAMP-induced inhibition of AE activity.

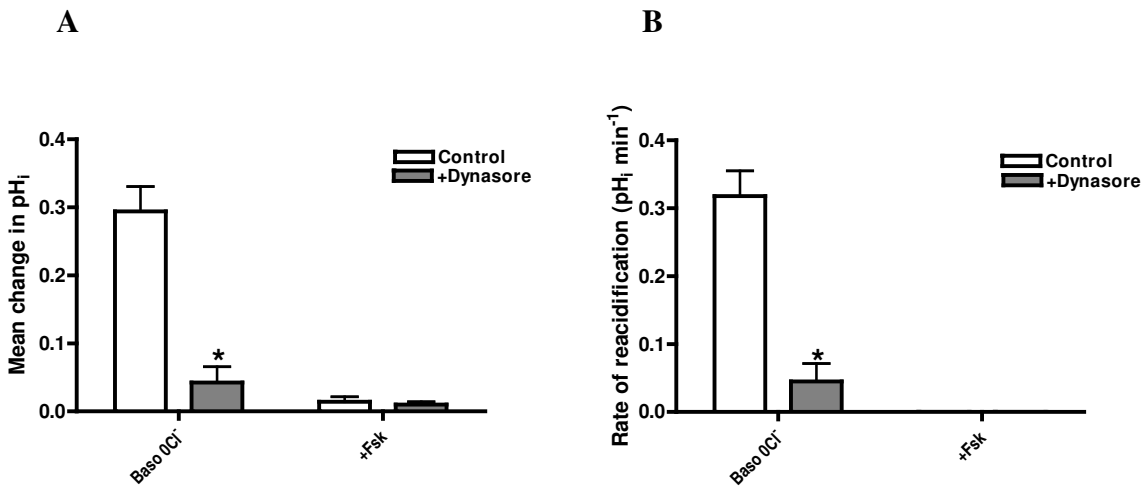


Figure 4. 29: **Inhibition of dynamin reduced basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.** Summary of the effect of dynamin disruption, by preincubation of cells with 80µM dynasore for 60 min, on mean alkalinisation in pH_i in response to basolateral Cl^- free removal (A) and the rate of reacidification following Cl^- readdition (B) under resting and Fsk stimulated conditions in Calu-3 cells. Data are shown as mean ±SEM, *P<0.001 compared to control, n=5 for Control, and n=4 for +Dynasore. Control cells run in parallel.

4.9 Role of the actin-cytoskeleton in the regulation of basolateral Cl^-/HCO_3^- anion exchanger activity

Since the actin cytoskeleton can bind directly to dynamin, through its actin binding domain (Gu et al., 2010), and dynamin inhibition significantly reduced the basolateral Cl^-/HCO_3^- AE activity (see Figure 4.29), my hypothesis was that an intact actin cytoskeleton would be essential for maintaining basolateral Cl^-/HCO_3^- AE activity. My hypothesis was further corroborated by the most recent finding that actin filaments participate in trafficking pathways of the secretory membrane vesicles from trans-Golgi network to the plasma membrane, and play an important role in the regulation of some ion pumps/channels (Egea *et al.*, 2015). To test this hypothesis, Calu-3 cells were preincubated with CytoD for 60 min, and then basolateral Cl^-/HCO_3^- AE activity was measured in response to basolateral Cl^- free solutions. Results showed that disruption of the actin cytoskeleton by CytoD significantly decreased the activity of the basolateral Cl^-

/HCO_3^- AE, both in the magnitude of alkalinisation produced by basolateral Cl^- removal, as well as the rate of reacidification following Cl^- readdition, compared to untreated cells. However, CytoD did not affect the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity after Fsk stimulation (Figure 4.30A and B). This implies that an intact actin-cytoskeleton plays an essential role in the resting activity of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells, but like dynamin, not in the cAMP-induced inhibition of AE activity.

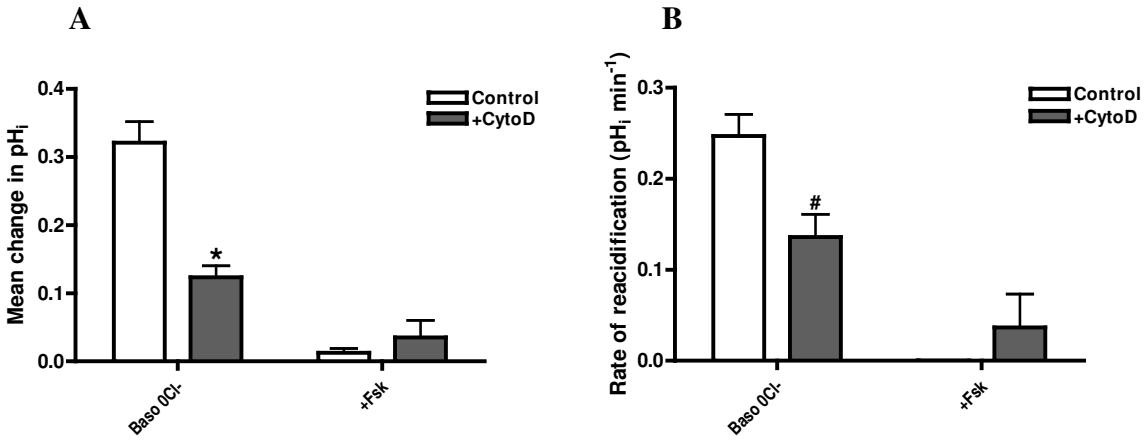


Figure 4. 30: Cytochalasin-D reduced basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. Summary of the effect of actin disruption, by preincubation of Calu-3 cells with 20µM CytoD for 60 min, on mean pH_i change (alkalinisation) caused by basolateral Cl^- removal (A) and the rate of reacidification upon Cl^- readdition (B) under resting and Fsk stimulated conditions. Data are shown as Mean±SEM. *P<0.001, #P<0.01 compared to Baso $0Cl^-$, n=10 for control Baso $0Cl^-$, n=12 for Baso $0Cl^-$+Cytochalasin-D, and n=4 for Control +Fsk.

4.10 Regulation of basolateral Cl^-/HCO_3^- anion exchanger activity by CK2

In order to further investigate the mechanism behind Ca^{2+}/CaM regulation of the basolateral Cl^-/HCO_3^- AE activity under resting conditions, another set of experiments were performed to assess the impact of CK2 on the basolateral AE activity as it has been reported that CK2 is the main serine/threonine kinase both

in vivo and in vitro that can phosphorylate CaM (Arrigoni *et al.*, 2004). Also, a recent study by Kang *et al*., (2014) has shown that phosphorylation of CaM by CK2 strengthens binding of CaM with the M-type potassium channel (Kang *et al.*, 2014). According to sequence analysis of SLC4A2 (see appendix), there are a number of potential CK2 phosphorylation sites present. It has been shown that CK2 plays an important role in the regulation of ion channels such as CFTR, and experimental evidence revealed that if CK2 is inhibited, PKA cannot activate CFTR (Mehta, 2008). My previous results showed that CK2 inhibition significantly reduced the apical Cl^-/HCO_3^- AE in Calu-3 cells (Chapter 3, Figure 3.19); however, it was unknown whether CK2 regulates the basolateral Cl^-/HCO_3^- AE activity under resting and/or cAMP stimulated conditions.

4.10.1 Effect of the CK2 inhibitor TBB:

In order to define the role of CK2 in the Ca^{2+}/CaM mediated regulation of the basolateral Cl^-/HCO_3^- AE activity, I first assessed the effect of CK2 inhibition, using TBB, on the mean pH_i change following Cl^- removal from the basolateral side of Calu-3 cells. For these experiments, cells were preincubated with 10μM TBB in both apical and basolateral compartments, for 60 min, and then perfused with KREBS solution containing TBB (Figure 4.31A). TBB caused a significant decrease in resting pH_i (6.8+0.08, $P<0.05$, n=5) compared to control untreated cells (7.5±0.07, $P<0.05$, n=6), which was recovered after washing off of the TBB. Results showed that CK2 inhibition caused a significant reduction in the magnitude of alkalinisation produced by basolateral Cl^- removal, and the rate of reacidification following Cl^- readdition, compared to control cells, while it did not affect the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity (Figure 4.31B and C). Inhibition of the basolateral AE activity, caused by CK2 inhibition, completely recovered after 25 min of washing off the TBB (Figure 4.31D and E), and was not significantly different to untreated cells. Also, CK2 inhibition caused a marked reduction in the rate of HCO_3^- flux produced by basolateral Cl^- removal AE activity compared to control untreated cells (Figure 4.31F).

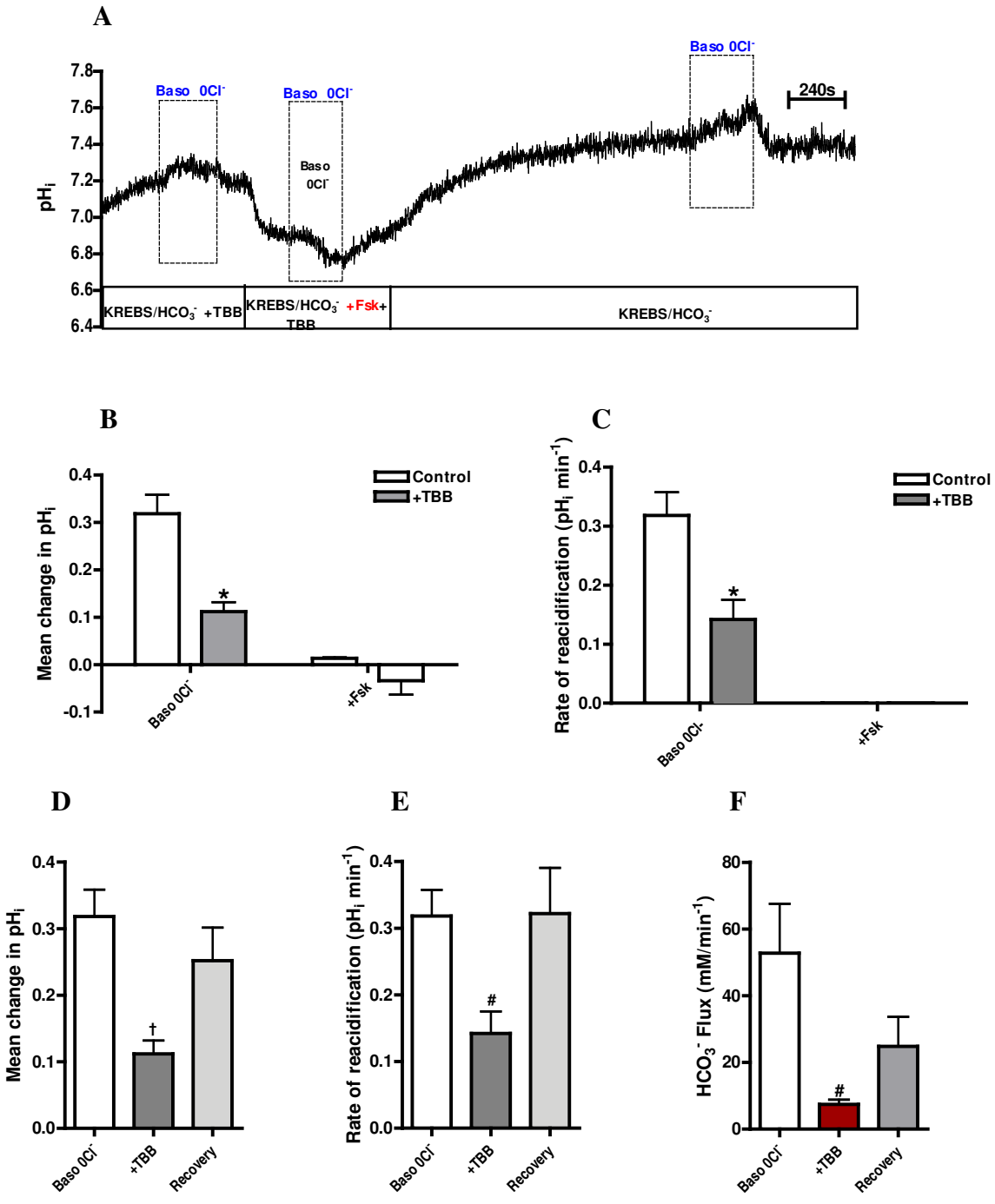
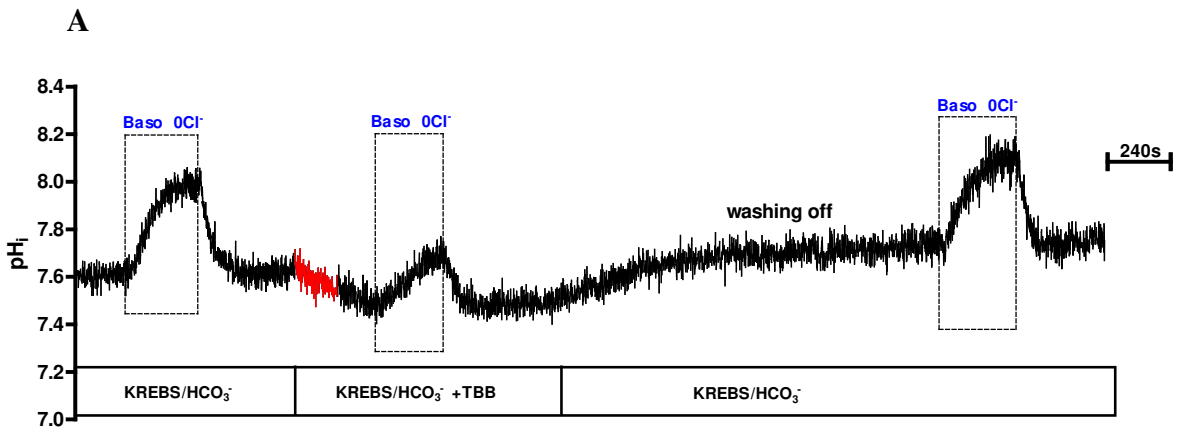


Figure 4. 31: Inhibition of basolateral Cl^-/HCO_3^- AE activity by TBB in Calu-3 cells. (A) Raw pH_i trace showing the effect of TBB preincubation and perfusion on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting and cAMP stimulated conditions. The inhibitory effect of TBB was recovered after 25 min washing off. Summary of the effect of CK2 inhibitor TBB (10µM preincubation and perfusion) on the basolateral AE activity on mean alkalinisation (pH_i) produced by basolateral Cl^- removal (B) and the rate of reacidification upon Cl^- readdition (C) under resting and Fsk stimulated conditions. Recovery of TBB-induced inhibition of the basolateral AE activity, both in the mean alkalinisation (D), and the rate of reacidification (E), in Calu-3 cells. (F) The rate of HCO_3^- flux in control and TBB treated cells. Data are shown as Mean±SEM. *P<0.001, #P<0.05 compared to control, n=6 for control, and n=5 for +TBB. Control cells run in parallel.

To further investigate the temporal effects of TBB-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity, Calu-3 cells were instead acutely exposed to KREBS/HCO_3^- solution containing 10µM TBB to both the apical and basolateral compartments, and then basolateral Cl^-/HCO_3^- AE activity measured in response to Cl^- free solutions (Figure 4.32.A). Acute TBB exposure caused an intracellular acidification (Figure 4.32A, red trace) and mean pH_i was significantly decreased from 7.59±0.02 to 7.49±0.01 ($P<0.05$, n=6) after TBB exposure. In addition there was a significant decrease in both the magnitude of alkalinisation produced by basolateral Cl^- removal, and the rate of reacidification upon Cl^- readdition compared by TBB exposure compared to control responses. Inhibition of the basolateral AE activity, caused by CK2 inhibition, completely recovered after 25 min washing off of the TBB (Figure 4.32B and C). Since the mean pH_i was within the normal range after TBB exposure, it was unlikely that pH_i itself caused the decrease in basolateral AE activity. CK2 inhibition also led to a marked reduction in the rate of HCO_3^- flux, compared to control untreated cells (Figure 4.32D). This suggests that CK2 is important to maintain the activity of the basolateral Cl^-/HCO_3^- AE under resting conditions.



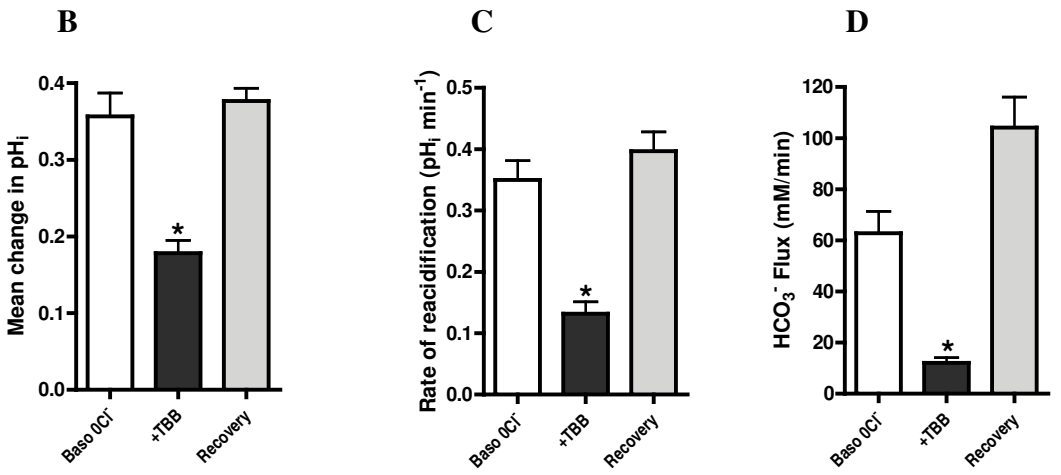


Figure 4. 32: Inhibition of basolateral Cl^-/HCO_3^- AE activity by acute exposure to TBB in Calu-3 cells. (A) Representative pH_i trace showing the effect of acute bilateral TBB exposure (10µM) on the basolateral Cl^-/HCO_3^- AE activity under resting conditions in Calu-3 cells. Note that washing off the inhibitor led to recovery of the basolateral Cl^-/HCO_3^- AE activity. Summary of the effect of acute exposure to TBB (10µM) on the mean alkalinisation (pH_i) produced by basolateral Cl^- removal (B) and the rate of reacidification upon Cl^- readdition (C) in control and TBB treated Calu-3 cells. (D) HCO_3^- efflux in control and TBB treated cells, basolateral Cl^-/HCO_3^- AE activity reversed after 25 min washing off the CK2 inhibitor. Data are shown as Mean±SEM.*P<0.001 compared to control and recovery, n=6 for each condition, paired observations.

In order to further investigate whether CK2 regulation of the basolateral Cl^-/HCO_3^- AE activity was direct or through CaM, in another series of experiments, Calu-3 cells were preincubated with the CaM inhibitor J-8 for 60 min, and then acutely exposed to bilateral TBB, and then the basolateral Cl^-/HCO_3^- AE activity was measured in response to Cl^- free solutions (Figure 4.33A). As shown in Figure 4.33B and C, TBB did not further decrease the activity of the basolateral AE in the presence of CaM inhibitor, with respect to both magnitude of alkalinisation produced by basolateral Cl^- removal, or the rate of reacidification following Cl^- readdition, compared to control responses. Also, TBB did not cause a further reduction in the % inhibition in the rate of reacidification in the presence of CaM inhibitor, but did it further reduce the % inhibition in mean pH_i change (Figure 4.33D and E). These results suggest that CK2 potentially controls the resting activity of the basolateral Cl^-/HCO_3^- AE through the downstream target CaM, in Calu-3 cells.

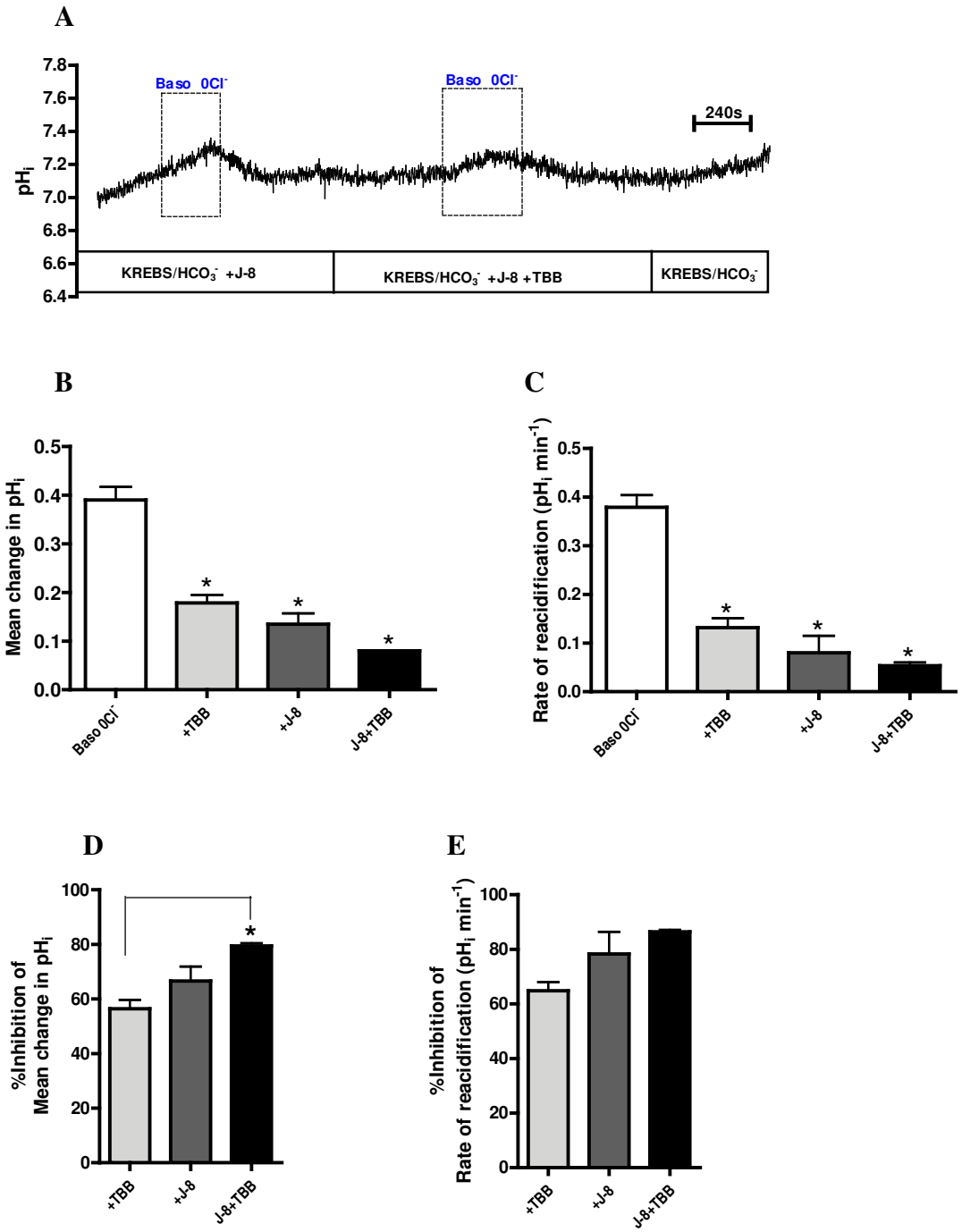


Figure 4. 33: Inhibition of basolateral Cl^-/HCO_3^- AE activity by J-8 preincubation and acute TBB exposure in Calu-3 cells. (A) Representative pH_i trace showing the effect of acute bilateral TBB exposure on the basolateral Cl^-/HCO_3^- AE activity in preincubated cells with J-8 under resting condition in Calu-3 cells. Summary of the effect of acute TBB exposure in J-8 preincubated Calu-3 cells on mean alkalinisation (pH_i) produced by basolateral Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C). % inhibition in mean pH_i change (D), and the rate of reacidification (E), in Calu-3 cells treated with TBB or J-8 alone, compared to J-8 and TBB treated cells. Data are shown as Mean±SEM.*P<0.001 compared to control, n=9 for control, n=6 for TBB and +J-8, and n=3 for TBB+J-8.

4.10.2 Effect of the CK2 inhibitor CX4945

In order to further investigate the role of CK2 in the regulation of basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, I used another CK2 inhibitor, 5-(3-chlorophenylamino) benzo[c][2,6]naphthyridine-8-carboxylic acid (CX4945), which has recently been shown to be a potent and selective ATP-competitive inhibitor of CK2 (Pierre *et al.*, 2011). Calu-3 cells were preincubated with bilateral 10μM CX4945 for 60 min, and then perfused with CX4945 containing KREBS solution (Figure 4.34A). CX4945 also caused a significant decrease in mean pH_i (6.8±0.05, n=3) compared to untreated cells (7.4±0.02, n=3, $P<0.001$). As shown in Figure 4.34B and C, CX4945 produced a significant decrease in the mean pH_i change produced by basolateral Cl^- removal, and the rate of reacidification following Cl^- readdition compared to control cells. However, it did not affect the subsequent Fsk-induced inhibition of the remaining basolateral Cl^-/HCO_3^- AE activity (Figure 4.34B and C). Inhibition of the basolateral Cl^-/HCO_3^- AE activity by CX4945 completely recovered after 25 min washing off of the inhibitor, as did the pH_i (7.5±0.06). HCO_3^- flux was also significantly reduced by CX4945, compared to untreated cells (Figure 4.34F).

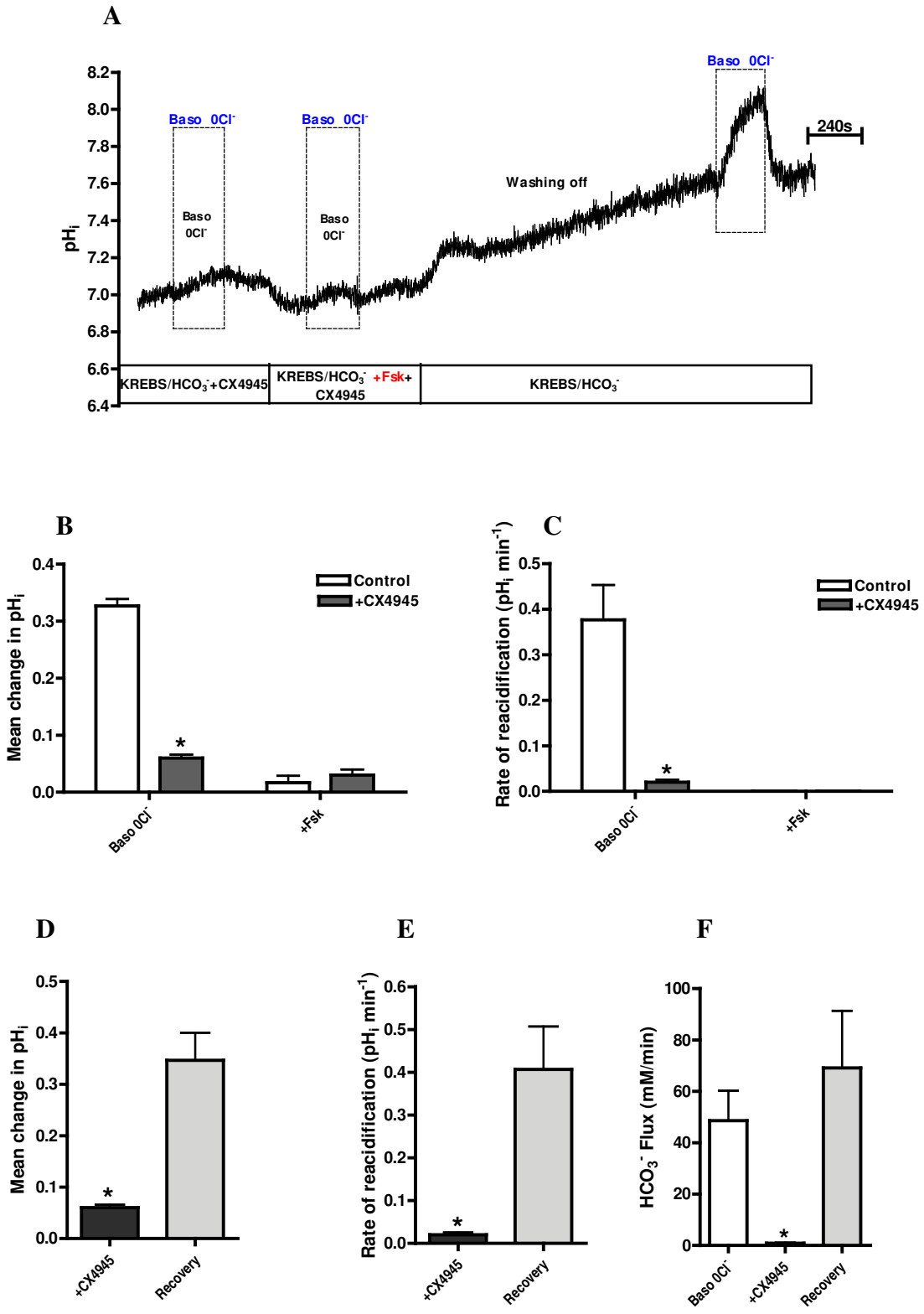
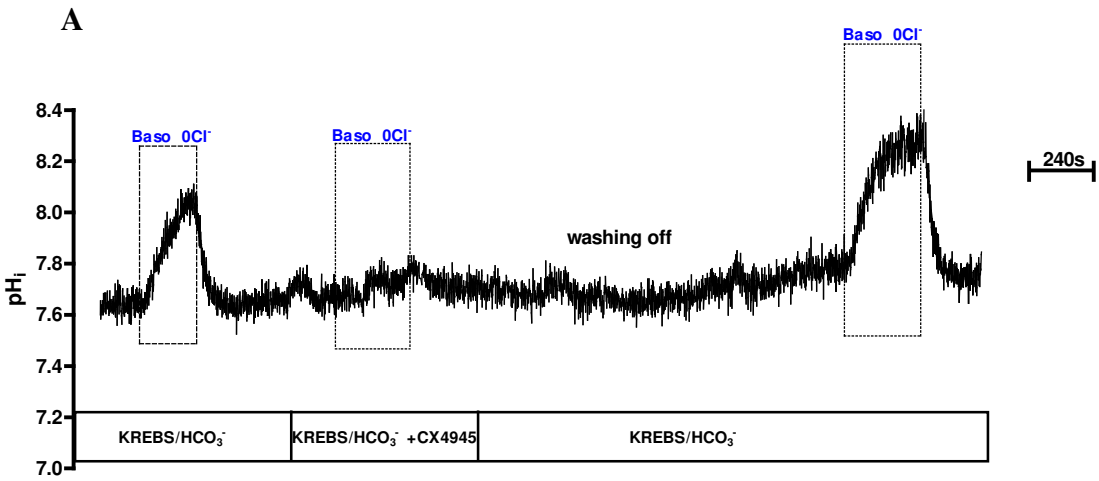


Figure 4. 34: Inhibition of basolateral Cl^-/HCO_3^- AE activity by CX4945 in Calu-3 cells. (A) Raw pH_i trace showing the effect of CX4945 preincubation and perfusion (10µM) on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting and Fsk stimulated conditions. The inhibitory effect of CX4945 was reversed after ~ 25 mins wash off. Summary of the effect of CK2 inhibitor CX4945 (10µM preincubation and perfusion) on the basolateral Cl^-/HCO_3^- AE activity on mean alkalinisation (pH_i) produced by basolateral Cl^- removal (B) and the rate of reacidification upon Cl^-

readdition (C), recovery of CX4945-induced inhibition of basolateral Cl^-/HCO_3^- AE in both mean alkalinisation (D) and the rate of reacidification (E) in Calu-3 cells. (F) HCO_3^- efflux in control and TBB treated cells. Data are shown as Mean±SEM. *P<0.001, #P<0.05 compared to control, n=3 for each condition.

Similarly, in another set of experiments, acute bilateral exposure to 10μM CX4945 nearly abolished the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells (Figure 4.35A). CX4945 caused a significant decrease in basolateral Cl^-/HCO_3^- AE activity in both the magnitude of alkalinisation produced by basolateral Cl^- removal, and the rate of reacidification upon Cl^- readdition compared to control responses (Figure 4.35B and C). Also, CK2 inhibition by CX4945 produced a significant decrease in the rate of HCO_3^- efflux compared to untreated cells (Figure 4.35D). However, unlike TBB, CX4945 did not produce an intracellular acidification, but like TBB, the effect of CX4945 was fully reversible on washing away the inhibitor (Figure 4.35B-D). Taken together, the above data clearly indicate that CK2 plays an essential, and novel, role in the regulation of the basolateral Cl^-/HCO_3^-AE activity under resting conditions in Calu-3 cells.



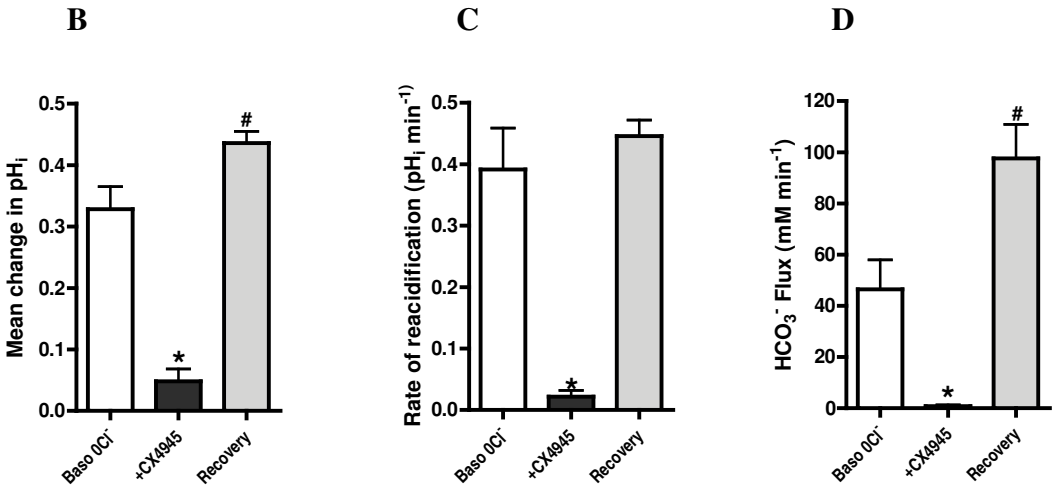


Figure 4. 35: Inhibition of basolateral Cl^-/HCO_3^- AE activity by acute exposure to CX4945 in Calu-3 cells. (A) Representative pH_i trace showing the effect of acute bilateral exposure of CX4945 on the basolateral Cl^-/HCO_3^- AE activity under resting condition in Calu-3 cells. Washing off CX4945 recovered the basolateral Cl^-/HCO_3^- AE activity. Summary of the effect of acute bilateral exposure of CX4945 (10μM) on the mean alkalinisation (pH_i) produced by basolateral Cl^- removal (B) and the rate of reacidification upon Cl^- readdition (C), HCO_3^- flux produced by basolateral Cl removal AE activity (D) in control response compared to CX4945 treated and recovery in Calu-3 cells. Data are shown as Mean±SEM. B and C: *P<0.001 compared to control and recovery, #P<0.05 compared to recovery. D: *P<0.05 to Baso $0Cl^-$, *P<0.001 to recovery, #P<0.05 compared to Baso $0Cl^-$, n=6 for each condition, except for recovery n=5.

4.11 Regulation of the basolateral Cl^-/HCO_3^- anion exchanger by protein phosphatase (PP1/PP2A) activity

Recently, it was shown that regulation of M-type potassium channels by CK2-mediated phosphorylation of CaM depends on CK2 and PP1 anchored to the M-type potassium channel (Kang *et al.*, 2014). In order to assess whether PP1/PP2A could be involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity by CK2 and CaM, I performed another series of experiments where Calu-3 cells were preincubated with 100nM okadaic acid (OA) and perfused with KREBS/HCO_3^- solution containing OA, and then basolateral Cl^-/HCO_3^- AE activity measured in response to Cl^- free solutions (Figure 4.36A). My results showed that 100nM OA significantly inhibited the basolateral Cl^-/HCO_3^- AE activity, both in the mean pH_i change in response to basolateral Cl^- free solution, and the rate of reacidification upon Cl^- readdition under resting condition compared to untreated Calu-3 (Figure 4.36B and C). This is consistent with the recent finding by Garnett *et al*., (2013) that PP1 inhibition by OA (but not PP2A), mimicked the cAMP-induced inhibition of the basolateral AE activity in Calu-3 cells (Garnett *et al*., 2013). These results suggest that PP1/2A keeps the basolateral anion exchanger active under resting conditions by dephosphorylating the exchanger, or an accessory/regulatory protein. The inhibition of the basolateral Cl^-/HCO_3^- AE activity by OA was only seen when Calu-3 cells were pretreated with OA and then perfused with OA containing KREBS solution, as perfusion of OA alone failed to produce any significant effect on AE activity. This suggests that OA needs time to produce its inhibitory effect on the basolateral Cl^-/HCO_3^- AE activity (Figure 4.36D, E and F).

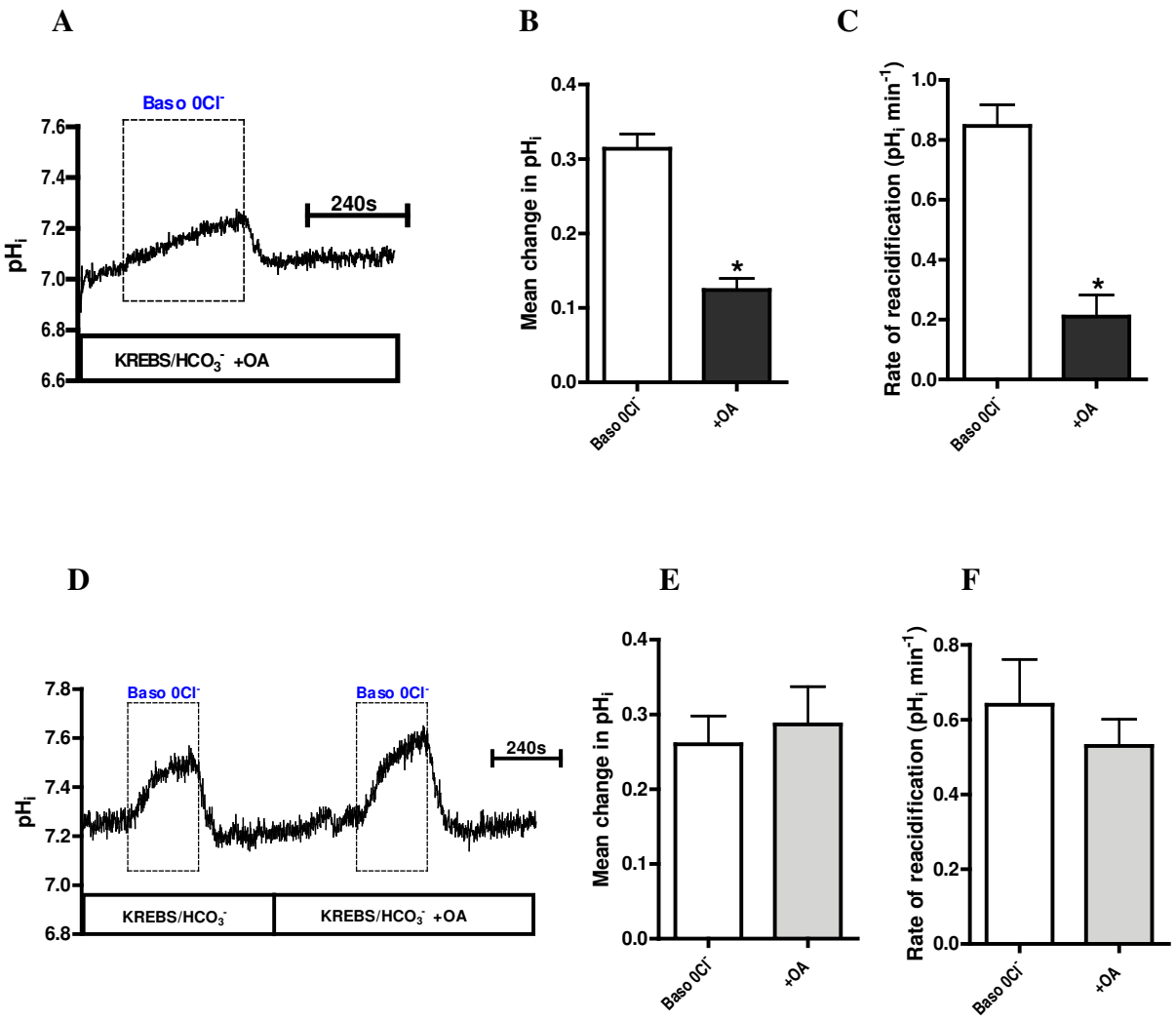


Figure 4. 36: Effect of okadaic acid (OA) on the activity of the basolateral anion exchanger. (A) Representative pH_i traces showing that OA preincubation and perfusion (100nM) reduced the activity of the basolateral anion exchanger in Calu-3 cells. Summary of the effect of OA preincubation and perfusion (100nM) on mean change in pH_i produced by basolateral Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C) compared to control responses. *P<0.05 compared to Baso $0Cl^-$, n=8 for control, and n=5 for OA treated cells. (D) Representative pH_i trace showing that acute OA exposure had no effect on the basolateral Cl^-/HCO_3^- AE activity. Summary of the effect of acute OA exposure on mean pH_i change (E), and the rate of reacidification (F), compared to control response in Calu-3 cells. Data are shown as mean ±SEM, no significant difference, n=3, paired observation.

4.12 Discussion

4.12.1 Inhibition of the basolateral AE activity by DIDS

Using real-time measurements of pH_i from polarised cultures of Calu-3 cells my results show that in the absence of cAMP stimulation these model human serous airway cells have a functional Cl^-/HCO_3^- anion exchanger on the basolateral membrane. The basic properties of this exchanger were consistent with SLC4A2, a Cl^-/HCO_3^- exchanger commonly referred to as AE2, which has previously been shown to be expressed at the basolateral membrane in Calu-3 cells by immunofluorescence (Loffing *et al.*, 2000). One of the goals of my project was to investigate the functional properties of the basolateral anion exchanger in more detail and its potential role in transepithelial HCO_3^- secretion. My results showed that the basolateral Cl^-/HCO_3^- AE activity was inhibited by the disulphonic stilbene DIDS, and that DIDS caused an increased amount of HCO_3^- to be secreted across the apical membrane without affecting the amount of fluid secreted over a 24 hr period. Therefore, these results suggest that inhibition of the basolateral Cl^-/HCO_3^- AE activity provides more HCO_3^- to accumulate inside the cells and this in turns increases the driving force for HCO_3^- secretion across the apical membrane in Calu-3 cells. The IC_{50} for DIDS inhibition of the basolateral anion exchanger was about 17 µM, which is in very good agreement with Humphreys *et al*., 1994 (Humphreys *et al.*, 1994) who obtained an IC_{50} of ~ 13 µM for DIDS block of human basolateral AE2 heterologously expressed in *Xenopus oocytes*. However, these authors (Humphreys *et al.*, 1994) found that 200 µM DIDS nearly abolished AE2 activity, whereas I found that a higher concentration (500 µM) was required to achieve complete inhibition. This suggests that other DIDS-sensitive base transporters in the basolateral membrane of Calu-3 cells may be present, along with AE2. These could be other members of the SLC4 family, or even the SLC26 family, since HCO_3^- transportation across the basolateral membrane of gastric parietal cells (Petrovic *et al.*, 2003) and intercalated cells of the outer medullary collecting duct (Petrovic *et al.*, 2004) are thought to be mediated by SLC26A7 (Rossmann *et al.*, 2001), which is expressed in Calu-3 cells and is

also sensitive to DIDS. However, based on the dose response for DIDS inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, a much lower concentration of DIDS was needed to block the basolateral AE activity, compared to DIDS inhibition of the SLC26A7, with an IC_{50} of 126 µM (Petrovic *et al.*, 2003). Also my results showed that the basolateral Cl^-/HCO_3^- AE activity is Cl^- and HCO_3^- dependent, in contrast to SLC26A7, which is impermeable to HCO_3^-, as the magnitude of Cl^- current in SLC26A7 transfected *Xenopus* oocytes and HEK cells was unaffected by HCO_3^- (Kim *et al.*, 2005). Therefore, based on my pharmacological data and the Cl^- and HCO_3^- dependency, my results are consistent with the functional expression of AE2 on the basolateral membrane of Calu-3 cells.

Although the exact role of AE2 in Calu-3 cells still needs to be determined, it is likely to be important in regulating resting intracellular pH via its ability to transport HCO_3^- across the basolateral membrane, as shown in my studies and by others (Inglis *et al.*, 2002; Garnett *et al.*, 2011). Also the study by Huang *et al.*, (2012) showed that intracellular alkalinisation produced by basolateral Cl^- removal was decreased by 80% in AE2 knock down Calu-3 cells (Huang *et al.*, 2012). In addition, through its transport activity under resting conditions, it will also act to accumulate Cl^- inside the cell, particularly when working in parallel with NKCC1 that facilitates the influx of Na^+, K^+ and $2Cl^-$ ions across the basolateral membrane of Calu-3 cells (Liedtke *et al.*, 2001). This is likely to be beneficial for transepithelial Cl^- secretion, prior to stimulation by cAMP agonists, where the activity of the basolateral Cl^-/HCO_3^- AE exchanger is subsequently reduced. As discussed in the introduction, AE2 may also have a role in accumulating Cl^- via coupled transport with the basolateral NBC, as recently suggested (Huang *et al.*, 2012; Shan *et al.*, 2012). However, my results which showed no effect of DIDS on fluid secretion, suggests this mode of coupling is not that important under resting conditions at least.

4.12.2 Role of cAMP and cGMP in the regulation of the basolateral AE activity

A recent study by Kim *et al*., (2014) demonstrated that apical HCO_3^- efflux via CFTR interferes with measuring basolateral AE2 activity, using pH_i methods, in Fsk stimulated Calu-3 cells. In this study, exposing cells to the apical CFTR inhibitor, $CFTR_{inh}$-172 after cAMP stimulation, unmasked basolateral AE2 activity such that basolateral Cl^- substitution caused a significant alkalinisation (Kim *et al.*, 2014). While I haven't specifically tested the effect of adding a CFTR inhibitor to the apical membrane on basolateral AE activity, I have used several PKA inhibitors to block apical AE/CFTR activity and failed to show any basolateral AE2 activity, in Fsk stimulated Calu-3 cells. Moreover, it has recently been shown that the basolateral Cl^-/HCO_3^- AE activity is not abolished by an increase of intracellular cAMP (using Fsk) in Calu-3 cells (Huang *et al.*, 2012). However, a study by Ishiguro *et al*., (2002) demonstrated that the basolateral Cl^-/HCO_3^- AE activity is abolished by intracellular elevation of cAMP following stimulation of guinea pig pancreatic duct cells with Fsk (Ishiguro *et al.*, 2002). My results showed that stimulation of Calu-3 cells by addition of cAMP agonists Fsk, ADO, dbcAMP or IBMX all inhibited the activity of the basolateral Cl^-/HCO_3^- AE activity. Further evidence that the activity of the basolateral Cl^-/HCO_3^- AE was affected by an increase of intracellular cAMP, comes from the finding that inhibition of the cAMP efflux transporter, most likely MRP4, mimicked the effect of cAMP agonists on basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

Additionally, I have also shown that intracellular elevation of cGMP did not affect either the basolateral Cl^-/HCO_3^- AE activity under resting and cAMP stimulated conditions. This suggests that intracellular elevation of cAMP blocks the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, consistent with the study by Garnett *et al*., (2013). Furthermore, the mechanism by which cAMP blocks the basolateral Cl^-/HCO_3^- AE activity was assessed in more detail by inhibition of the downstream targets of cAMP. My results showed that inhibition of PKA, Epac, CNG channels and mTOR kinase, failed to overcome the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. This clearly suggests that cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity is through a

PKA, Epac, CNG channel, and mTOR-independent mechanism. Although Epac inhibition did not remove the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity, it significantly reduced the resting activity of the basolateral Cl^-/HCO_3^- AE, suggesting, for the first time, that Epac plays an essential role in maintaining the resting basolateral Cl^-/HCO_3^-AE activity in Calu-3 cells. Since Epac plays an important role in Ca^{2+} release in cardiac myocytes (Oestreich *et al.*, 2009), the effect of Epac inhibition on the basolateral Cl^-/HCO_3^- AE activity might be through an intracellular Ca^{2+} signaling pathway as it has been shown that Epac regulation of human intestinal Cl^- secretion was completely abolished by BAPTA-AM (Kazi Mirajul Hoque, 2009). Future experiments should test these conditions on the basolateral AE activity in Calu-3 cells.

4.12.3 Role of Ca^{2+} in the regulation of the basolateral AE activity

4.12.3.1 Intracellular Ca^{2+}

To focus on the mechanism of cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, the potential role of intracellular and extracellular Ca^{2+} was assessed under resting and cAMP stimulated conditions, as it has been shown that changes in intracellular Ca^{2+} concentration affect cAMP production, either through inhibition or stimulation of Ca^{2+}-sensitive ACs (Willoughby and Cooper, 2007). Calu-3 cells were stimulated with the Ca^{2+} agonists Cch and thapsigargin that increase $[Ca^{2+}]_i$ by releasing Ca^{2+} from ER stores into the cytosol (Thastrup *et al.*, 1990; Mayerhofer *et al.*, 1992). Results showed that basolateral Cl^-/HCO_3^- AE activity was not changed by elevation of intracellular Ca^{2+} under both resting and cAMP-stimulated conditions. However, under Fsk stimulation basolateral Cl^- removal in the presence of Cch produced a transient acidification in pH_i in a Ca^{2+}-dependent manner, and BAPTA-AM abolished the Cch-induced transient acidification. This suggests that elevation of intracellular Ca^{2+} does not play a critical role in the regulation of basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

Although it has been shown that cAMP and Ca^{2+} cooperate to simulate HCO_3^- secretion, my results showed that intracellular depletion of Ca^{2+}, using BAPTA-AM, did not alter the cAMP-induced inhibition of basolateral Cl^-/HCO_3^- AE activity suggesting that intracellular Ca^{2+} is not involved in the cAMP-mediated inhibition of the basolateral Cl^-/HCO_3^- AE activity. However, a decrease in $[Ca^{2+}]_i$ caused a marked reduction in basolateral Cl^-/HCO_3^- AE activity, highlighting the potential importance of maintaining normal $[Ca^{2+}]_i$ levels for optimal basolateral Cl^-/HCO_3^- AE activity under resting conditions in Calu-3 cells. Consistent with this, perfusion of thapsigargin in BAPTA-AM loaded Calu-3 cells, partially removed the inhibitory effect of BAPTA-AM on the basolateral Cl^-/HCO_3^- AE activity, suggesting that thapsigargin caused a small increase in intracellular Ca^{2+} even in the presence of Ca^{2+} chelator, BAPTA-AM. Furthermore, elevating cAMP and decreasing $[Ca^{2+}]_i$ simultaneously, produced a further reduction in the activity of the basolateral Cl^-/HCO_3^- AE, indicating that cAMP and Ca^{2+} regulate the basolateral Cl^-/HCO_3^- AE activity via different signaling pathways, which is an interesting area for further investigation. This could involve determining the Ca^{2+} sensitive protein that regulates the basolateral Cl^-/HCO_3^- AE activity under cAMP stimulated conditions in Calu-3 cells.

4.12.3.2 Extracellular Ca^{2+}

Extracellular Ca^{2+} sensing receptor (CaSR) plays an important role in the regulation of anion and fluid secretion in human epithelial cells (Racz *et al.*, 2002). It has been shown that a decrease in extracellular Ca^{2+} concentration reduces the CaSR activity (Brown, 2007). In Calu-3 cells, removing of extracellular Ca^{2+} concentration, either apically or basolaterally, failed to inhibit basolateral Cl^-/HCO_3^- AE activity, or overcome the cAMP-induced inhibition of the basolateral anion exchanger, suggesting that apical or basolateral extracellular Ca^{2+} sensing receptor alone is not involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. However, bilateral removal of extracellular Ca^{2+} significantly reduced the cAMP-induced inhibition of the basolateral anion exchanger, although the resting activity did not change. This

suggests that bilateral Ca^{2+} removal alters CaSR which somehow alters cAMP signaling and changes basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. This might be because in the absence of extracellular Ca^{2+}, CaSR interferes with cAMP production, thereby partially overcoming the cAMP-induced inhibition of the basolateral anion exchanger. Consistent with this, it has been shown that cAMP production is decreased in the absence of extracellular Ca^{2+} concentration (Ferreira *et al.*, 1998). Although removing extracellular Ca^{2+} alone did not affect the resting basolateral Cl^-/HCO_3^- AE activity, my results showed that in the absence of extracellular Ca^{2+}, ER Ca^{2+}-store depletion (induced by thapsigargin), caused a significant inhibition of basolateral Cl^-/HCO_3^- AE activity, and interestingly produced a transient intracellular acidification, potentially through stimulation of the apical AE activity. However, cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity was not changed. Thus, a possible explanation would be that a combination of extracellular Ca^{2+} removal combined with ER Ca^{2+}-store depletion, increased intracellular cAMP levels and thereby blocked the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. It has been reported that ER Ca^{2+}-store depletion and absence of extracellular Ca^{2+} enhance AC and increase $[cAMP]_i$ through the ER Ca^{2+}-sensor STIM, which couples ER Ca^{2+} level to cAMP production (Lefkimmiatis *et al.*, 2009). Future experiments could involve measuring $[Ca^{2+}]_i$ and ER $[Ca^{2+}]$ in the absence of extracellular Ca^{2+} to support the role of Ca^{2+} in the regulation of the basolateral Cl^-/HCO_3^- AE activity.

4.12.4 Role of CaM and CaMK in the regulation of the basolateral AE activity

Since depletion of $[Ca^{2+}]_i$ significantly reduced the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, suggested that an intracellular calcium-sensor was involved, and therefore the role of CaM was assessed, because it has been shown that there is a strong relationship between CaM activation and intracellular Ca^{2+}concentration (Keller *et al.*, 2008). CaM inhibition caused a marked reduction in the basolateral Cl^-/HCO_3^- AE activity under resting conditions, but it did not prevent the remaining activity to be inhibited by Fsk. Although there is

well described cross-talk between Ca^{2+}/Calmodulin dependent protein kinase (CaMK) and some cAMP dependent signaling pathways (Soderling, 1999), and normal intracellular Ca^{2+} concentration is also required to maintain the resting activity of the basolateral Cl^-/HCO_3^- AE, inhibition of CaMK (CaMKI, CaMKII and CaMKIV) produced little effect on basolateral Cl^-/HCO_3^- AE activity, and did not prevent the cAMP-mediated inhibition of the basolateral Cl^-/HCO_3^- AE activity. This suggests that CaM-dependent kinases were not involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity under resting and cAMP stimulated conditions in Calu-3 cells. These data indicate that although CaM is not involved in cAMP-mediated inhibition of the basolateral Cl^-/HCO_3^- AE activity, it does play an important role in regulating the resting activity, through a CaMK-independent mechanism. This is further supported by the effect of BAPTA-AM, which also reduced the basolateral Cl^-/HCO_3^- AE activity (see section 4.6.1.2, figure 4.18). Taken together, decreasing $[Ca^{2+}]_i$ potentially blocks the basolateral Cl^-/HCO_3^- AE activity through a CaM dependent pathway in Calu-3 cells. Consistent with this, it has been reported that CaM plays an essential role in transduction of Ca^{2+}-mediated signaling pathways in all eukaryotic cells (O'Day, 2003). Interestingly, in the presence of a CaM inhibitor, elevating cAMP (by inhibition of MRP4), did cause a further reduction in the resting activity of the basolateral Cl^-/HCO_3^- AE, suggesting that both CaM and cAMP induce their effects via separate regulatory mechanism.

4.12.5 How does actin-cytoskeleton disruption and dynamin inhibition lead to the marked decrease in the basolateral AE activity

Another hypothesis to explain the effect of $[Ca^{2+}]_i$ depletion on resting basolateral Cl^-/HCO_3^- AE activity could be provided by an effect on dynamin mediated endocytosis, as it has been reported that this process is controlled via a Ca^{2+}-sensing mechanism (Lai *et al.*, 1999). Although blocking dynamin in Calu-3 cells did not affect the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^-

AE activity, the resting basolateral Cl^-/HCO_3^- AE activity was significantly decreased, which could potentially be through inhibition of supply of new proteins from the trans-face of Golgi complex, as dynamin plays an important role in the fission of newly formed vesicles, and regulates the kinetics of released protein to the plasma membrane (Anantharam *et al.*, 2011). This highlights the importance of dynamin in the regulation of the basolateral Cl^-/HCO_3^- AE under resting conditions in Calu-3 cells. However, exactly how this occurs is unclear, but one possible explanation could be via the actin cytoskeleton, since there is a direct interaction between dynamin and the actin cytoskeleton (Gu *et al.*, 2010), and recent findings showed that actin filaments plays a vital role in transferring secretory vesicles from the trans-Golgi network to the plasma membrane, and thereby regulation of some ion transporter activity ((Egea *et al.*, 2015), see Figure 4.37). Consistent with this, I showed that disruption of the actin cytoskeleton in Calu-3 cells produced a marked reduction in the basolateral Cl^-/HCO_3^- AE activity under resting conditions, but it did not overcome the cAMP-induced inhibition. Taken together, the above data imply that both an intact actin cytoskeleton and dynamin are required to maintain the normal resting activity of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells, potentially through a Ca^{2+}-dependent mechanism.

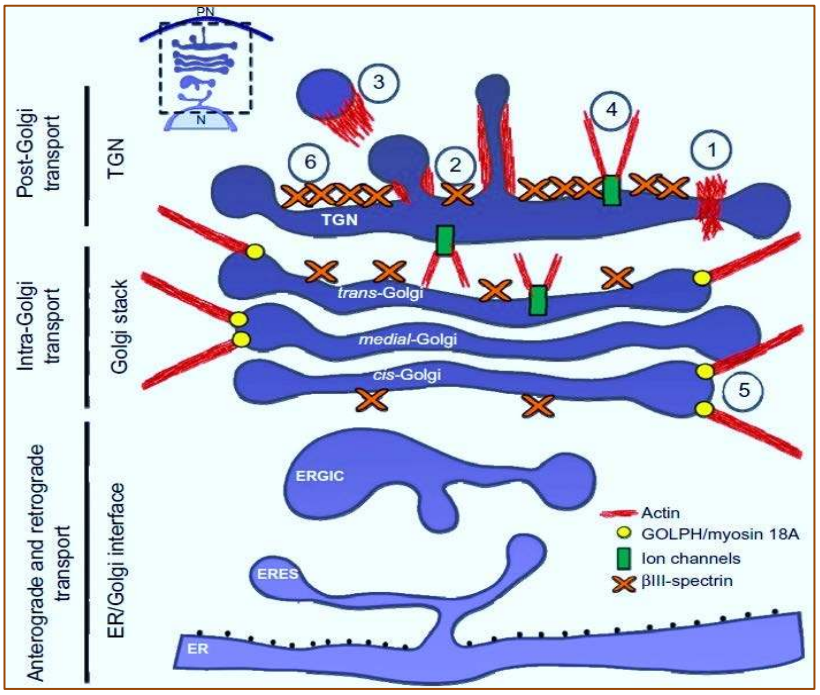


Figure 4. 37: Schematic illustration of actin participation in the trafficking pathway of secretory vesicles from the Golgi complex to the plasma membrane. Actin polymerization plays a vital role in scission (1), pulling (2), and forward movement (3) of carrier vesicles, and regulation of ion transporter function (4), and/or being part of cytoskeleton (6), and keeping the Golgi extended (5). Diagram taken from (Egea *et al.*, 2015).

4.12.6 Role of CK2 in the regulation of the basolateral anion exchanger

To further understand the mechanism of CaM-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells under resting conditions, the role of CK2 was assessed since there are a number of putative CK2 phosphorylation sites in SLC4A2 (see appendix), and it has been shown that CK2 is the main kinase that can phosphorylate CaM (Arrigoni *et al.*, 2004) via three physiological CK2-phosphorylation acceptor sites in CaM (Quadroni *et*

al., 1994; Arrigoni *et al.*, 2004). Interestingly, treatment of Calu-3 cells with two different selective CK2 inhibitors, TBB and CX4945, either by preincubation or acute exposure of cells with the inhibitors, caused a significant decrease in the basolateral Cl^-/HCO_3^- AE activity under resting conditions, while CK2 inhibition failed to overcome the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. This clearly indicates that CK2 plays an important role in the regulation of the basolateral Cl^-/HCO_3^- AE activity under resting conditions, and also implies that CK2 regulates the basolateral AE activity potentially through a CaM-dependent mechanism, based on the fact that CaM is a known downstream target of CK2. This is further supported by my results when Calu-3 cells were treated simultaneously with a J-8 as well as TBB, which did not produce a further decrease in the basolateral Cl^-/HCO_3^- AE activity, compared to the presence of CaM inhibitor or CK2 inhibitor alone. My results are also consistent with recent findings that showed CK2-mediated phosphorylation of CaM plays an important role in the regulation of K^+-channels by strengthening CaM binding to the K-channels, which was shown to regulate channel trafficking and stabilize its activity in HEK293A cells (Kang *et al.*, 2014).

4.12.7 Impact of PP1 on the regulation of the basolateral AE activity

It has been previously reported from our group that PP1 inhibition leads to stimulation of apical AE activity and inhibition of the basolateral Cl^-/HCO_3^- AE activity, in Calu-3 cells (Garnett *et al.*, 2013). I also showed that PP1 inhibition by OA markedly decreased the basolateral Cl^-/HCO_3^- AE activity under resting conditions, suggesting that dephosphorylation of the basolateral Cl^-/HCO_3^- AE, or other regulatory proteins might be responsible for maintaining the resting activity of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells. The exact mechanism as to how PP1 regulates basolateral Cl^-/HCO_3^- AE activity is still unclear; however, it has been shown that CK2 can phosphorylate and activate PP1 (Van Eynde *et al.*, 1994), suggesting there exists a link between active PP1 and CK2. My results are consistent with this as I showed that CK2 inhibition markedly

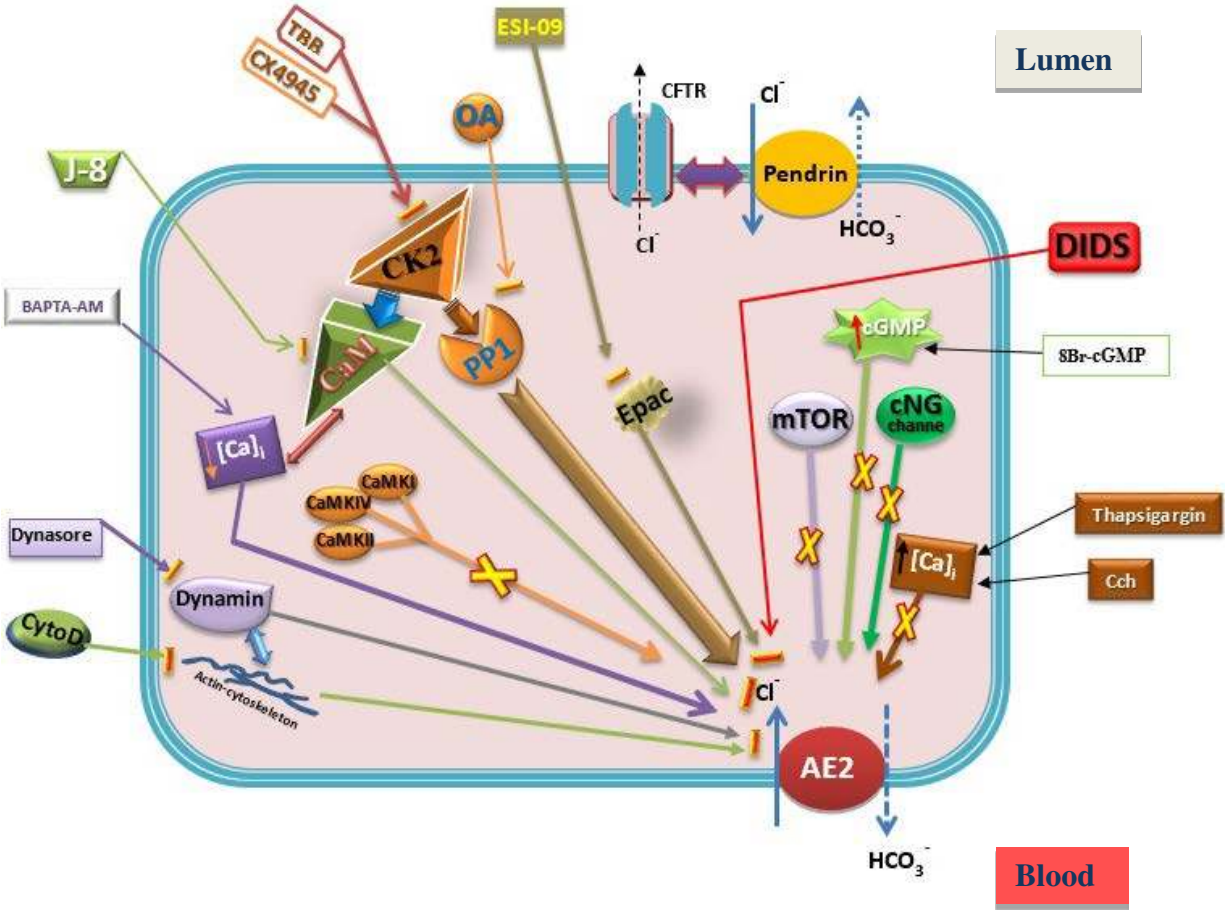
reduced basolateral Cl^-/HCO_3^- AE activity, similar to PP1 inhibition. A possible explanation could involve CK2 phosphorylation of PP1, which would help maintain the resting activity of the basolateral anion exchanger, while blocking the apical AE activity, under resting conditions. A recent study demonstrated that regulation of K^+-channel activity by CK2-mediated phosphorylation of CaM in HEK293A cells, depends on the binding of CK2 and PP1 to the K^+ channels (Kang *et al.*, 2014). In accordance with this, my results showed that inhibition of CK2, CaM and PP1 all significantly reduced the resting activity of the basolateral Cl^-/HCO_3^- AE; therefore, regulation of the basolateral Cl^-/HCO_3^- AE activity via CK2-mediated phosphorylation of CaM might need a physical interaction between CK2 and PP1 and the basolateral Cl^-/HCO_3^- AE in Calu-3 cells. Taken together, this suggests that under normal condition CK2 mediated phosphorylation of PP1 is potentially responsible for keeping the basolateral Cl^-/HCO_3^- AE active under resting condition, while inhibiting apical AE activity in Calu-3 cells. Future experiments could involve measuring basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells treated with CaM and PP1 inhibitors simultaneously, to test whether CaM is involved in the potential CK2-mediated phosphorylation of PP1. It could also look at binding of CK2/PP1 to AE2 by co-immunoprecipitation.

The key findings of this chapter are summarized below (see Figure 4.38):

- DIDS inhibits the basolateral Cl^-/HCO_3^- AE activity with an IC_{50} of 17µM, which is consistent with SLC4A2 underlying this anion exchange activity.
- Intracellular elevation of cAMP is responsible for abolishing the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells through a mechanism that is independent of direct downstream targets of cAMP.
- Changes in the levels of [cGMP] not involved in the regulation of the basolateral Cl^-/HCO_3^- AE activity.

- Inhibition of Epac protein markedly decreased the resting activity of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells.
- Decreasing intracellular Ca^{2+} concentration by BAPTA-AM loading, significantly reduced the basolateral Cl^-/HCO_3^- AE activity, which was dependent on CaM, but independent of several CaMKs.
- Acute increase in $[Ca^{2+}]_i$ did not affect the basolateral Cl^-/HCO_3^- AE activity.
- An intact actin cytoskeleton and active dynamin are essential in maintaining the activity of the basolateral Cl^-/HCO_3^- AE in Calu-3 cells.
- I have identified a novel role for CK2 in regulating the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells, potentially through a CaM and PP1-dependent mechanism.

Resting condition:



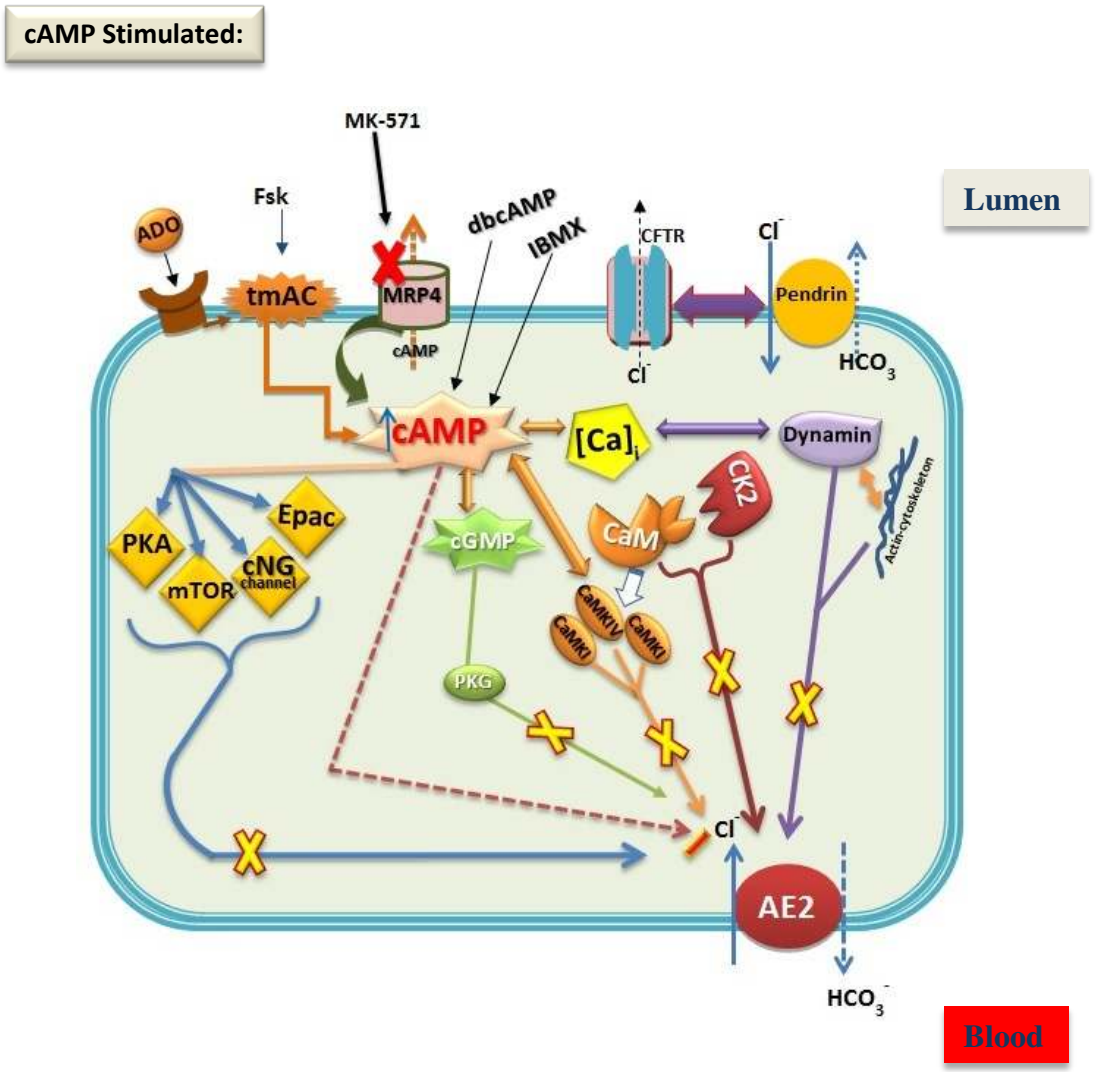


Figure 4. 38: Diagram to show which signaling pathway regulate the basolateral AE activity under resting and stimulated conditions in Calu-3 cells. Under resting conditions: CK2 showed a novel role in the regulation of the basolateral AE activity potentially through a CaM and PP1 dependent mechanism. Decreasing $[Ca^{2+}]_i$ also significantly reduced the basolateral AE activity but through CaMK independent mechanisms. Under cAMP stimulated conditions; increasing $[cAMP]_i$ by adenylyl cyclase (tmAC) activators, forskolin or adenosine, and the membrane permeable cAMP analogue, dibutyryl cAMP or the phosphodiesterase inhibitor, IBMX, significantly inhibited basolateral AE activity, but this did not involve several well-known downstream targets of cAMP. Chelation of intracellular Ca^{2+} by BAPTA-AM, or elevation of $[Ca^{2+}]_i$ by thapsigargin had no effect on basolateral AE activity. Blocking CK2 and CaMK failed to overcome the cAMP-induced inhibition of the basolateral AE activity. (-) denotes inhibition, (+) denotes stimulation, and (X) denotes not contribute.

Chapter 5 Regulation of heterologously expressed mouse AE2 in transiently transfected HEK-293T cells

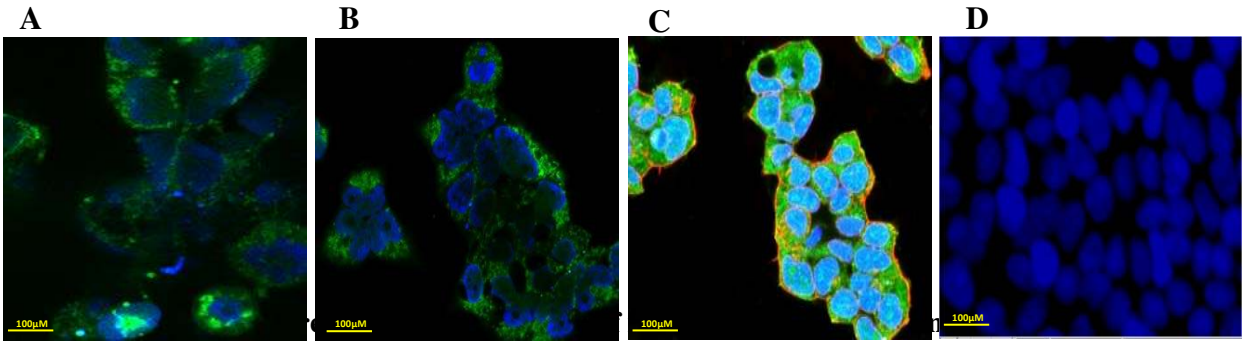
5.1 Introduction

My results described in Chapter 4 showed for the first time that the activity of the basolateral anion exchanger in polarised cultures of Calu-3 cells was regulated by a number of intracellular messengers and signalling pathways, including cAMP/PKA/Epac, resting calcium concentration and calmodulin, as well as via a novel CK2-dependent pathway. I also showed that CFTR appeared to be involved in regulating AE activity, consistent with previous results from our lab (Garnett *et al.*, 2011). Although at this stage I cannot be certain about the molecular identity of the basolateral AE activity in Calu-3 cells, my own results, and those of others (Loffing *et al.*, 2000; Al-Bazzaz *et al.*, 2001; Romero *et al.*, 2004; Garnett *et al.*, 2011; Huang *et al.*, 2012; Shan *et al.*, 2012; Garnett *et al.*, 2013; Kim *et al.*, 2014) provide strong support that the anion exchanger is AE2 (SLC4A2). In order to provide further evidence that these various signaling pathways were capable of regulating AE2 activity, I have transiently transfected Human Embryonic Kidney-293T cells (HEK293T) cells with cDNA for mouse AE2 (mAE2; slc4A2) and studied the effect of altering the same signaling messengers/pathways on mAE2 mediated anion transport. I have also investigated the potential role of CFTR in regulating anion exchange activity by cotransfecting HEK-293T cells with mAE2 and human CFTR. I used mouse AE2, rather than human AE2, for these studies as I was not able to obtain full-length human AE2. Nonetheless, based on the amino acid sequence of mAE2, this protein shows a great deal of sequence homology to human AE2, and in particular contains many potential CK2 phosphorylation sites similar to human AE2 (see appendix). The HEK-293 cell line has been commonly used to overexpress many different types of proteins to study their function and molecular regulation (Domingue *et al.*, 2014). HEK-293 cells have a wide range of advantages, such as high transfection yields and adaptation to growth in

serum-free media and suspension culture (Graham, 1987; Garnier *et al.*, 1994). The HEK-293T cells are derived from the HEK-293 parent (Kim *et al.*, 1997) and have been extensively used in electrophysiological studies of heterologously expressed ion channels (Senatore *et al.*, 2011). Heterologous transfection and expression of cDNA in cells, over a short period of time, is known as "transient expression" (Kaufman, 1997), which can be detected for 1-4 days after transfection. (Colosimo *et al.*, 2000). This transient expression system has also been used to study the function of anion exchangers (AEs) (Lee *et al.*, 1991). It has been reported that HEK293 cells have a low level of endogenous Cl^-/HCO_3^- AE activity (Sterling *et al.*, 2002). Immunofluorescence and pulse-chase labelling experiments demonstrated that transient expression of murine AE2 can be detected both at the cell surface and in intracellular compartments, potentially the ER, of transfected HEK cells (Ruetz *et al*., 1993). Here, they also found that transient expression of murine AE2 in HEK cells, functions as a plasma membrane anion exchanger, by producing a significant increase in Cl^--driven SO_4^- efflux, which was inhibited by 200µM DIDS. Moreover, it has been shown that transient transfection of rat AE2 showed a high level of expression and Cl^-/HCO_3^- transport activity, in HEK-293 cells, which was also inhibited by DIDS, with an IC_{50} of 142µM (Fujinaga *et al.*, 2003). Domingo *et al*., (2014) used HEK-293 cells as a useful model to overexpress CFTR, to study the signaling pathway that regulate CFTR activity, as HEK-293 cells neither express CFTR mRNA nor protein (Domingue *et al.*, 2014). Therefore, in this chapter I have further investigated the role of cAMP, cytosolic calcium, CFTR and CK2 in the regulation of mouse AE2 (mAE2) activity, which was studied after being transiently transfected into HEK-293T cells.

5.2 Properties of mAE2 expressed in HEK-293T cells

To characterise the biophysical properties of mAE2, I have performed a series of experiments in HEK-293T cells transiently transfected with mAE2 cDNA, and compared the results to control, non-transfected, HEK293T cells. pH_i measurement in control cells showed that these cells possessed an endogenous Cl^-/HCO_3^- exchange activity based on the results from the standard Cl^- removal/readdition protocol employed in chapters 3 and 4. However, my results showed that Cl^-/HCO_3^- exchange activity was significantly increased in mAE2 transfected HEK-293T cells after two days of transfection, both in the magnitude of alkalinisation in response to Cl^- free solution (0.68±0.02, n=10, compared to control cells, 0.42±0.02, n=10, $P<0.05$), and the rate of reacidification upon Cl^- readdition (0.56±0.06, n=10, compared to control cells, 0.24±0.02, n=10, $P<0.05$). Also, I performed immunocytochemistry on HEK-293T transfected with HA-tagged mAE2 to confirm the successful transfection and expression of mAE2 in these cells. mAE2 expression was detected using an anti-HA antibody that was FITC-conjugated, and therefore produces a green colour when visualized by confocal microscopy. Results showed that mAE2 transfected cells exhibit strong green fluorescence, both at the plasma membrane and within the cells (Figure 5.1A and B), compared to control non-transfected cells (Figure 5.1D). Moreover, I further investigated mAE2 localization in transfected cells by staining the plasma membrane with wheat germ agglutinin (WGA), a red plasma membrane marker (Yacoub *et al*., 2006). mAE2 was shown to be highly expressed in the plasma membrane as indicated by the yellow colour around the cells produced from the combination of green mAE2 protein and red WGA (Figure 5.1C).



293T cells. HEK293T cells were transfected with HA-tagged mAE2 cDNA and studied two days post-transfection. (A) and (B) show confocal images where mAE2 was labelled with FITC-conjugated fluorophore (green). Significant intracellular as well as plasma membrane staining was observed. (C) Confocal localization of mAE2 in transfected HEK-293T cells, stained with the red plasma membrane marker, WGA. Plasma membrane localisation of mAE2 was confirmed by yellow staining, (D) control non-transfected cells stained with HA primary antibody and FITC-conjugated fluorophore. Images are representative of 3 independent experiments for mAE2 transfected cells, 2 for control, non-transfected cells. DAPI (4',6-diamidino-2-phenylindole) was used for staining nucleus (blue color).

I then investigated the DIDS-sensitivity of the endogenous AE activity and compared this to mAE2 transfected cells. In order to test the effect of DIDS on the endogenous Cl^-/HCO_3^- exchange activity in non-transfected HEK-293T cells, I measured the pHi response of the HEK293T cells to a Cl^- free solution, in the presence of 25µM DIDS, and compared this to mAE2 transfected HEK-293T cells. My results showed that perfusion of a Cl^- free solution, containing 25µM DIDS, produced almost complete inhibition of the endogenous Cl^-/HCO_3^- AE activity (Figure 5.2A), with a significant decrease in both the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification after Cl^- readdition (Figure 5.2C and D). However, 25µM DIDS did not affect the magnitude of alkalinisation following Cl^- removal in mAE2 transfected HEK cells (Figure 5.2B and C), but did significantly reduce the rate of reacidification upon Cl readdition (Figure 5.2D). Therefore, I measured mAE2 activity in transfected HEK-293T cells in the presence of 25µM DIDS in order to remove most of the contribution of the endogenous Cl^-/HCO_3^- exchange activity, so that the resulting pH_i changes were mainly due to the heterologously expressed mAE2 activity.

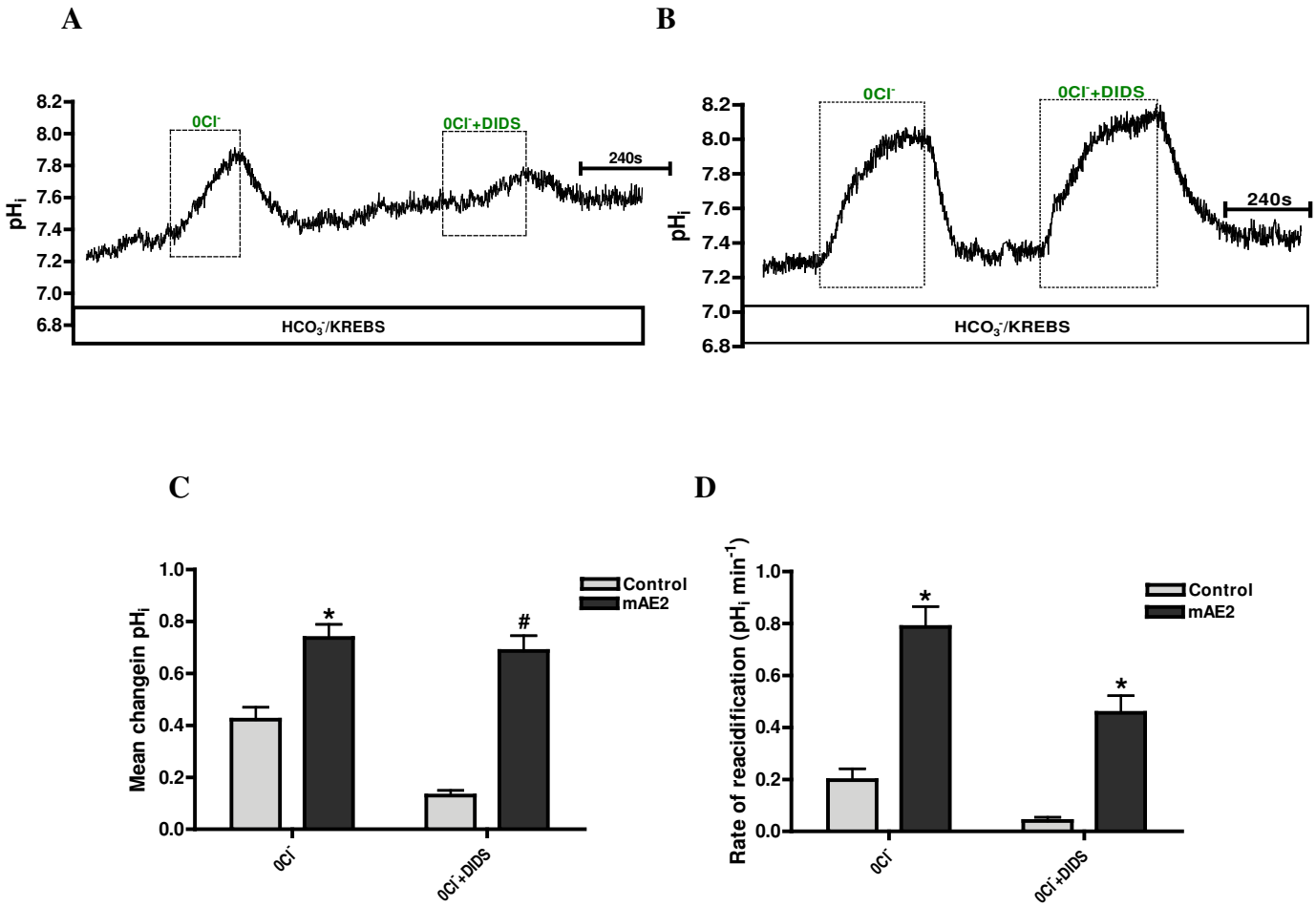


Figure 5. 2: Effect of 25µM DIDS on the Cl^-/HCO_3^- AE activity in control and mAE2 transfected HEK-293T cells. (A) and (B) show representative pH_i traces which show that 25µM DIDS significantly reduced mAE2 activity in transfected HEK-293T cells compared to control, non-transfected, HEK-293T cells, respectively. Summary data showing the effect of 25µM DIDS on mean alkalinisation in pH_i in response to Cl^- free solution (C) and the rate of reacidification upon Cl^- readdition (D) in control and mAE2 transfected HEK-293T cells. Data are shown as Mean±SEM. C: *P<0.01, #P<0.001 compared to control. D: *P<0.001 compared to control, n=4 for control, and n=3 for +mAE2. Control experiments run in parallel.

My previous results showed that 100µM DIDS significantly reduced the basolateral AE activity in Calu-3 cells (see chapter 4, section 4.02). To further investigate the functional properties of mAE2 activity in transfected HEK-293T cells, I performed another set of experiments where different concentrations of

DIDS (25µM and 200µM DIDS) were present in the standard Cl^- free solution (Figure 5.3A). Results showed that 25µM did not affect the magnitude of alkalinisation produced by mAE2 activity in transfected cells, but did significantly reduce the rate of reacidification upon Cl^- readdition (Figure 5.3A and B). However, the higher concentration of DIDS, 200µM, caused a significant decrease in mAE2 activity, both in the mean change in pH_i in response to Cl^- free solution, and the rate of reacidification following Cl^- readdition, compared to the control responses (Figure 5.3A and B). In another set of experiments, 100µM DIDS caused a marked reduction in mAE2 activity, both in the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition, while 50µM DIDs only significantly decreased the rate of reacidification in mAE2 transfected HEK cells (Figure 5.4A and B). Consistent with this, it has been shown that 200µM DIDS significantly inhibited mAE2 in transfected HEK-293 cells (Ruetz *et al.*, 1993). The effect of different concentrations of DIDS is summarized in figure 5.4A and B.

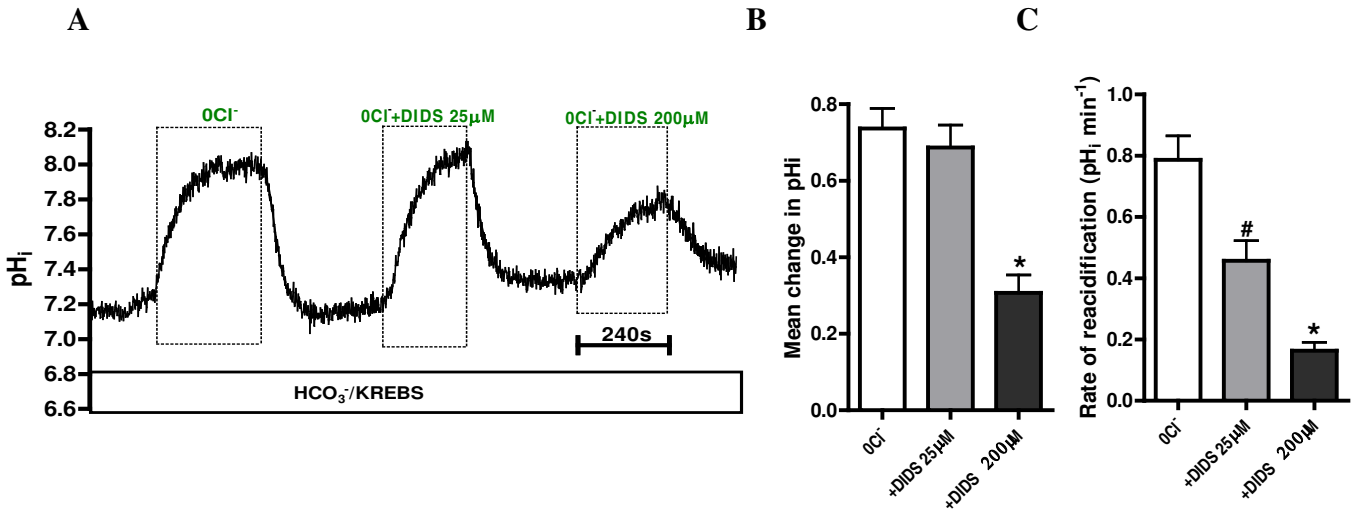


Figure 5. 3: Impact of DIDS on the mAE2 activity in transfected HEK-293T cell. (A) Representative pH_i trace showing that 25µM DIDS had no effect on the mean alkalinisation in pH_i in response to Cl^- free solution but did inhibit rate of reacidification upon Cl^- readdition , while 200µM DIDS significantly reduced both parameters in transfected HEK293T cells, compared to the response in the absence of DIDS. (B) Summary of the effect 25µM and 200 µM DIDS on mean alkalinisation in pH_i in response to Cl^- free solution and (C), the rate of reacidification upon Cl^- readdition. Data are shown as Mean±SEM. B: *P<0.001 compared to DIDS 25µM and $0Cl^-$. C: *P<0.001 compared to DIDS 25µM and P<0.001 compared $0Cl^-$, #P<0.01 compared to $0Cl^-$, n=3 for each condition, paired observations.

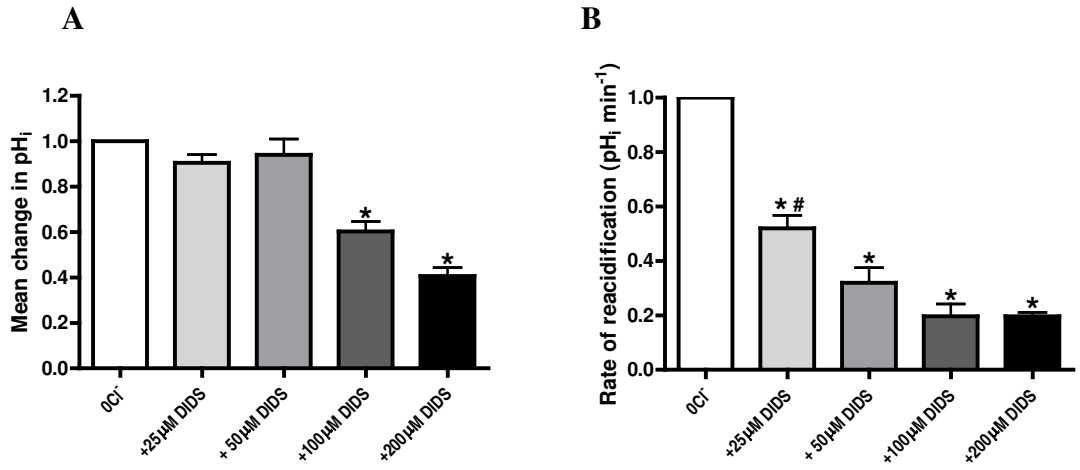


Figure 5. 4: Effect of different concentrations of DIDS on mAE2 activity in transfected HEK-293T cells. Summary of mean change in pH_i (alkalinisation) caused by Cl^- removal (A) and rate of reacidification after Cl^- readdition (B), produced by transfected mAE2 activity in HEK-293T cells in response to different concentration of DIDS, when normalized to the control response in the absence of DIDS. Data are shown as Mean±SEM.*P<0.001 compared to control, $^{\#}$P<0.05 compared to 50µM, and $^{\#}$P<0.001 compared to 100µM and 200µM DIDS, n=6 for 0Cl and 25µM DIDS; n=3 for 50µM, 100µM, and 200µM DIDS.

5.3 Effect of cAMP agonist, Fsk, on mAE2 activity in transfected HEK-293T cells

My previous experiments in Calu-3 cells showed that the basolateral Cl^-/HCO_3^- AE activity was almost completely inhibited following an intracellular increase of cAMP. In contrast to Calu-3 cells, the cAMP agonist, Fsk, did not affect mAE2 activity (Figure 5.5D-F). Interestingly, elevation of intracellular cAMP also did not affect the endogenous Cl^-/HCO_3^- activity in control cells, both in the magnitude of alkalinisation, and the rate of reacidification (Figure 5.5A-C).

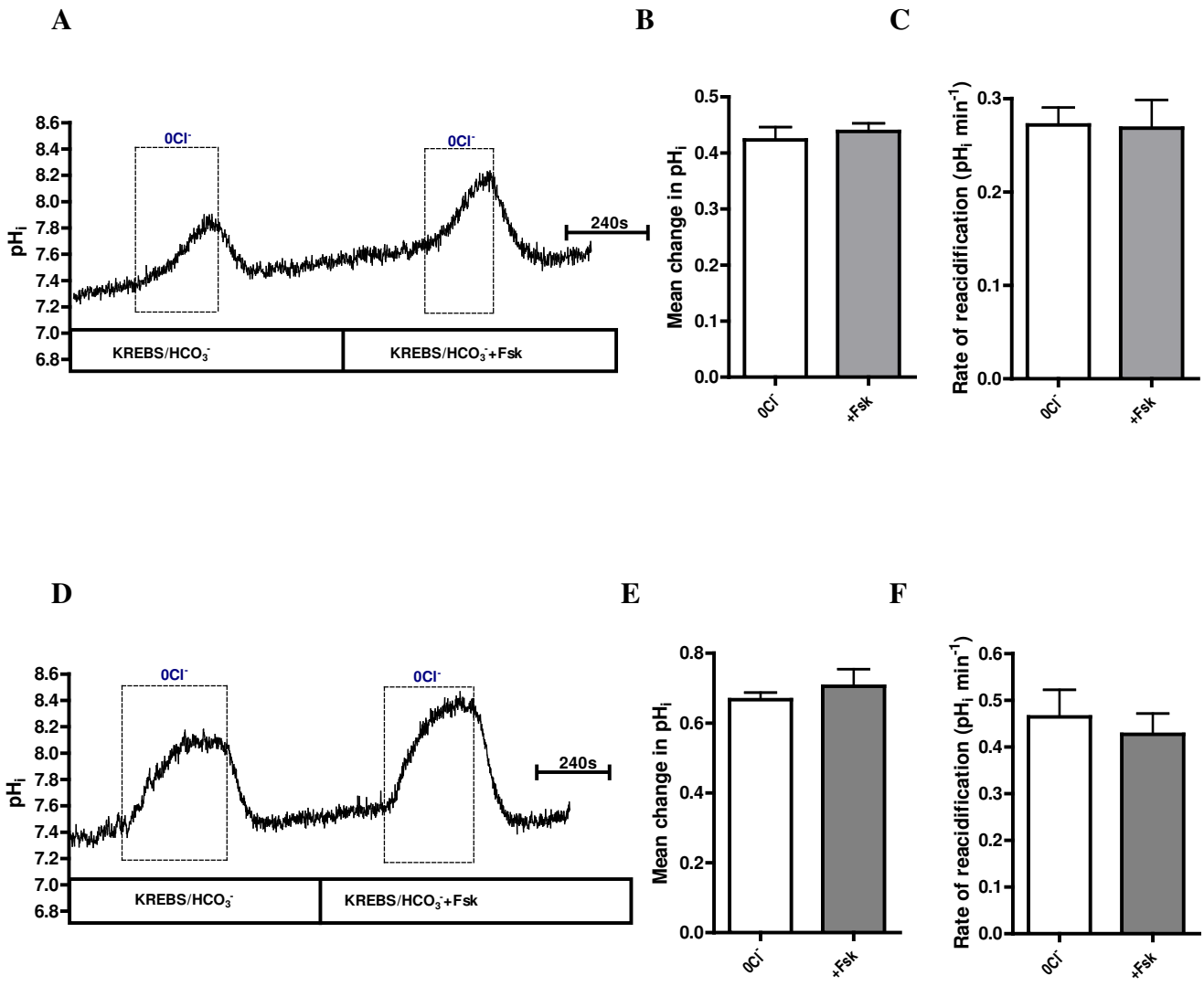


Figure 5. 5: Impact of cAMP on endogenous Cl^-/HCO_3^- anion exchange activity in non-transfected HEK-293T cell and mAE2 activity in transfected cells. Representative pH_i traces showing that an increase in intracellular cAMP, using 5µM Fsk, did not affect the endogenous Cl^-/HCO_3^- AE activity in non-transfected cells (A), or mAE2 activity in transfected HEK-293T cells (D). Summary of the effect of Fsk (5µM) on mean alkalinisation in response to Cl^- free solutions (B), and the rate of reacidification following Cl^- readdition (C) in non-transfected HEK-293T cells. No significant difference (P>0.05), n=7 for each condition, paired observations. Effect ofFsk (5µM) on mean alkalinisation in response to Cl^- free solution (E), and the rate of reacidification following Cl^- readdition (F) in mAE2 transfected HEK-293T cells. Data are shown as Mean±SEM. No significant difference (P>0.05), n=6 for each condition, paired observations.

In another set of experiments, the effect of Fsk on mAE2 activity was assessed in the presence of 25µM DIDS in the Cl^- free solution in order to remove the contribution of the endogenous Cl^-/HCO_3^- AE activity to the pH_i responses in transfected HEK-293T cells (Figure 5.6A). Results also showed that Fsk stimulation did not affect mAE2 activity, as both the mean alkalinisation in response to a Cl^- free solution, and the rate of reacidification upon Cl^- readdition, were not different to the control response (Figure 5.6B and C), even where the endogenous Cl^-/HCO_3^- AE activity had been removed by DIDS. One possible explanation for the apparent lack of effect of cAMP on AE2 activity could be the absence of CFTR in these cells and/or difference between mAE2 and human AE2 sensitivity to cAMP/Fsk. I therefore next investigated the effect of co-expressing CFTR and mAE2 on the response of mAE2 to cAMP stimulation.

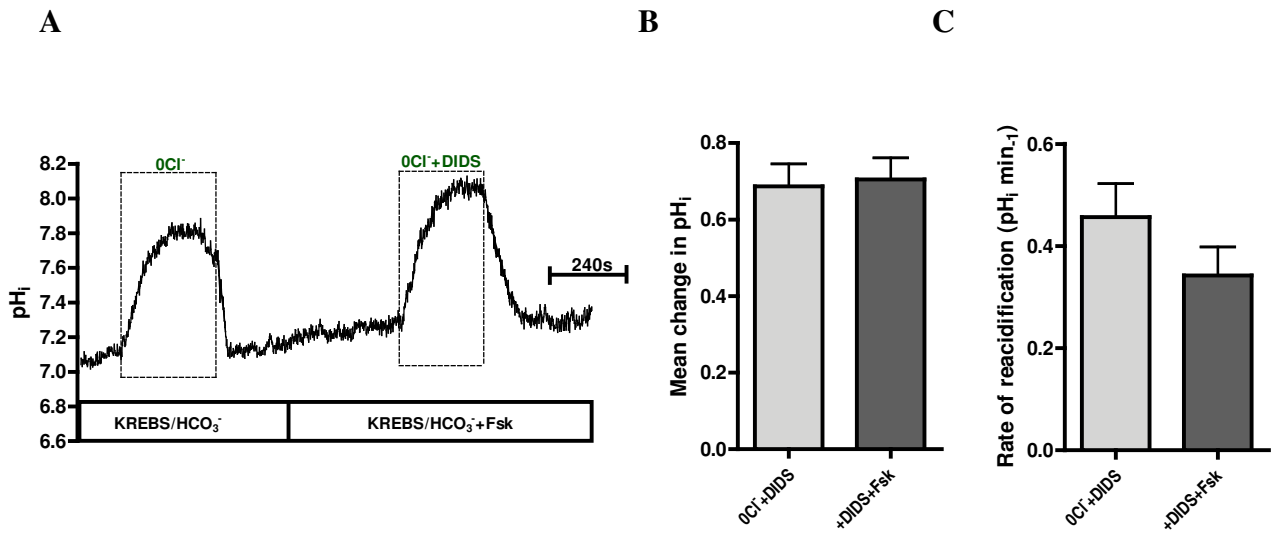


Figure 5. 6: Forskolin did not affect mAE2 activity in transfected HEK-293T cells. (A) Representative pH_i trace showing that increasing $[cAMP]_i$ did not affect mAE2 activity in the presence 25µM DIDS, compared to the control response. Summary data showing the effect of increasing intracellular cAMP, using 5µM Fsk, on mAE2 activity in transfected HEK-293T cells on mean alkalinisation in response to Cl^- free solution (B), and the rate of reacidification upon Cl^- readdition (C). Data are shown as Mean±SEM. P>0.05 no significant difference compared to control, n=3 for $0Cl^-$+DIDS, and n=4 for +DIDS+Fsk.

5.4 Role of CFTR in the regulation of mAE2 activity in transfected HEK-293T cells

My results in Chapter 4 showed that a rise in cAMP led to a marked inhibition in the activity of the basolateral anion exchanger. Previous results from our lab found that the inhibition of the basolateral exchanger appeared to be dependent on the expression of CFTR, since the degree of inhibition by cAMP was significantly reduced in CFTR-KD Calu-3 cells (Garnett *et al.*, 2013). However, it should be noted that a recent study by Kim *et al*., (2014), found no evidence that an increase in cAMP led to the inhibition of the basolateral anion exchanger in polarised cultures of Calu-3 cells (Kim *et al.*, 2014). Indeed these authors found that when CFTR was activated by cAMP, HCO_3^- transport via apical-located CFTR obscured the activity of the basolateral anion exchanger, when assessed by pH_i measurements using the Cl^- removal/readdition method. In order to further investigate the potential regulatory effect of CFTR on AE2 activity, I have measured mAE2 activity in HEK293T cells after co-expression of CFTR. However, I first needed to determine whether CFTR itself contributed to Cl^--coupled HCO_3^- transport in HEK293T cells, when assessed using the standard Cl^- removal/readdition technique.

Results summarised in Figure 5.7 show that expression of CFTR in HEK-293T cells produced a significant increase in the mean pH_i alkalinisation in response to a Cl^- free solution, compared to non-transfected cells. Although the mean rate of reacidification also increased following Cl^- readdition in CFTR transfected cells, it was not significantly different compared to non-transfected cells (Figure 5.7A and B). This suggests that CFTR may be involved in Cl^--coupled HCO_3^- transport in transfected HEK293T cells. However, in order to further investigate this possibility, HEK-293T cells transfected with CFTR were studied in the presence of 25µM DIDS to remove the contribution from the endogenous Cl^-/HCO_3^- AE, as well as in the presence of a CFTR blocker (Figure 5.8A). Interestingly, results showed that there was an increase in mean intracellular pH upon cell perfusion with a Cl^- free solution, in the presence of 25µM DIDS, which was significantly reduced by the specific CFTR inhibitor, $CFTR_{inh}$-172 (Figure 5.8B). The rate of reacidification in CFTR transfected cells was almost

completely inhibited by $CFTR_{inh}$-172, but it was not significantly different compared to the rate in the presence of DIDS alone (Figure 5.8C). Nonetheless, these results indicate that (i) CFTR is active under basal conditions and (ii) contributes to Cl^--coupled HCO_3^- transport in HEK293T cells. The fact that CFTR was active in the absence of a cAMP agonist suggests that HEK-293T cells have enough intracellular cAMP under resting conditions to activate CFTR, although the reason for this is unknown. An alternative explanation is that expression of CFTR (rather than activity) led to the activation of another Cl^- dependent HCO_3^- transporter in HEK293T cells which was also blocked by the CFTR inhibitor.

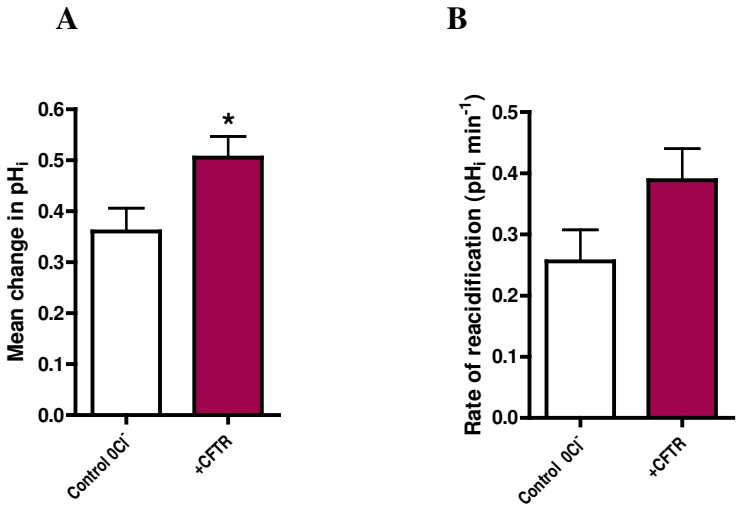


Figure 5. 7: Role of CFTR in HCO_3^- transport in HEK-293T cells transfected with CFTR, compared to control cells. Summary data shows the effect of expression CFTR on Cl^--coupled HCO_3^- transport in CFTR transfected HEK-293T cells, compared to untransfected cells. (A) Mean change in pH_i in response to a Cl^- free solution, and (B) the rate of reacidification following Cl^- readdition (B). $^*P<0.05$ compared to control, n=5 for control, and n=8 for +CFTR.

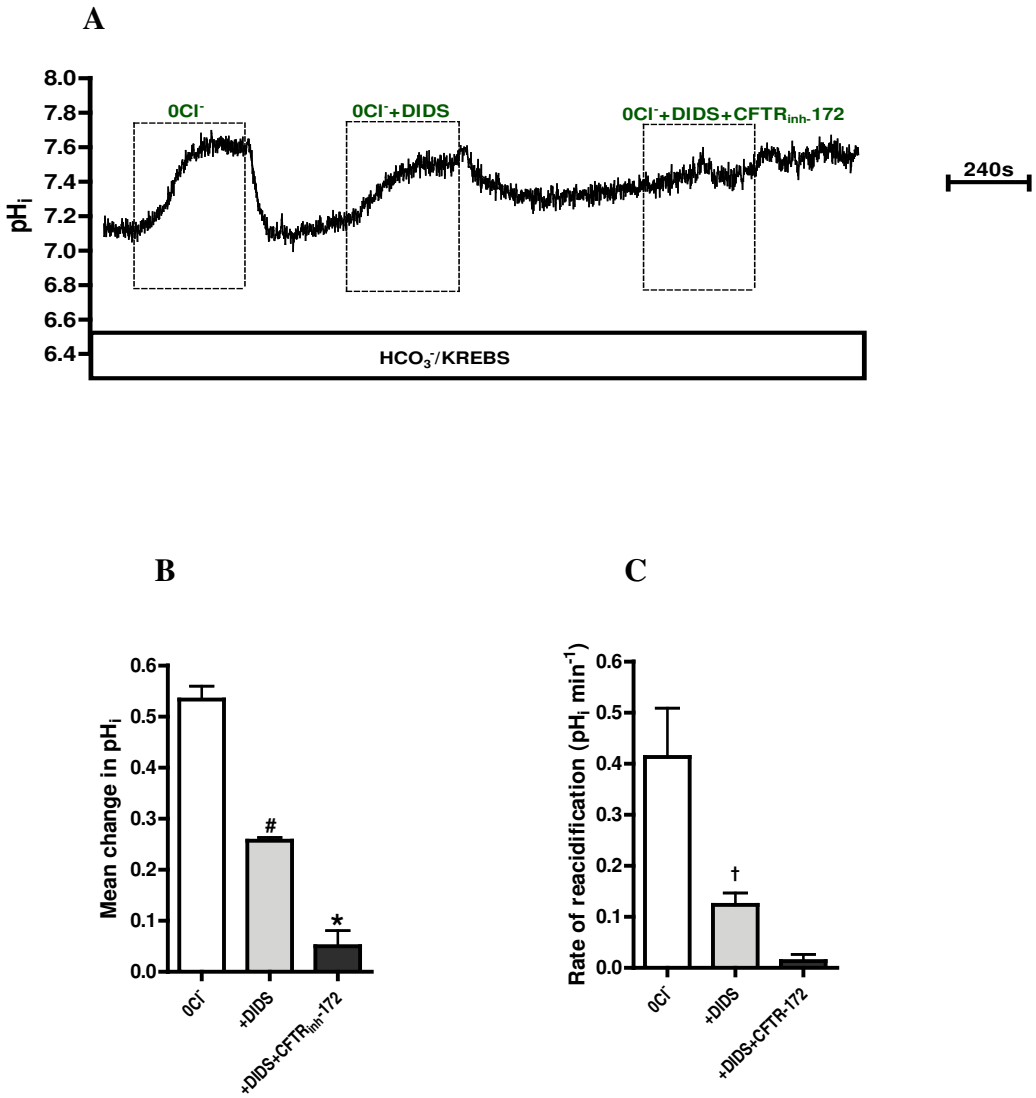
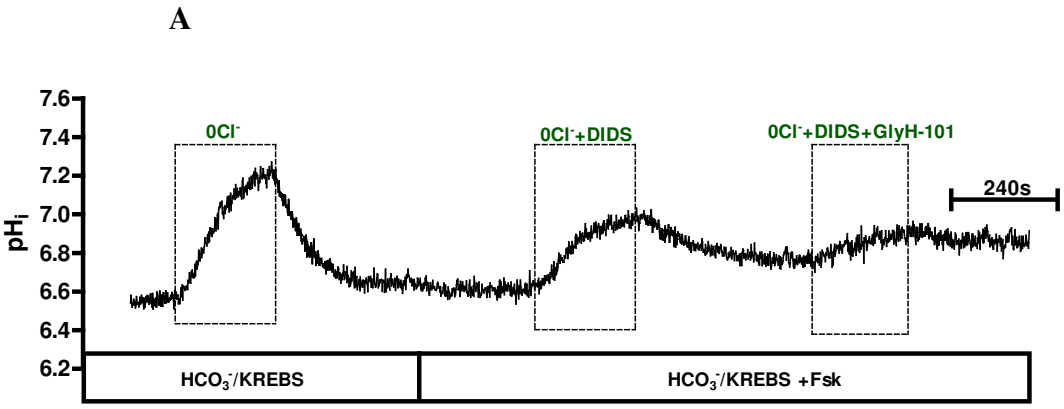


Figure 5. 8: Effect of CFTR expression $0Cl^-$-coupled HCO_3^- transport in HEK-293T cells transfected with CFTR under resting conditions. (A) Representative pH_i trace showing CFTR-dependent HCO_3^- transports in CFTR transfected HEK-293T cells, in the presence of 25µM DIDS. Note that CFTR-dependent Cl^--coupled HCO_3^- transport was almost completely inhibited by $CFTR_{inh}$-172. Summary data showing the mean alkalinisation in pH_i in response to Cl^- free solution (B), and the rate of reacidification upon Cl^- readdition (C) in CFTR transfected HEK293T cells. Data are shown as Mean±SEM. $^{\#}P<0.001$, $^{\dagger}P<0.05$ compared to $0Cl^-$, *P<0.001 compared to DIDS, n=3 for each condition, Paired observations.

It has been found that heterologous CFTR expression in HEK-293 cells, significantly increased intracellular pH after stimulation with Fsk in Cl^- free solutions (Ko *et al.*, 2002). To investigate if cAMP stimulation also affected Cl^--coupled HCO_3^- transport by CFTR in HEK293T cells, I studied the response of the CFTR-transfected cells before and after stimulation with Fsk, in the presence of 25µM DIDS (Figure 5.9). Overall, a comparison of the data in Figures 5.08 and 5.09 showed that there was no significant difference in mean pH_i change in CFTR transfected HEK-293T cells under Fsk stimulation compared to unstimulated cells, both in the magnitude of alkalinisation (0.25±0.01, n=5, compared to the unstimulated response of 0.25±0.00, n=3, P>0.05), nor in the rate of reacidification (0.10±0.01, n=5, compared to the unstimulated response of 0.12±0.02, n=3, P>0.05). The most likely explanation for the lack of effect of cAMP stimulation would be that the transfected HEK-293T cells have enough endogenous cAMP to fully activate CFTR, which is in contrast to the results obtained by Ko *et al*., (2002).



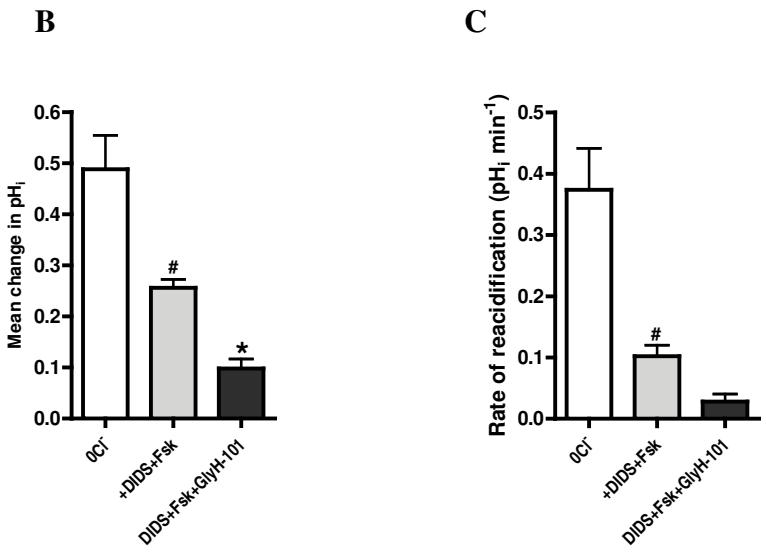


Figure 5. 9: Effect of CFTR expression on Cl^--coupled HCO_3^- transport in HEK-293T cells transfected with CFTR under Fsk stimulated conditions. (A) Representative pH_i trace showing the effect of Fsk (5µM) stimulation on HCO_3^- transport in CFTR transfected HEK-293T cells in the presence of 25µM DIDS. CFTR activity was nearly completely inhibited by the CFTR inhibitor, GlyH-101. Summary data showing the mean alkalinisation in pH_i in response to Cl^- free solution (B), and the rate of reacidification following Cl^- readdition (C). GlyH-101 significantly reduced HCO_3^- transport, under Fsk stimulated condition. Data are shown as Mean±SEM.*P<0.05 compared to control, n=5 for each condition, Paired observations.

5.5 Role of CFTR in the regulation of mAE2 activity

5.5.1 Under cAMP stimulated conditions

Although mAE2 activity in transfected HEK-293T cells was not inhibited by Fsk in the presence of 25µM DIDS (Figure 5.6), it was unknown whether the presence of CFTR would alter the response of mAE2 to cAMP stimulation. Therefore, HEK-293T cells were co-transfected with CFTR and mAE2, and then perfused with a Cl^- free solution in the presence of 25µM DIDS and 5µM Fsk (Figure 5.10A). Results showed that exposure to Fsk significantly reduced the mean change in pH_i in response to a $0Cl^-$ solution, in the presence of 25µM DIDS, although the rate of reacidification was not changed, compared to the

control response (Figure 5.10B and C), suggesting that cAMP induced inhibition of mAE2 requires the presence of CFTR. This result is consistent with the recent finding in our laboratory which showed that intracellular elevation of cAMP, using Fsk, did not abolish the basolateral AE activity in CFTR KD Calu-3 cells, compared to WT Calu-3 cells (Garnett *et al.*, 2013). Results also showed that the mean alkalinisation under Fsk stimulated conditions was significantly reduced by the CFTR inhibitor GlyH-101, which indicates that CFTR and mAE2 contribute equally to HCO_3^- transport in co-transfected HEK-293T cells (Figure 5.10B and C).

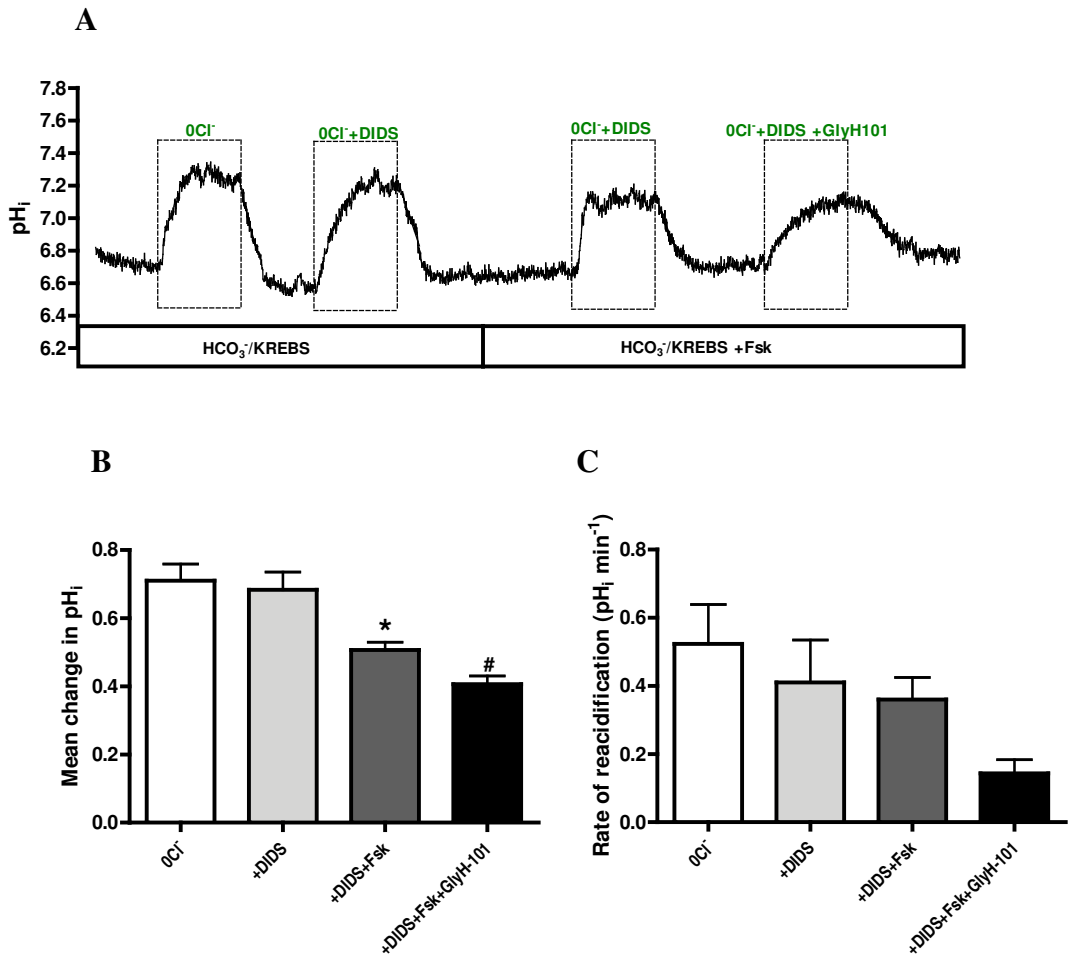


Figure 5. 10: Role of CFTR in the regulation of mAE2 activity in HEK-293T cells co-transfected with CFTR and mAE2. (A) Representative pH_i trace showing the

effect of Fsk in HEK-293T cells co-transfected with CFTR and mAE2, in the presence of 25μM DIDS. Summary data shows although Fsk significantly reduced mAE2 activity, as measured by the mean pH_i change induced by zero Cl^- (B), it did not affect rate of reacidification (C). The CFTR inhibitor GlyH-101, significantly reduced the mean alkalinisation in pH_i under cAMP stimulated conditions in co-transfected cells (B), although the rate of reacidification did not change (C). Data are shown as Mean±SEM. B: *P<0.05 compared to DIDS and #P<0.001 compared to +DIDS +FSK. C: no significant difference (P>0.05), n=3 for mAE2 0Cl^- and +DIDS, and n=6 for other conditions.

In addition, HEK-293T cells co-transfected with CFTR and mAE2 showed a significant increase in the magnitude of the alkalisation in response to a Cl^- free solution, and the rate of reacidification upon Cl^- readdition, compared to HEK-293T cells transfected with CFTR only (Figure 5.11A and B), but less activity compared to mAE2 only transfected cells. This further supports the idea that CFTR expression/activity down regulates mAE2 activity in HEK293T, and possibly Calu-3 cells.

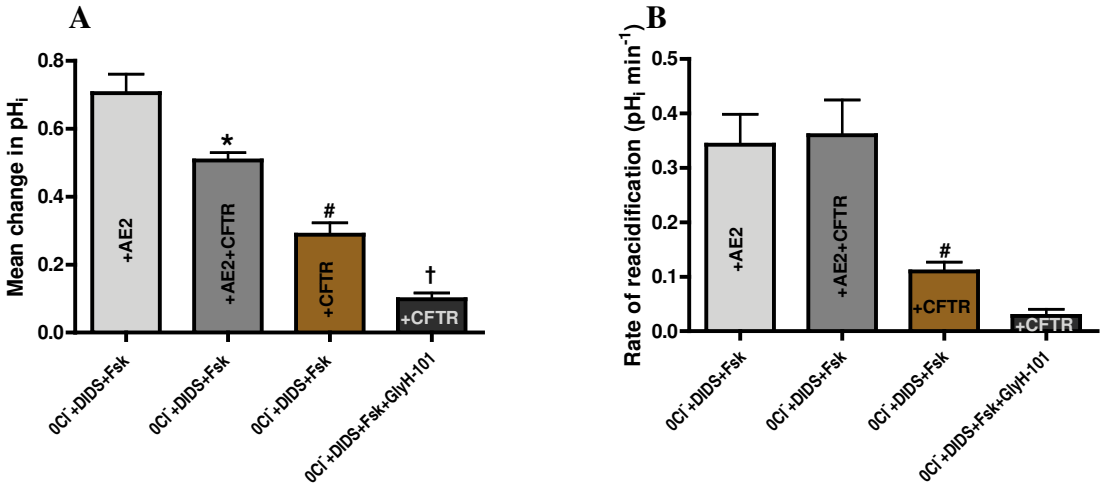


Figure 5. 11: Role of CFTR in HCO_3^- transport in transfected HEK-293T cells. HEK293T cells co-transfected with CFTR and mAE2 caused a significant increase in the mean pH_i change in response to Cl^- free solution (A), and the rate of reacidification upon Cl readdition (B), compared to CFTR-only transfected cells, under Fsk stimulation. Data are shown as Mean±SEM. A: *P<0.01 compared to (AE2), #P<0.001 compared to (AE2+CFTR), †P<0.01compared to (CFTR). B: #P<0.01 compared to (AE2+CFTR), n=4 for (AE2), n=6 for (CFTR) and (CFTR+AE2), and n=5 for (CFTR) +GlyH-101.

In order to investigate whether the CFTR inhibitors, GlyH-101 and $CFTR_{inh}$-172, could potentially affect mAE2 in the absence of CFTR, mAE2 activity was measured in response to a Cl^- free solution, in the presence of 25µM DIDS, under cAMP stimulated conditions (Figure 5.12A). Results showed that both CFTR inhibitors had no effect on mAE2 activity, (Figure 5.12B and C). This is consistent with my previous results in Calu-3 cells where I showed that GlyH-101 did not affect the basolateral AE activity.

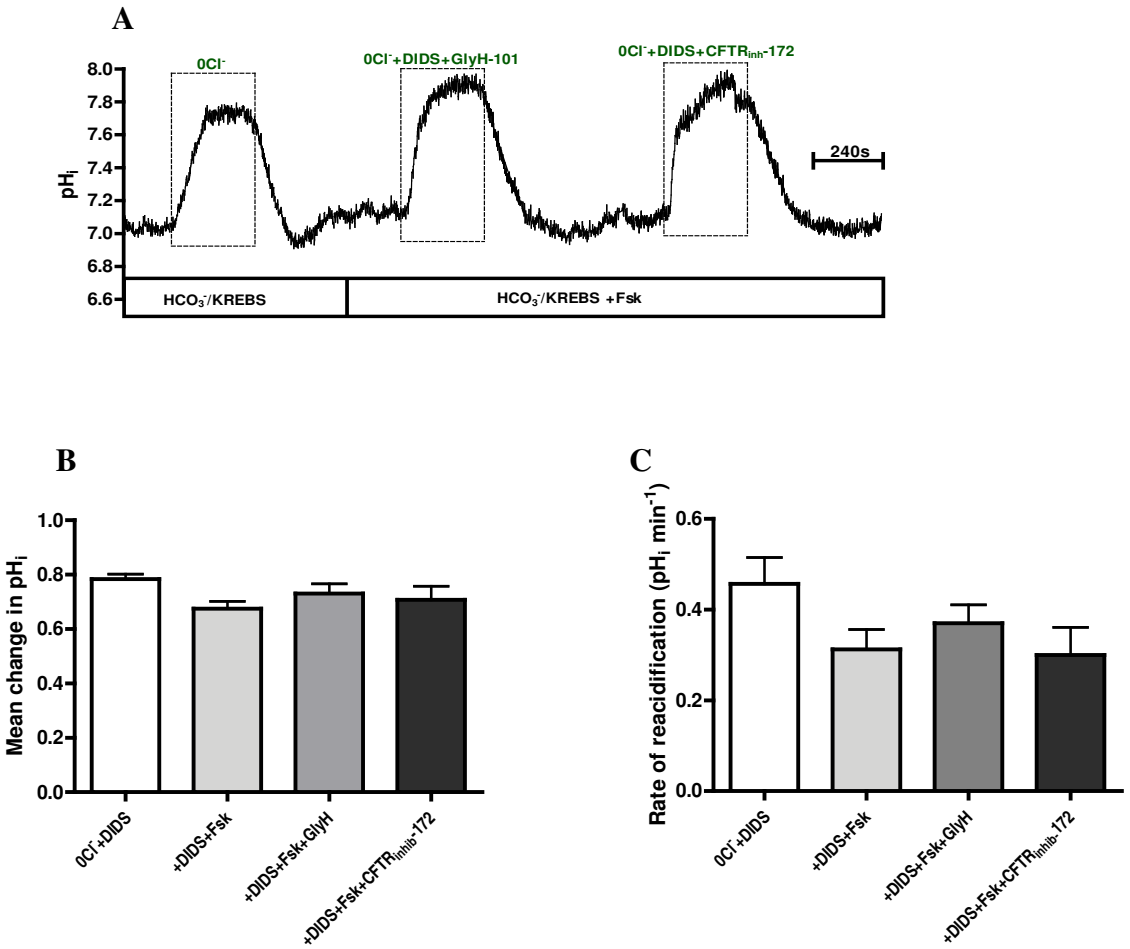


Figure 5. 12: CFTR inhibitors, GlyH-101 and $CFTR_{inh}$-172, did not affect mAE2 activity in transfected HEK-293T cells. (A) Representative pH_i trace showing that the CFTR had no effect on mAE2 activity when expressed in in transfected HEK-293T cells, in the presence of 25µM DIDS. Summary of the effect of both CFTR inhibitors, GlyH-101 and $CFTR_{inh}$-172, on mean alkalinisation in pH_i in response to Cl^- free solution (B) and the rate of reacidification upon Cl^- readdition (C), compared to control response. Data are shown as Mean±SEM. No significant difference (P>0.05) compared to control, n=3 for $0Cl^-$, n=5 for +DIDS+Fsk (control), and n=5 for CFTR inhibitor treated cells.

5.6 Role of Exchange protein directly activated by cAMP (Epac) in the regulation of mAE2 activity

I have shown in Chapter 4 (section 4.5.2) that Epac inhibition caused a significant decrease in basolateral AE activity in Calu-3 cells. To further clarify the role of Epac in the regulation of mouse anion exchange activity, mAE2 transfected cells were preincubated with the specific Epac inhibitor, ESI-09, (Almahariq *et al.*, 2013) for 60 mins. mAE2 activity was then measured under resting conditions in the presence of 25μM DIDS, (Figure 5.13B) and compared to untreated mAE2 transfected cells (Figure 5.13A). Results showed that Epac inhibition caused a marked reduction in the rate of reacidification, compared to untreated cells (Figure 5.13D). Although the mean alkalinisation was decreased, it was not significantly different to control cells (Figure 5.13C). The percent inhibition of AE2 activity by Epac inhibition in HEK-293T cells was significantly lower than the percent inhibition of AE2 in Calu-3 cells (Table 5.01). This suggests that Epac plays an important role in the regulation of AE2 activity under resting conditions similar to results obtained in Calu-3 cells.

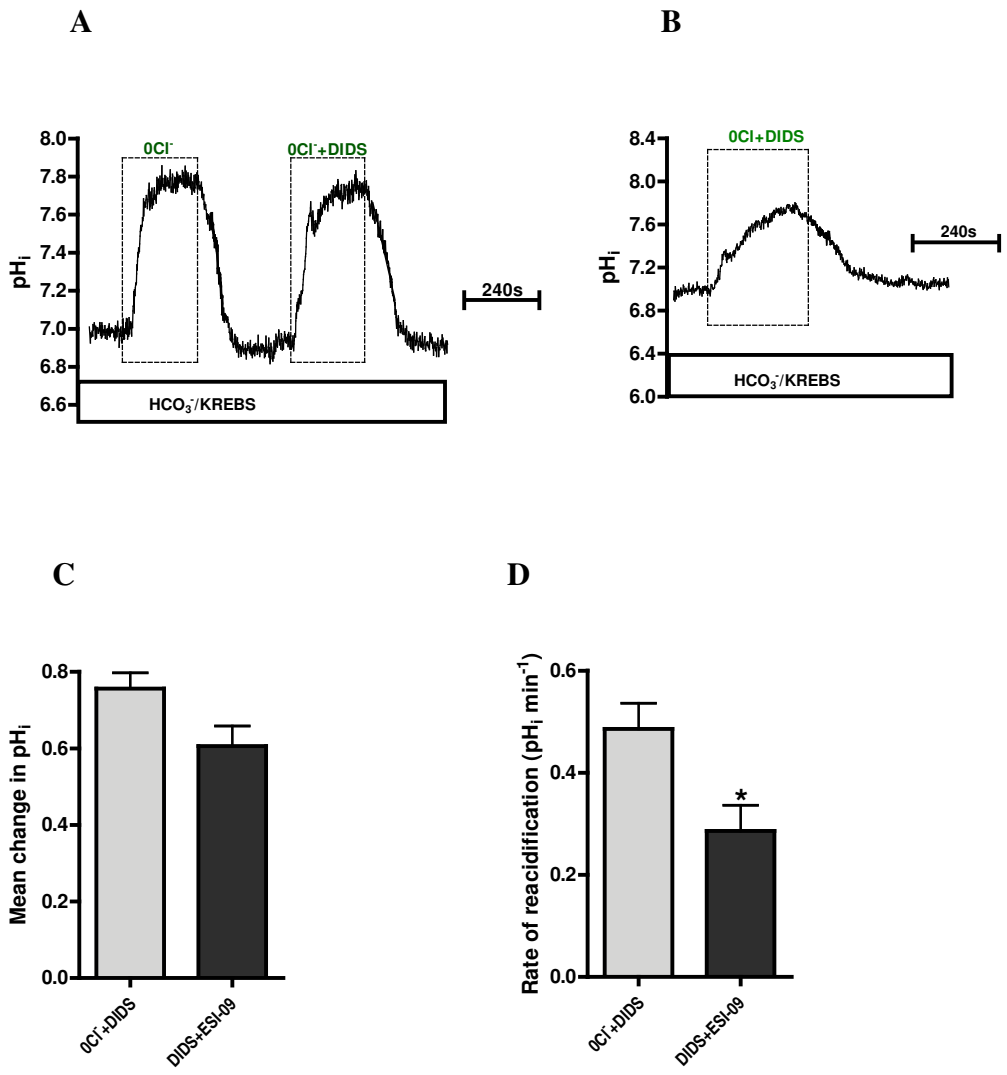


Figure 5. 13: Epac inhibitor, ESI-09, reduced mAE2 activity in transfected HEK-293T cells. Representative pH_i traces showing the effect of ESI-09 (10µM, preincubated for 60 min) on mAE2 activity in transfected HEK-293T cells (B) compared to untreated mAE2 transfected cells (A), in the presence of 25µM DIDS. Summary of the effect of preincubation of cells with 10µM ESI-09 for 60 min, on mean alkalinisation in pH_i (C) and the rate of reacidification upon Cl^- readdition (D). Data are shown as Mean±SEM.*P<0.05 compared to +DIDS, n=5 for each condition.

It has been shown that Epac plays an important role in the regulation of CFTR (Kazi Mirajul Hoque, 2009), and I demonstrated that Epac was required for maintaining the resting activity of mAE2 in Calu-3 cells (Chapter 4). I therefore investigated if CFTR expression could modulate the regulation of mAE2 by Epac. Here, HEK-293T cells were co-transfected with CFTR and mAE2, and preincubated with ESI-09 for 60 min. Cells were then perfused with a Cl^- free solution in the presence of 25μM DIDS (Figure 5.14B), and results compared to control cells co-transfected with mAE2 and CFTR (Figure 5.14A). Surprisingly, my results showed that in co-transfected cells the Epac inhibitor did not affect either the mean pH_i change in response to $0Cl^-$ or the rate of reacidification upon Cl^- readdition (Figure 5.14C & D). The results in Figures 5.13 and 5.14 therefore suggest that the presence of CFTR appears to prevent the Epac inhibitor from reducing mAE2 activity or alternatively that the Epac might not be involved in the regulation of CFTR under basal conditions.

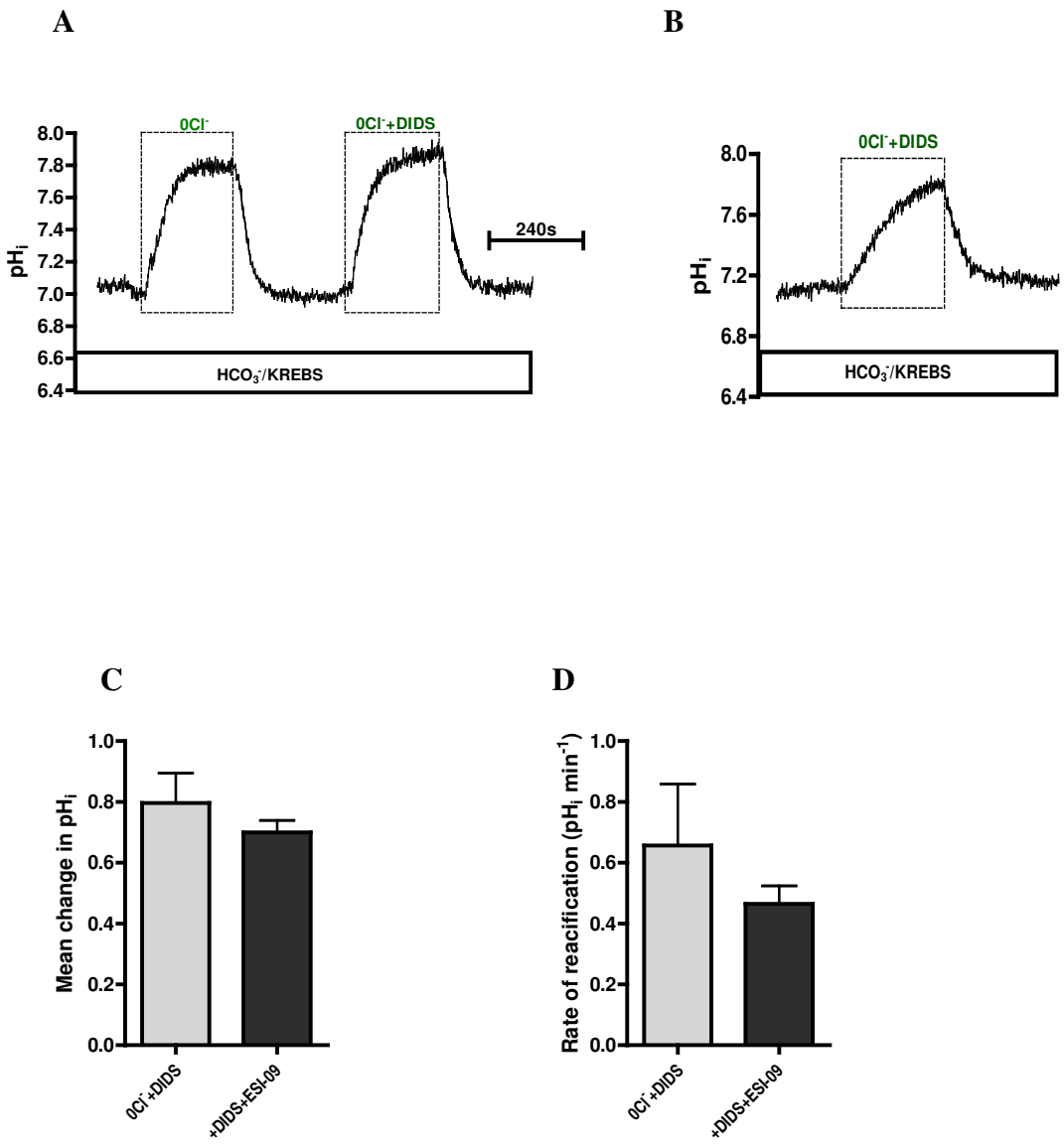


Figure 5. 14: Epac inhibitor, ESI-09, did not affect mAE2 activity in HEK-293T cells co-transfected with mAE2 and CFTR. Representative pH_i traces in which the effect of Epac inhibitor ESI-09 (10µM, 60min preincubation) was assessed in HEK-293T cells co-transfected with CFTR and mAE2 (B), compared to control (untreated) HEK-293T cells co-transfected with CFTR and mAE2 (B). Summary of the effect of preincubation of cells with 10µM ESI-09 on mean alkalinisation in pH_i (C), and the rate of reacidification upon Cl^- readdition (D), in ESI-09 treated and non-treated HEK-293T cells co-transfected with CFTR and mAE2. Data are shown as Mean±SEM.*P<0.05 compared to +DIDS, n=3 for control +DIDS, and n=4 for +DIDS+ESI-09 treated cells.

5.7 Role of intracellular Ca^{2+} in the regulation of mAE2 activity

My previous results in Calu-3 cells showed that a decrease in $[Ca^{2+}]_i$ markedly reduced the basolateral Cl^-/HCO_3^- AE activity under resting conditions (see chapter 4, section 4.6.1.2). To further investigate the role of intracellular Ca^{2+} in the regulation of Cl^-/HCO_3^- AE activity, mAE2 transfected HEK-293T cells were preincubated with 50μM BAPTA-AM for 60 min (Figure 5.15B), and then mAE2 activity was measured in the presence of 25μM DIDS, and compared to untreated mAE2 transfected cells (Figure 5.15A). BAPTA-AM preincubation caused a significant decrease in mAE2 activity, both in the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition, compared to the control (non-BAPTA-AM treated cells) response (Figure 5.15C and D). The BAPTA-AM induced inhibition of the magnitude of alkalinisation was significantly lower in mAE2 transfected HEK-293T cells, compared to the inhibition in Calu-3 cells. However, the BAPTA-AM induced inhibition of the rate of reacidification was not significantly different in HEK-293T compared to Calu-3 cells (Table 5.01). This further supports the results from Chapter 4 that intracellular Ca^{2+} concentration plays a significant role in maintaining AE2 active under resting conditions.

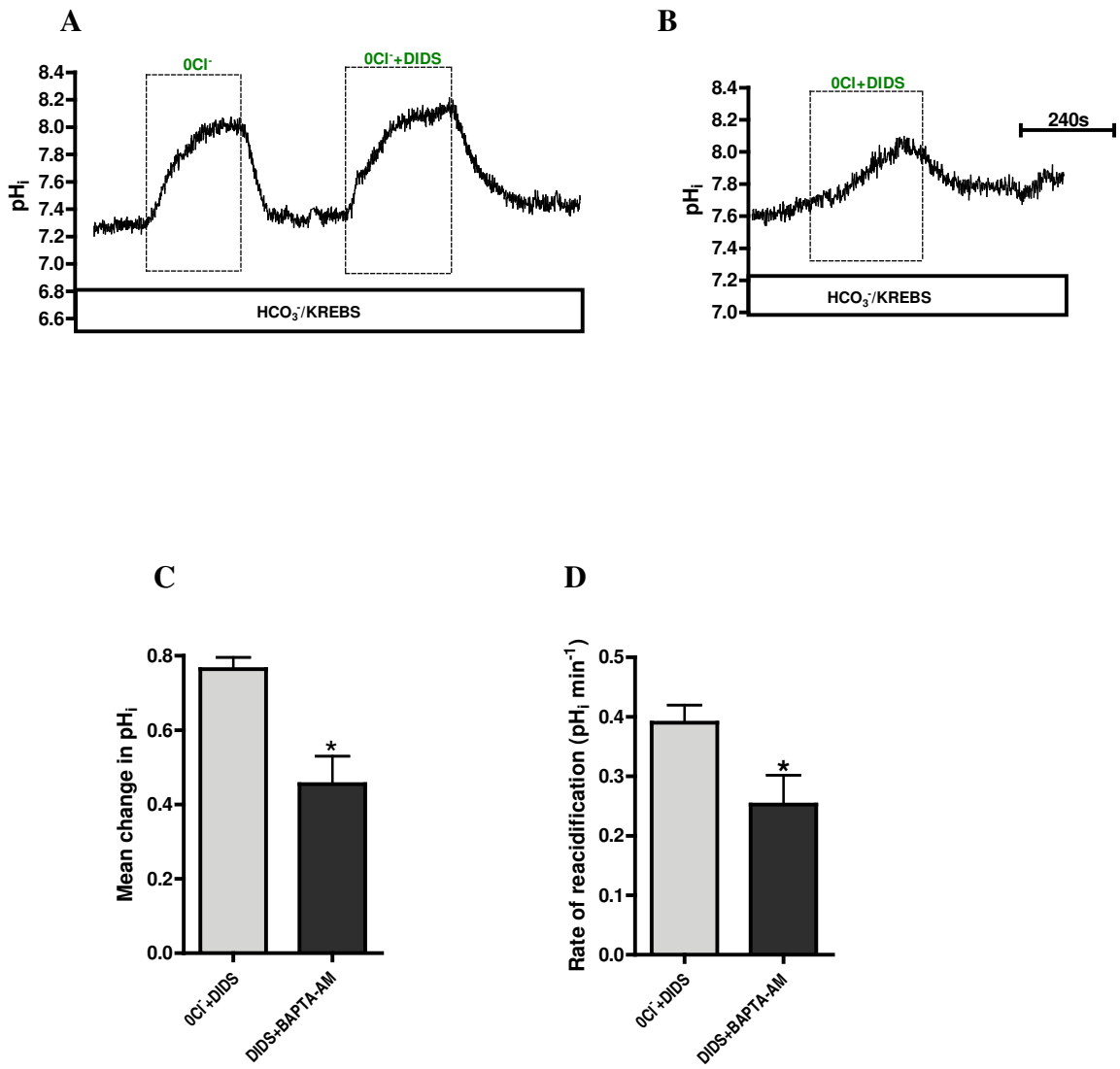
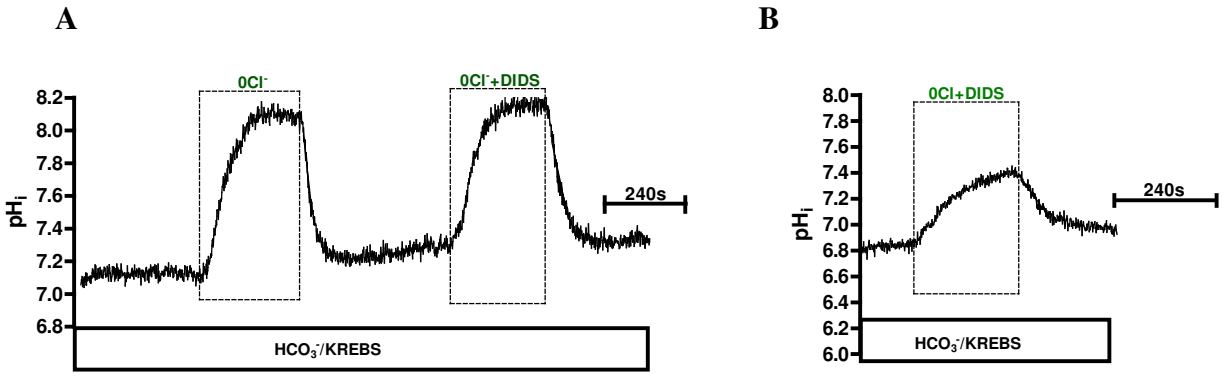


Figure 5. 15: BAPTA-AM reduced mAE2 activity in transfected HEK-293T cells. Representative pH_i traces showing the effect of Ca^{2+} chelator BAPTA-AM, (50µM, preincubated for 60 min) on mAE2 activity in transfected HEK-293T cells (B) compared to untreated mAE2 transfected cells (A), in the presence of 25µM DIDS. Summary of the effect of BAPTA-AM (50µM) preincubation for 60 min, in mAE2 transfected HEK-293T cells, on both the mean pH_i change in response to Cl^- removal (C), and the rate of reacidification upon Cl^- readdition (D). Data are shown as Mean±SEM. *P<0.05 compared to control, n=7 for AE2+DID, and n=4 for +DIDS+BAPTA-AM.

5.8 Role of CaM in the regulation of mAE2 activity

As described in my previous results in chapter 4, CaM inhibition by 50µM J-8 produced a significant decrease in the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. The mean alkalinisation in response to Cl^- removal was reduced by 50±6.9% and the rate of reacidification by 59.3±16.6% (see table 5.01). To further investigate the role of CaM in the regulation of anion exchange activity, mAE2 transfected cells were preincubated with 50µM J-8 for 60 min, and then mAE2 activity measured in Cl^- free solutions in the presence of 25µM DIDS (Figure 5.16A), compared to control untreated mAE2-transfecterd cells (Figure 5.16B). Results showed that CaM inhibition caused a significant decrease in mAE2 activity, both in the mean pH_i change (56±7% decrease) in response to a Cl^- free solution, and the rate of reacidification, which declined by 70.8±8.8%, upon Cl^- readdition, compared to untreated mAE2 transfected cells (Figure 5.16C and D). The J-8 induced inhibition of mAE2 activity was not significantly different compared to the J-8 induced inhibition of the basolateral AE activity in Calu-3 cells, with respect to the mean alkalinisation in response to Cl^- free solution and the rate of reacidification upon Cl^- readdition (see Table 5.01). This further supports a role for CaM in the regulation of AE activity under resting conditions, potentially through a Ca^{2+}-dependent pathway.



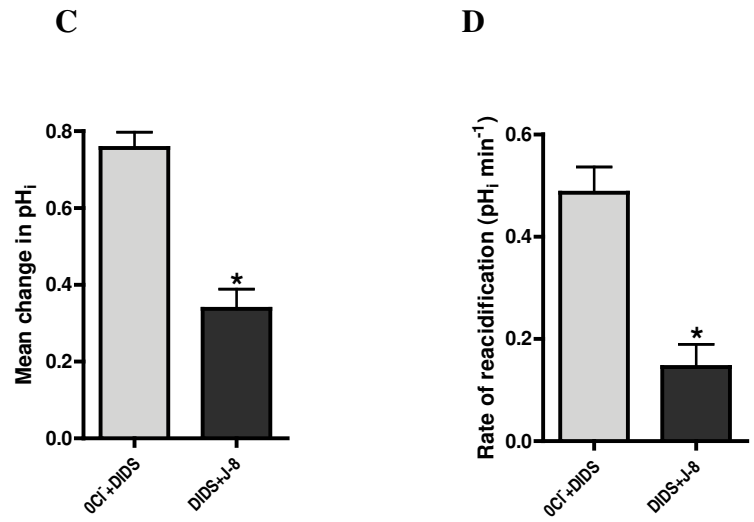


Figure 5. 16: CaM inhibitor, J-8, markedly reduced mAE2 activity in transfected HEK-293T cells. Representative pH_i traces showing the effect of J8 (50µM J-8 preincubated for 60 min) on mAE2 activity in transfected HEK-293T cells (B) compared to untreated mAE2 transfected cells (A), in the presence of 25µM DIDS. Summary of the effect of J-8, on the mean change in pH_i in response to Cl^- free solution (C), and the rate of reacidification upon Cl^- readdition (D). Data are shown as Mean±SEM.*P<0.05 compared to control, n=5 for control $0Cl^-$+DIDS, and n=4 for J-8 treated cells.

5.9 Role of protein phosphatase 1 (PP1/2A) in the regulation of mAE2 activity

I have shown that inhibition of PP1/2A in Calu-3 cells with 100nM OA caused a significant decrease in basolateral AE activity. To further investigate the role of PP1/2A in the regulation of mAE2 activity, HEK-293T cells transfected with mAE2 were preincubated with 100nM OA for 60 min, and then perfused with a Cl^- free solution in the presence of 25µM DIDS to measure mAE2 activity (Figure 5.17A), and compared to untreated cells (Figure 5.17B). Results showed that PP1/2A inhibition caused a significant decrease in the mean change in response to $0Cl^-$, although the rate of reacidification did not change, compared to untreated cells (Figure 5.17C and D). However, the OA-induced inhibition of mAE2 activity in transfected HEK-293T cells was significantly different to the OA-induced inhibition of basolateral AE activity in Calu-3 cells, both in the magnitude of alkalinisation and the rate of reacidification (Table 5.01).

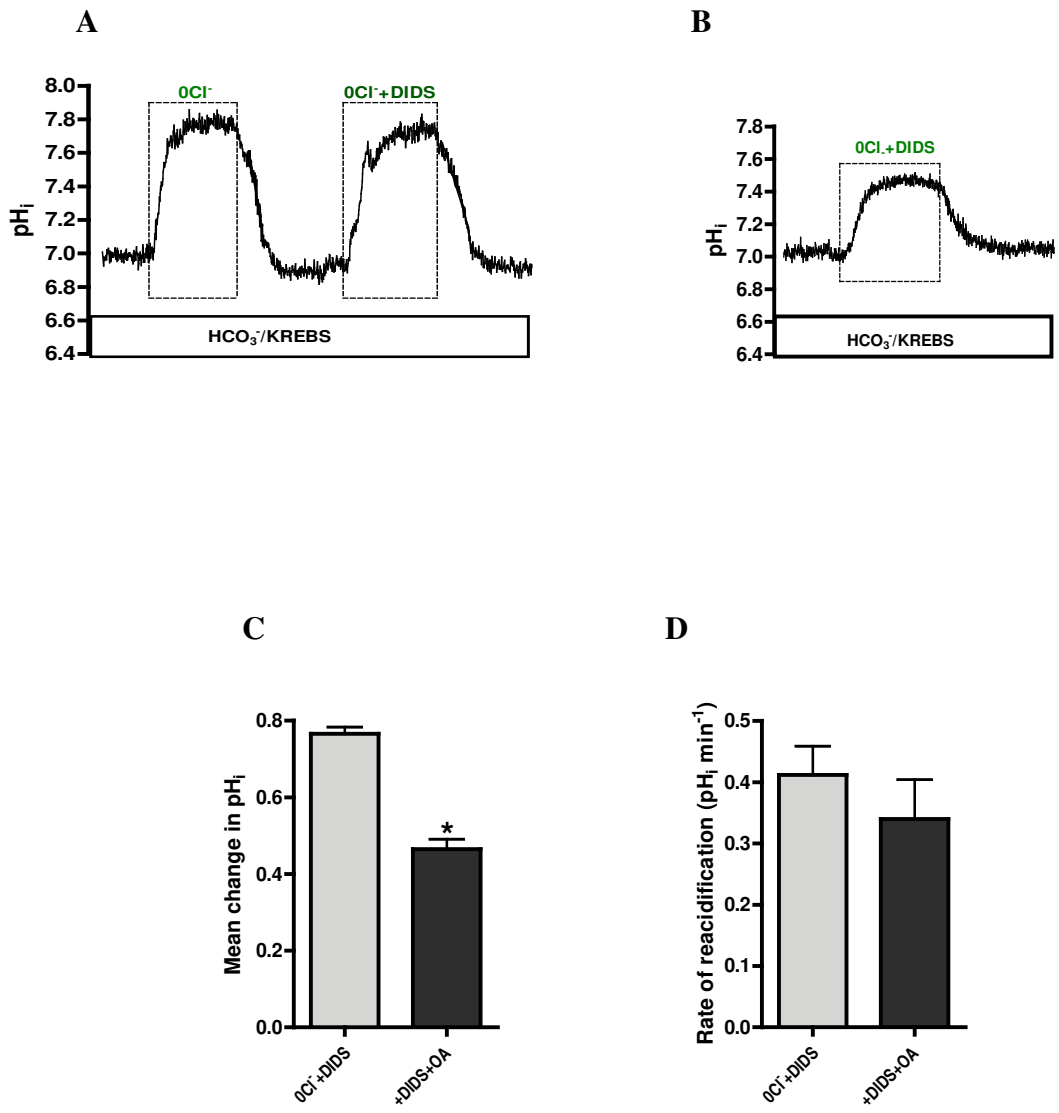


Figure 5. 17: PP1/2A inhibition reduced mAE2 activity in transfected HEK-293T cells. pH_i traces in which the effect of PP1/2A inhibitor, OA (100nM, preincubation for 60 min), was assessed in mAE2 transfected HEK-293T cells (B), compared to untreated mAE2 transfected HEK-293T cells (A). Summary of the effect of okadaic acid on the magnitude of alkalinisation in response to Cl^- removal (C), and the rate of reacidification upon Cl^- readdition (D). Data are shown as Mean±SEM.*P<0.05 compared to $0Cl^-$+DIDS, n=5 for $0Cl^-$+DIDS, and n=4 for +DIDS+OA.

5.10 Role of CK2 in the regulation of mouse AE2 activity

5.10.1 Effect of acute exposure to the CK2 inhibitor TBB on endogenous Cl^-/HCO_3^- AE activity and mouse AE2 activity expressed in HEK 293T cells

Since CK2 showed a novel role in the regulation of the basolateral AE activity in Calu-3 cells, the effect of CK2 inhibition was also tested on mAE2 activity in transfected HEK-293T cells. First, I investigated if CK2 inhibition had any effect on the endogenous Cl^-/HCO_3^- exchanger. For these experiments, non-transfected cells were acutely exposed to TBB and then endogenous Cl^-/HCO_3^- AE activity measured in Cl^- free solutions (Figure 5.18A). Results showed that CK2 inhibition caused almost complete loss of endogenous Cl^-/HCO_3^- AE activity, both in the magnitude of alkalinisation in Cl^- free solution, and the rate of reacidification upon Cl^- readdition, compared to untreated cells (Figure 5.18B and C). This indicates that endogenous Cl^-/HCO_3^- exchange activity was clearly CK2 dependent. Interestingly, and similar to Calu-3 cells, application of TBB caused an intracellular acidification in non-transfected HEK-293T cells (Figure 5.18A, red trace), which was not significantly different compared to the TBB-induced acidification in mAE2 transfected cells, (see Fig. 5.19A) both in the magnitude of alkalinisation (control 0.60±0.06, n=3; mAE2 transfected,0.44±0.06, n=6, P>0.05), and the rate of reacidification (control, 0.17±0.02, n=3; mAE2 transfected, 0.12±0.01, n=6, P>0.05).

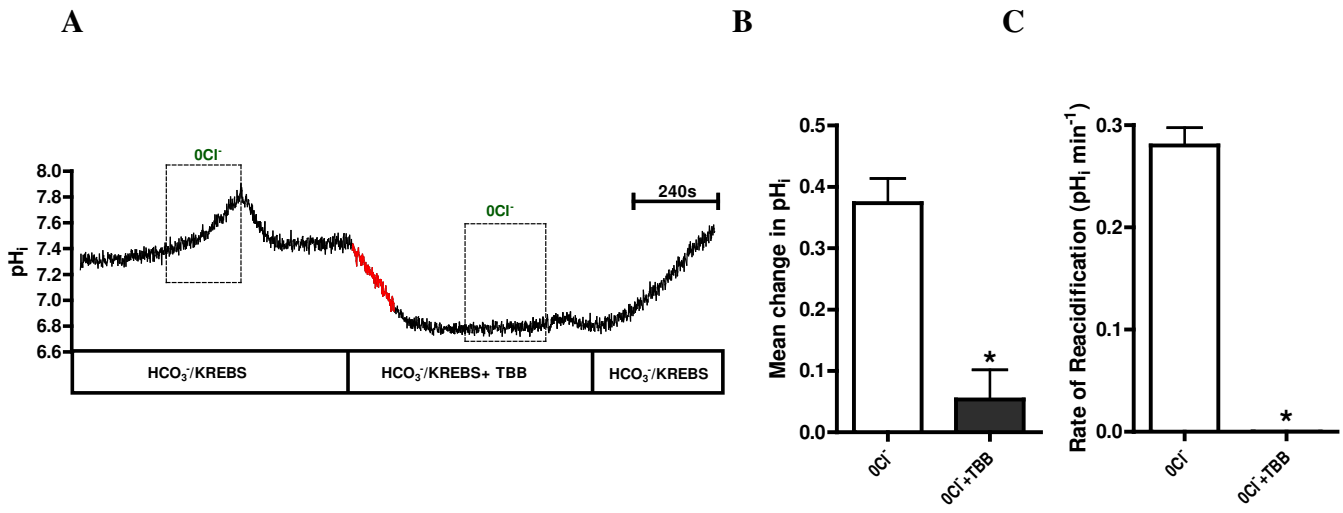


Figure 5. 18: Impact of the CK2 inhibitor, TBB, on endogenous Cl^-/HCO_3^- anion exchange activity in non-transfected HEK-293T cells. (A) Representative pH_i trace showing that acute TBB exposure (10µM) inhibited the endogenous Cl^-/HCO_3^- AE activity in non-transfected HEK-293T cells. Summary of the effect of acute exposure to TBB on mean pH_i change (alkalinisation) following Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C) in non-transfected HEK-293T cells. Data are shown as Mean±SEM.*P<0.05 compared to control $0Cl^-$. n=3 for each condition, paired observations.

To further investigate the role of CK2 in the regulation of AE2 activity, mAE2 transfected cells were acutely exposed to TBB in the presence of 25µM DIDS (Figure 5.19A). Results showed that CK2 inhibition significantly reduced mAE2 activity, both the mean alkalinisation in response to Cl^- free solution (by 62.5±5.1%), and the rate of reacidification upon Cl^- readdition by (53.3±6.9%) (Figure 5.19B and C), which were not significantly different compared to TBB-induced percent inhibition of the basolateral AE activity in Calu-3 cells (Table 5.01). This is consistent with my previous result in Calu-3 cells which showed that acute exposure to TBB significantly reduced the basolateral AE activity. This implies that CK2 plays an important role in the regulation of mAE2 activity under resting conditions in transfected HEK-293T cells.

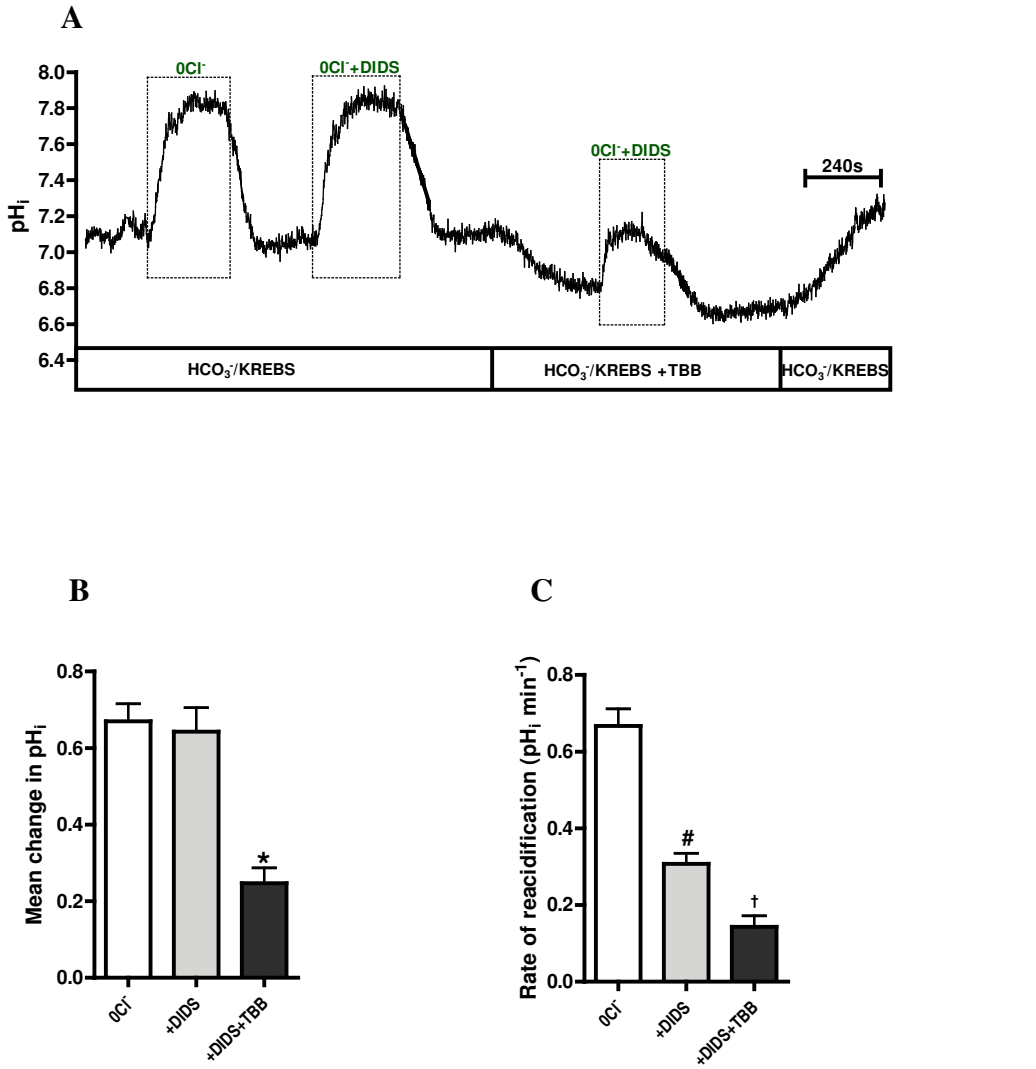


Figure 5. 19: CK2 inhibitor, TBB, significantly reduced mAE2 activity in transfected HEK-293T cells. (A) Representative pH_i trace showing that acute exposure to TBB (10µM), in the presence of 25µM DIDS, inhibited mAE2 activity in transfected HEK-293T cells. Summary data showing that acute exposure of 10µM TBB significantly reduced mAE2 activity, both the mean pH_i alkalinisation in response to Cl^- free solution (B), and the rate of reacidification after Cl^- readdition (C), in the presence 25µM DIDS, in transfected HEK-293T cells. Data are shown as Mean±SEM. *P<0.001 compared to +DIDS, $^{\#}$P<0.001 compared to $0Cl^-$, †P<0.01 compared to +DIDS, n=7 for each condition, Paired observations.

In order to further investigate the role of CK2 in the regulation of mAE2 activity, HEK-293T cells were co-transfected with mAE2 and the alpha catalytic (α)-subunit of wild type (WT) CK2 (see Methods), and then cells were acutely exposed to 10µM TBB in the presence of 25µM DIDS (Figure 5.20A). TBB

significantly decreased mAE2 activity, both the magnitude of alkalinisation in Cl^- free solution, and the rate of reacidification following Cl^- readdition, in HEK-293T cells co-transfected with mAE2 and WT-CK2 (Figure 5.20B and C). Also, the TBB induced percent inhibition of mAE2 activity in co-transfected HEK-293T cells was equivalent to the percent inhibition observed in mAE2 transfected HEK-293T cells, having only endogenous CK2 (Figure 5.20D and E). This suggests that CK2 levels were not limiting in HEK-293T cells, as overexpression of the active alpha CK2 subunit did not alter the ability of TBB to block AE2 activity. Taken together, these results clearly indicate that keeping CK2 in an active state plays an essential role in maintaining both the endogenous, as well as mAE2 activity, under resting conditions.

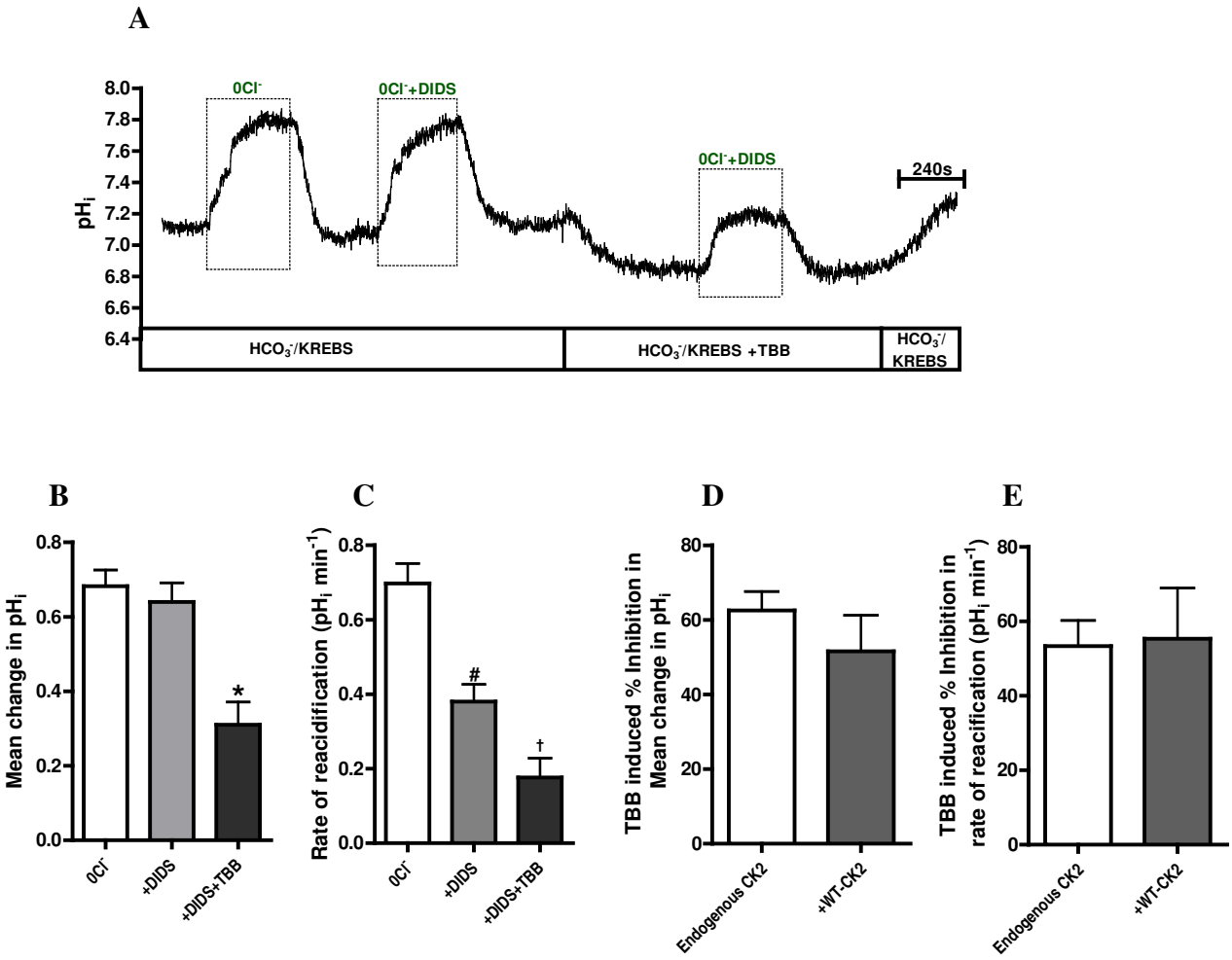


Figure 5. 20: CK2 inhibitor, TBB, markedly reduced mAE2 activity in HEK-293T cells co-transfected with mAE2 and WT-CK2. (A) Representative pH_i trace showing

the inhibition of mAE2 activity, by acute exposure to TBB (10μM) in the presence of 25μM DIDS, in HEK-293T cells co-transfected with mAE2 and WT-CK2. Summary of the effect of acute exposure to the CK2 inhibitor, TBB (10μM), on mAE2 activity, both the mean alkalinisation in pH_i in response to Cl^- free solution (B), and the reacidification following Cl^- readdition in co-transfected HEK-293T cells. Data are shown as Mean±SEM. B: *P<0.01 compared to +DIDS, $^{\#}$P<0.01 compared to $0Cl^-$, †P<0.05 compared to +DIDS, n=6 for each condition, except for $0Cl^-$ n=4. Summary of the percent inhibition of mAE2 activity by acute exposure to 10μM TBB in HEK-293T cells co-transfected with mAE2 and WT-CK2 compared to the effect of TBB on endogenous CK2 in mAE2 only transfected cells, both on the mean alkalinisation in pH_i in response to Cl^- free solution (D), and the rate of reacidification upon Cl^- readdition (E). Data are shown as Mean±SEM, no significant difference (P>0.05) compared to control, n=7 for mAE2 (), and n=6 for co-transfected cells (+WT-CK2).

However, in another set of experiments HEK-293Tcells were co-transfected with mAE2 and the alpha subunit of CK2 containing a double mutation, V66A & I174A, (DM-CK2), which has previously been shown to make CK2 much less sensitive than WT-CK2 to TBB inhibition, without altering its catalytic activity (Sarno *et al.*, 2005). Expression of the DM-CK2 mutant protein partially reduced the effect of TBB on mAE2 activity in co-transfected cells (Figure 5.21A). In the presence of DM-CK2, TBB had no effect on the rate of reacidification following Cl^- readdition, compared to the control response, but it still caused a significant decrease in the magnitude of alkalinisation (Figure 5.21B and C). Interestingly, there was a significant decrease in the percent inhibition of mAE2 activity by TBB in HEK-293T cells co-transfected with AE2 and DM-CK2, both in the mean pH_i change and the rate of reacidification, compared to HEK-293T transfected with mAE2 only (Figure 5.21D and E). This result suggests that having the TBB-insensitive DM-CK2 present was able to maintain mAE2 activity even in the presence of the CK2 inhibitor TBB.

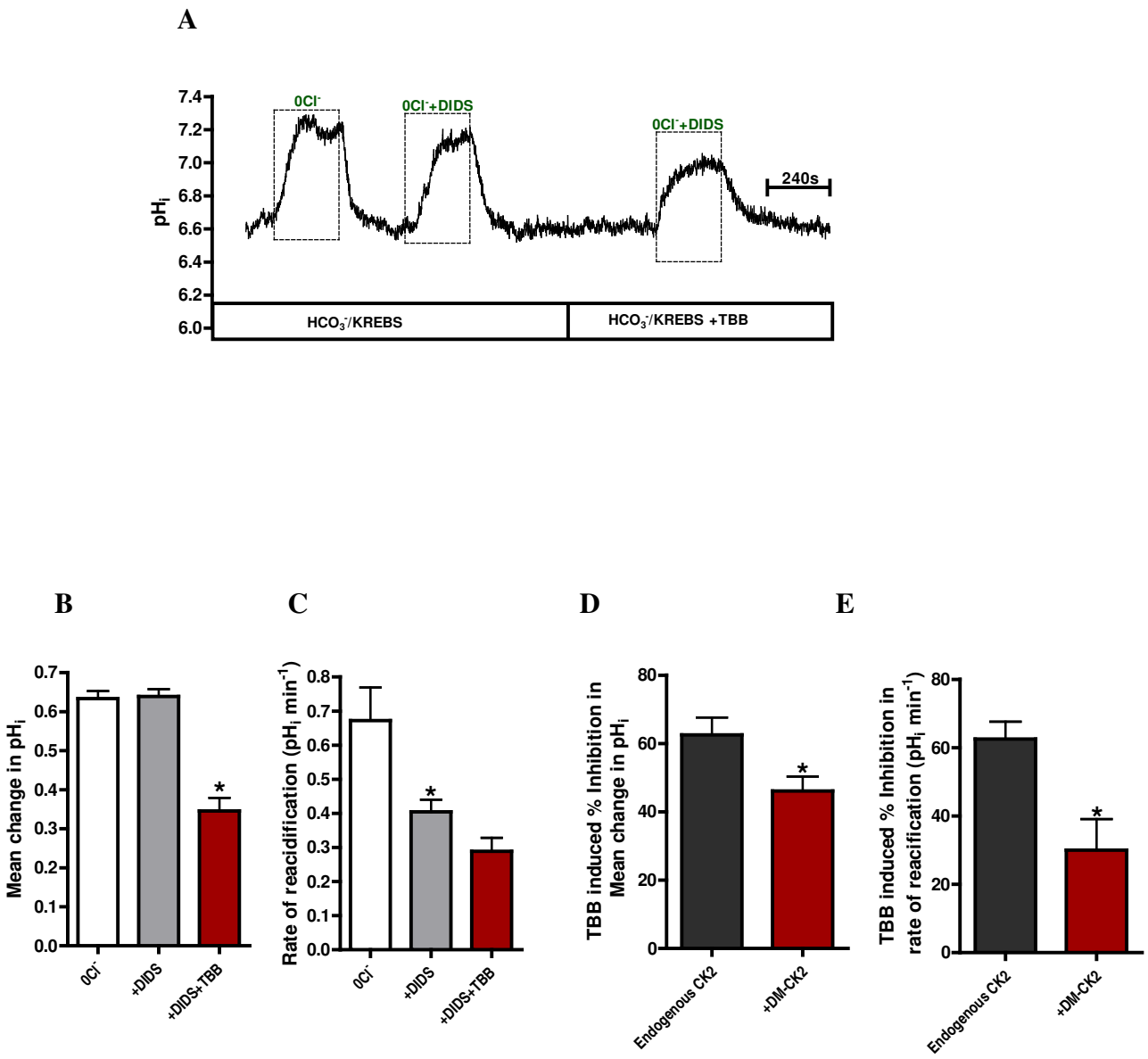
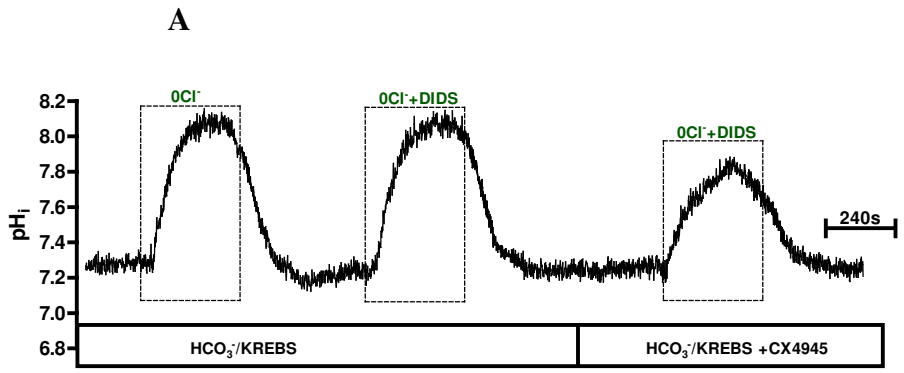


Figure 5. 21: Impact of the CK2 inhibitor TBB on mAE2 activity in HEK-293T cells co-transfected with mAE2 and DM-CK2. (A) Representative pHi trace showing the effect of acute exposure to TBB (10µM) on mouse AE2 activity, in the presence of 25µM DIDS, in HEK-293T cells co-transfected with mAE2 and DM-CK2. Summary of the effect of acute exposure to TBB on mouse AE2 activity in HEK-293T cells co-transfected with mAE2 and DM-CK2 on mean alkalinisation in pH_i in response to Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C). *P<0.05 compared to control (+DIDS), n=9 for each condition, paired observations. TBB-induced percent inhibition of mAE2 activity, both the magnitude of alkalinisation in response to Cl^- free solution (D), and the rate of reacidification upon Cl^- readdition (E) in HEK-293T cells co-transfected with mAE2 and DM-CK2, compared to endogenous CK2 in mAE2 only transfected cells. Data are shown as Mean±SEM.*P<0.05 compared to control, n=7 for endogenous CK2, and n=9 for +DM-CK2.

5.10.2 Effect of acute exposure to the CK2 inhibitor, CX4945, on mAE2 activity expressed in HEK-293T cells

To provide further support for CK2 regulation of mAE2 in transfected HEK-293T cells, CX4945, another potent and selective CK2 inhibitor was tested (Kim and Hwan Kim, 2013). Results showed that acute exposure to CX4945 significantly reduced mAE2 activity, in the presence of 25µM DIDS (Figure 5.22A), both the magnitude of alkalinisation in response to Cl^- free solution (by 34.2±7.3%), and the rate of reacidification upon Cl^- readdition (by 53.6±7.8%), compared to the control response (Figure 5.22B and C). However, the CX-4945 induced inhibition of mAE2 in HEK-293T cells was significantly lower than the CX-4945 induced inhibition of the basolateral AE activity in Calu-3 cells, both in the magnitude of alkalinisation and the rate of reacidification (Table 5.01). These results support my previous observations in Calu-3 cells, where acute exposure to CX4945 caused a significant decrease in the basolateral AE activity, but suggest that either mAE2 is less sensitive to the inhibitor.



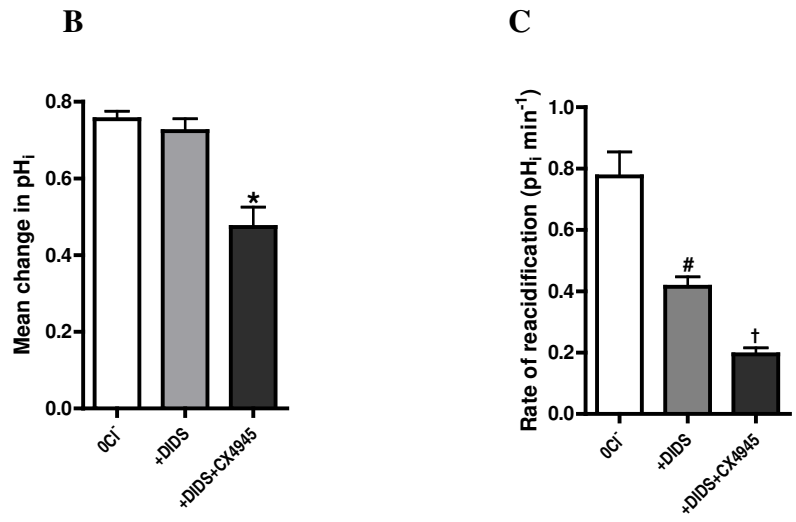


Figure 5. 22: CK2 inhibitor, CX4945, significantly reduced mAE2 activity in transfected HEK-293T cells. (A) Representative pH_i trace showing the effect of acute exposure to CX4945 (10μM) on mAE2 activity, in the presence of 25μM DIDS, in mAE2 transfected HEK-293T cells. Summary of the effect of acute exposure to CX4945 on mean alkalinisation in pH_i in response to Cl^- removal (B), and the rate of reacidification upon Cl^- readdition (C) compared to control response. Data are shown as Mean±SEM. (B) *P<0.001 compared to +DIDS, (C) $^{\#}$P<0.001 compared to $0Cl^-$, †P<0.05 compared to +DIDS, n=9 for each condition.

To investigate the effect of CX4945 further, HEK-293T cells were also co-transfected with mAE2 and DM-CK2, and then acutely exposed to CX4945, and mAE2 activity studied using Cl^- free solutions containing 25μM DIDS (Figure 5.23A). Results showed that the presence of DM-CK2 partially reduced the ability of CX4945 to inhibit mAE2 activity as CX4945 failed to affect the mean change in pH_i in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition (Figure 5.23B and C). Taken together, the above data suggest that CK2 plays an important role in the regulation of mAE2 activity in transfected HEK-293T cells under resting conditions, which further supports my previous results from Calu-3 cells.

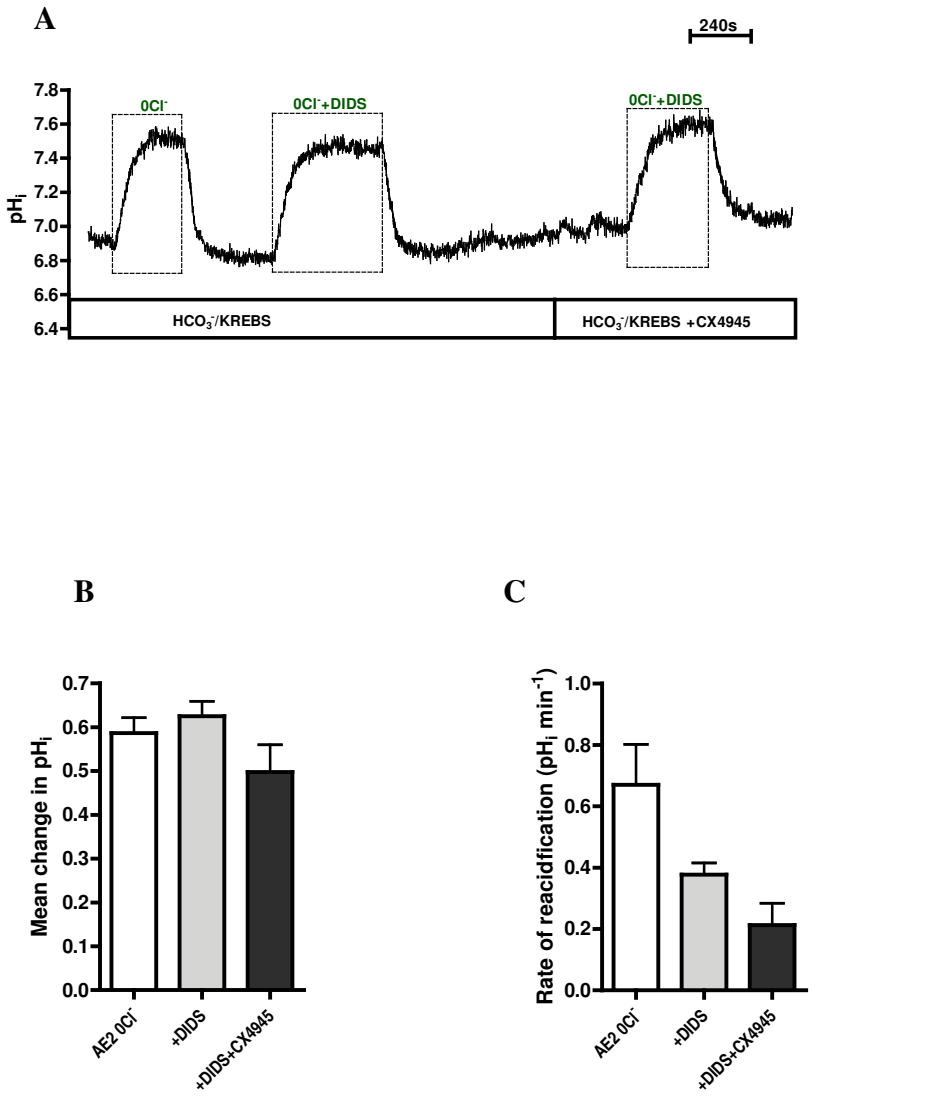


Figure 5. 23: Impact of the CK2 inhibitor CX4945 on mAE2 activity in HEK-293T cells co-transfected with mAE2 and DM-CK2. (A) Representative pH_i trace showing the effect of acute exposure of 10µM CX4945 on mAE2 activity in the presence of 25µM DIDS, from HEK-293T cells co-transfected with mAE2 and DM-CK2. Summary of the effect of acute exposure of 10µM CK2 inhibitor CX4945 on mAE2 activity in HEK-293T cells co-transfected with mAE2+DM-CK2 on mean alkalinisation in pH_i in response to Cl^- removal (B), and the rate of reacidification after Cl^- readdition (D). Data are shown as Mean±SEM. (B). No significant difference (P>0.05); n=4 for +DIDS and +DIDS +CX4945, and n=3 for AE2 $0Cl^-$.

In order to further substantiate a role for CK2 in the regulation of mAE2, I performed additional experiments utilising CK2-knockout (KO) HEK-293T cells in which either the αCK2 subunit or the α-prime CK2-had been KO (see Methods for further details about these cells). For these experiments, mAE2 was transfected into both types of CK2-KO HEK-293T cells, and then anion exchange activity measured in response to a Cl^- free solution in the presence of 25μM DIDS. Results showed that mAE2 activity was significantly decreased in both the αCK2-KO and α-prime CK2-KO HEK-293T transfected cells, as both the magnitude of alkalinisation in response to Cl^- free solution as well as the rate of reacidification upon Cl^- readdition were decreased compared to the control mAE2 transfected cells containing endogenous CK2 (Figure 5.24A and B). Since the reduction in mAE2 activity was similar in both types of CK2 KO cells, this implies that αCK2 as well as α-prime CK2 subunits play equal roles in regulating mAE2 activity, and that maintaining CK2 in active state is essential for mAE2 activity. Unfortunately, double α /α -prime KO cells were not available for my studies.

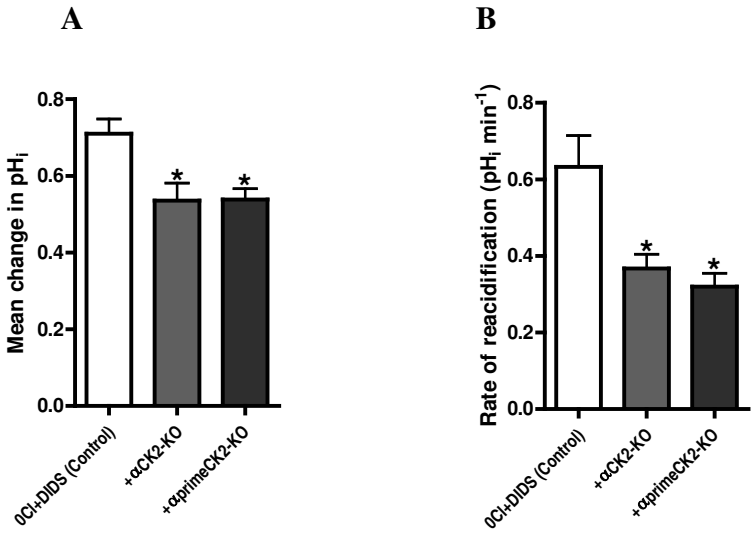


Figure 5. 24: mAE2 activity was significantly reduced in CK2-KO HEK-293T cells, transfected with mAE2 compared to control mAE2 transfected cells. Summary of mAE2 activity in CK2-KO mAE2 transfected HEK-293T cells. Data shows (A) Mean change in pH_i in response to Cl^- free solution, and (B) the rate of reacidification upon Cl^- readdition. Data are shown as Mean±SEM, *P<0.05 compared to control mA2 transfected cells, n=4 for control, n=7 for αCK2-KO, n=6 for αprime CK2-KO.

However, as shown in figure 5.25A and B (black bar graph), acute TBB exposure of α-prime CK2-KO HEK cells, transfected with mAE2, produced a similar level of inhibition of mAE2 activity, compared to the inhibition in control HEK-293T TBB treated cells (red bar graph), both in the magnitude of alkalinisation, and the rate of reacidification. This result may be because TBB blocked the other 'normal' alpha CK2 subunit that is active in the α-prime cells. Interestingly, a very similar reduction in the rate of reacidification was observed in CK2-KO cells compared to that seen with TBB exposure (Figure 5.25B, green bar graphs *vs*. black and red bar graphs). However, the percent inhibition in the mean pH_i change produced by CK2-KO cells was significantly less, compared to the percent inhibition caused by TBB in control mA2 transfected cells (Figure 5.25A, green bar graph *vs.* red bar graph). This apparent lack of inhibition might be because both CK2 α-subunits need to be KO to produce the same effect as TBB on mAE2 activity.

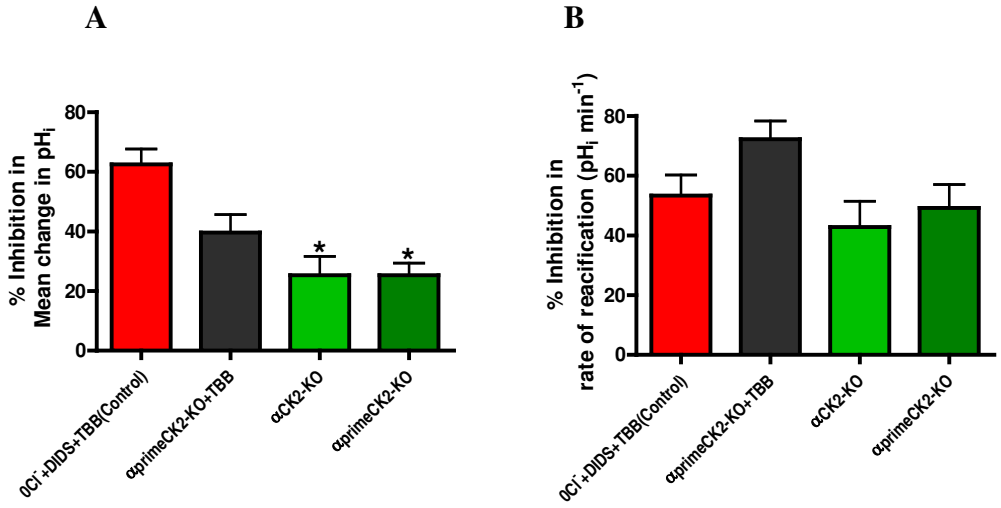


Figure 5. 25: The percent inhibition of mAE2 activity in control and CK2-KO HEK-293T cells. Summary of percent inhibition of mAE2 activity in control HEK-293T cells, compared to CK2-KO HEK-293T cells with and without TBB, both in the magnitude of alkalinisation in response to Cl^- free solution (A), and the rate of reacidification upon Cl^- readdition (B). Data are shown as Mean±SEM, *P<0.05 compared to control responses, n=7 for control, n=7 for αCK2-KO, n=6 for αprime CK2-KO, and n=3 for αprime CK2+TBB.

To focus on the role of CK2 subunits in the regulation of the mAE2 activity further, αCK2 KO HEK-293T cells were co-transfected with mAE2 and α-CK2 (WT-CK2), and then mAE2 activity measured in response to Cl^- free solutions, in the presence of 25μM DIDS. Results showed that transfection of α-CK2 into α-CK2-KO HEK-293T cells significantly recovered mAE2 activity, as shown by changes in both the mean pH_i response to Cl^- free solutions, and the rate of reacidification following Cl^- readdition, compared to mAE2 activity in αCK2-KO HEK-293T cells co-transfected with empty plasmid (Figure 5.26A and B). Taken together, all the above data clearly indicate that CK2 is a protein kinase that plays a novel role in the regulation of mAE2 activity.

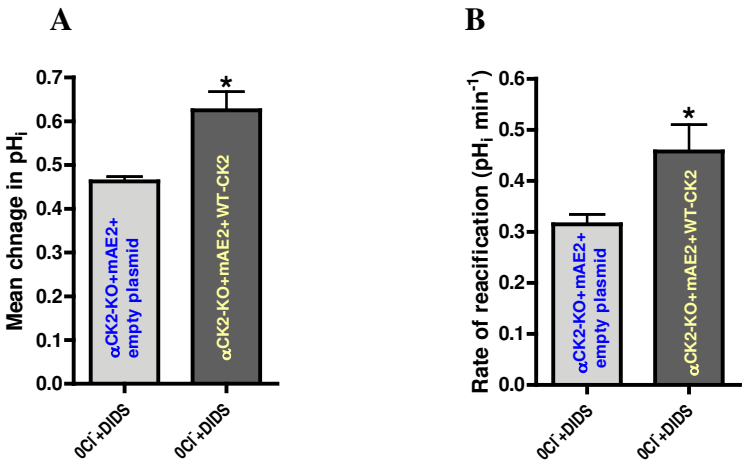


Figure 5. 26: mAE2 activity in α-CK2-KO HEK-293T cells cotransfected with mAE2 and α-CK2, compared to α-CK2-KO HEK-293T cells co-transfected with mAE2 and empty plasmid. Summary of the effect of transfection of mAE2 and α-CK2 into α-CK2-KO HEK cells on mean pH_i change in response to Cl free solution (A), and the rate of reacidification upon Cl readdition (B), compared to cells cotransfected with mAE2 and empty plasmid. Data are shown as Mean±SEM.*P<0.05 compared to control $0Cl^-$+DIDS, n=4 for each condition.

5.11 Discussion

5.11.1 Inhibition of mAE2 activity by DIDS

Although HEK293 cells have a low endogenous Cl^-/HCO_3^- AE activity (Sterling *et al.*, 2002), my results showed for the first time that 25µM DIDS almost completely abolished this endogenous AE activity in HEK-293T cells. My results also showed that the mAE2 activity in transfected HEK-293T cells was sensitive to different concentrations of DIDS. Although 25µM and 50µM DIDS did not block the magnitude of alkalinisation produced by mAE2 activity in response to Cl^- free solutions, the two concentrations significantly inhibited the rate of reacidification upon Cl^- readdition. However, 100µM and 200µM DIDS caused a marked reduction in both parameters, with 200µM DIDS significantly inhibiting the mean alkalinisation by 57.7±5.8%. Consistent with my results, it has been shown that transfected murine AE2 activity was significantly inhibited by 68.5% at 300µM DIDS and by 74.2% by 400µM DIDS in HEK-293 cells (Ruetz *et al.*, 1993). In contrast to mAE2, my previous results in Calu-3 cells showed that 100µM DIDS almost completely inhibited the basolateral AE activity (see chapter 4, section 4.2). The different sensitivities to DIDS between Calu-3 cells and HEK cells transfected with mAE2 might be due to differences in amino acid sequence between mAE2 and the human Calu-3 basolateral AE, particularly with regard to the lysine-residue that DIDS forms a covalent bond with, and which causes inhibition of the AE2 by DIDS (Lee *et al.*, 1991).

5.11.2 Role of cAMP in the regulation of mAE2

Although my results clearly showed that intracellular elevation of cAMP caused almost complete inhibition of the basolateral AE activity in Calu-3 cells (see chapter 4, section 4.4.1.), mAE2 activity was not altered by an increase in $[cAMP]_i$, compared to control responses. Consistent with this, it has been previously found that AE2 was shown to be active under cAMP-stimulated condition in the proximal colon of mouse (Gawenis *et al.*, 2010). However, a

recent finding in our laboratory demonstrated that an increase in $[cAMP]_i$, did not fully inhibit the basolateral AE activity in CFTR knock down Calu-3 cells, compared to WT Calu-3 cells (Garnett *et al.*, 2011; Garnett *et al.*, 2013). Therefore, one possible explanation could be the lack of CFTR expression in HEK-293T cells (Domingue *et al.*, 2014). Consistent with this, mAE2 activity was found to be significantly reduced (rate of reacidification following Cl^- readdition) in HEK-293T cells cotransfected with mAE2 and CFTR, compared to control responses. This implies that CFTR was required to enable cAMP to inhibit mAE2 activity. Exactly how this occurs is not known. Furthermore, Epac inhibition did not affect mAE2 activity in HEK-293T cells cotransfected with mAE2 and CFTR, which suggests that the presence of CFTR potentially abolished the effect of Epac inhibition on the mAE2 activity, because I have shown that Epac inhibition caused a marked decrease in the rate of reacidification produced by mAE2 activity in transfected HEK-293T cells, compared to untreated cells. The underlying mechanism for the effect of CFTR expression is not known, but Epac is known to interact with CFTR (Sun *et al.*, 2000; Hochbaum *et al.*, 2011), and therefore overexpression of CFTR could potentially lead to Epac 'depletion' inside HEK-293T cells. However, I would have predicted that if this was case then this would lead to a reduction in mAE2 activity, similar to Epac inhibition itself.

5.11.3 Role of CFTR in regulating mAE2 activity

It is well known that there is a physical and functional interaction between CFTR and several members of the SLC26A family of Cl^-/HCO_3^- AE (Ko *et al.*, 2004) present in the apical membrane of many epithelial cells. A recent study by Garnett *et al*., (2011) demonstrated Cl^- dependent HCO_3^- transport across the apical membrane of Calu-3 cells via a CFTR-dependent anion exchanger, known as Pendrin (SLC26A4) (Garnett *et al.*, 2011). However, most recently, Kim *et al*., (2014) found that CFTR is the predominant pathway for HCO_3^- secretion in Calu-3 cells (Kim *et al.*, 2014). This apparent difference in interpretation is not resolved but maybe due to differences in methodologies between our lab and

theirs. In addition, it has been shown that heterologous expression of CFTR in HEK-293 cells caused a significant increase in Cl^- and HCO_3^- dependent transport, as substitution of a Cl^- free solution caused an increase in pH_i, under Fsk stimulated conditions (Ko *et al.*, 2002). However, we have also shown that CFTR can also regulate the basolateral AE activity through an unknown mechanism. Consistent with this finding, my results showed that CFTR transfection caused a significant increase in mean pH_i change in response to Cl^- free solution, and the rate of reacidification following Cl^- readdition in HEK-293T cells, under Fsk stimulated condition, which was almost completely inhibited by the CFTR pore blocker GlyH-101. Also, the mean pH_i change in response to Cl^- free solution was significantly larger in CFTR transfected HEK-293T cells, compared to non-transfected cells. Surprisingly, CFTR transfection into HEK-293T cells also produced an increase in HCO_3^- transport, under resting (non-cAMP stimulated) conditions, which was also significantly reduced by the specific CFTR inhibitor, $CFTR_{inh}$-172. This result suggested that CFTR was fully active in HEK cells in the absence of exogenous cAMP agonists. One possible explanation for this might be that under resting conditions, HEK-293T cells have enough intracellular cAMP to activate CFTR which has been observed by others (Caci *et al.*, 2003; Moran and Zegarra-Moran, 2005). In addition, both CFTR inhibitors, GlyH-101 and $CFTR_{inh}$-172 did not affect mAE2 activity in transfected HEK-293T cells, which is consistent with my previous results in Calu-3 cells where GlyH-101 did not affect the basolateral AE activity.

5.11.4 Role of Ca^{2+} and CaM in the regulation of mAE2 activity

My work is the first to assess the effect of changes in resting Ca^{2+} concentration in the regulation of mAE2 activity in HEK-293T cells. In BAPTA-AM loaded HEK-293T cells, mAE2 activity was significantly decreased, both in the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition, compared to untreated cells. Consistent with

this, Chernova *et al*., (2010) have also shown that chelation of intracellular Ca^{2+} by BAPTA-AM significantly reduced the Cl^- transport by murine AE2, when expressed in *Xenopus* oocytes (Chernova *et al.*, 2003).This is consistent with my previous results in Calu-3 cells (see chapter 4, section 4.6.1.2.), and further supports an important role for normal intracellular Ca^{2+} concentration to maintain the resting activity of AE2. In addition, inhibition of CaM caused a marked reduction in mAE2 activity in HEK-293T transfected cells, both in the magnitude of alkalinisation and the rate of reacidification. A very similar level of inhibition was observed in Calu-3 cells. Consistent with this, Stewart *et al.*, (2007) and Chernova *et al*., (2010) demonstrated that mouse AE2 was inhibited by the calmodulin inhibitor, calmidazolium, when expressed in *Xenopus* oocytes (Chernova *et al.*, 2003; Stewart *et al.*, 2007). Collectively, these results indicate that CaM maintains normal AE2 activity potentially through a Ca^{2+}-dependent pathway, under resting conditions. It has been shown that inhibition of CaM-dependent kinase, CaMKII, did not affect the murine AE2 activity in transfected *Xenopus* oocytes (Stewart *et al.*, 2007), and my previous results in Calu-3 cells showed that inhibition of CaMKI, CaMKII, and CaMKIV were not involved in the regulation of the basolateral AE activity (see chapter 4, section 4.7.2-3). Since there is no stable interaction between CaM and murine AE2 (Chernova *et al.*, 2003), it would be of interest to investigate the role of CaM-dependent kinases, CaMKI, CaMKII, and CaMKIV, in the regulation of mAE2 activity in HEK-293T cells.

5.11.5 Role of PP1 in the regulation of mAE2 activity

As described in chapter 4, inhibition of PP1/2A in Calu-3 cells markedly reduced basolateral AE activity. Here, I have also shown that treating mAE2 transfected HEK-293T cells with okadaic acid caused a significant decrease in the mean pH_i response to a Cl^- free solution. This further support the role of dephosphorylation in the regulation of mAE2 activity by PP1/2A, potentially as a downstream target of CK2, because it has been shown that CK2 plays a critical role in the phosphorylation and activation of PP1 (Van Eynde *et al.*, 1994).

Further experiments are therefore required to fully deduce the role of PP1 signaling in regulating mAE2 activity, such as (i) testing mAE2 activity in cells treated with TBB and OA (ii), assessing mAE2 activity in PP1 KD cells.

5.11.6 Role of CK2 in the regulation of mAE2 activity

This is the first study to demonstrate that CK2 regulates mAE2 anion exchange activity in transfected HEK-293T cells. Interestingly, acute TBB exposure significantly reduced mAE2 activity, both the mean alkalinisation in response to Cl^- free solution, by 62.5±5.1%, and the rate of reacidification upon Cl^- readdition by 53.3±6.9% following Cl readdition. Also, acute exposure by another CK2 inhibitor, CX4945, caused a significant decrease in mAE2 activity, both the mean change in pH_i in response to Cl^- free solution by 34.2±7.3%, and the rate of reacidification upon Cl^- readdition by 53.6±7.8%, compared to control, untreated responses. This is consistent with my previous results in Calu-3 cells, which showed that acute exposure to both TBB and CX4945 significantly reduced basolateral AE activity. However, CX4945 was significantly more effective in inhibiting the basolateral AE activity in Calu-3 cells than mAE2 activity in HEK-293T cells. The reason for this is unknown but may be due to species differences (human *vs* mouse). Nonetheless, the CK2 inhibitor data supports a novel role for CK2 in the regulation of mAE2 activity under resting conditions. Moreover, co-transfection of HEK-293T cells with mAE2 and WT-CK2 did not affect the TBB induced inhibition of mAE2 activity, compared to mAE2 transfected HEK-293T cells expressing only endogenous CK2. However, co-transfection of HEK-293T cells with mAE2 and DM-CK2, a mutant that is much less sensitive to TBB inhibition (Sarno *et al.*, 2005), partially overcame the inhibitory effect of TBB. In addition, the presence of DM-CK2 partially prevented the ability of CX4945 to inhibit mAE2 activity, particularly in the mean pH_i change in response to Cl^- free solution. This implies that the presence of DM-CK2 in the transfected HEK293T cells potentially

blocked the inhibitory effect of CK2 inhibitors on mAE2 activity, which further supports a role for CK2 in the regulation of AE2 activity.

Further support for a role of CK2 in regulating anion exchange activity, came from results where mAE2 transport was measured in CK2-KO HEK-293T cells (αCK2-KO and α-prime CK2-KO), which showed that mAE2 activity was significantly decreased, both in the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition, in both types of CK2-KO HEK-293T cells, compared to transfected cells with mAE2 alone. This strongly suggests that both catalytic subunits were involved in regulating mAE2 activity. Interestingly, acute TBB exposure of α-prime CK2-KO HEK cells transfected with mAE2 produced a similar level of inhibition of mAE2 activity to the TBB-induced inhibition of mAE2 in wild type HEK-293T transfected cells, both in the magnitude of alkalinisation and the rate of reacidification. This indicates that TBB potentially blocked the alpha subunit in α-prime CK2-KO HEK cells. Moreover, the percent inhibition of mAE2 activity by TBB in normal HEK293T transfected cells was significantly greater than the inhibition of mAE2 activity in CK2-KO cells, at least with regard to the mean pH_i change in response to $0Cl^-$. This result might be due to the fact that both α-CK2 subunits need to be KO to produce the same effect as TBB on mAE2 activity. To further support this hypothesis, α-CK2 KO HEK-293T cells were cotransfected with mAE2 and α-CK2, and results showed that mAE2 activity was significantly recovered, both in the mean pH_i change in response to Cl^- free solution, and the rate of reacidification following Cl^- readdition, compared to control co-transfected cells with mAE2 and empty plasmid. Taken together, all the above data clearly indicate that CK2 plays a critical role in the regulation of mAE2 activity under resting conditions.

The key findings of this chapter are summarized below:

- HEK293T cells express an endogenous Cl^-/HCO_3^- AE activity that can be effectively inhibited by a low concentration of DIDS, and which is regulated by endogenous CK2.
- Heterologous expression of mouse AE2 confers a robust Cl^-/HCO_3^- AE activity which is blocked by DIDS in excess of 25 μM.
- mAE2 was not inhibited by an increase in $[cAMP]_i$ in HEK-293T cells, but mAE2 activity was significantly reduced by cAMP in mAE2/CFTR cotransfected cells.
- Epac plays an important role in the regulation of the resting level of mAE2 activity.
- A decrease of cytosolic Ca^{2+} caused a marked reduction in mAE2 activity, as did the inhibition of calmodulin.
- CK2 (both catalytic subunits) plays a novel and essential role in the regulation of mAE2 activity in HEK-293T cells.

Table 5.01 shows the summary of percent inhibition in AE2 activity, by different pharmacological agents, in HEK-293T cells and Calu-3 cells:

Pharmacological agents	% Inhibition of AE2 activity			
	in HEK-293T cells		in Calu-3 cells	
	ΔpH_i	Rate of reacidification (pH_i min^{-1})	ΔpH_i	Rate of reacidification (pH_i min^{-1})
ESI-09	20±7.8% *	41.6±11.9% *	85.7±7.5%	97.7±2%
BAPTA-AM	40.7±9.4% *	35.8±1.8% non	65.1±2.8%	57.1±8.1%
J-8	56±7% non	70.8±8.8% non	50±6.9%	59.3±16.6%
Okadaic acid	39.4±2.6% *	17±16.5% *	61.2±3.3%	75±8.5%
TBB	62.5±5.1% non	53.3±6.9% non	51.4±4.9%	62.8±4.2%
CX-4945	34.2±7.3% *	53.6±7.8% *	87.5±6%	94.8±2.6%

Table 5.1: Summary of percent inhibition in AE2 activity, by different pharmacological agents, in HEK-293T cells and Calu-3 cells. *P<0.05 significant difference compared to results in Calu-3 cells.

Chapter 6 Concluding Discussion

6.1 Summary of main findings

The aim of the current work was to investigate the signalling mechanisms that regulate the CFTR-dependent apical Cl^-/HCO_3^- anion exchanger, as well as the basolateral Cl^-/HCO_3^- AE, in a model human airway cell line, which secretes a HCO_3^- rich fluid in response to an increase in $[cAMP]_i$. I have utilized a dynamic, non-invasive, method to study the activity of the apical and basolateral exchangers independently, using polarized cultures of human airway Calu-3 epithelial cells combined with real-time pH_i measurements. This experimental set up is representative of the physiological condition, and thus makes the findings potentially transferable into an *in vivo* setting. I have also investigated the properties and regulation of mAE2 (SLC4AE2) after being transiently transfected into the HEK-293T cells, in order to compare the properties of the heterologously expressed transporter with results from the cultured Calu-3 cells.

6.1.1 Apical Cl^-/HCO_3^- anion exchanger in Calu-3 cells

The results of this current study have shown that the apical Cl^-/HCO_3^- anion exchanger, known as pendrin (SLC26A4), was enhanced in Calu-3 cells by a variety of cAMP agonists, such as Fsk, db-cAMP and IBMX, but not by cGMP. The mechanism by which increased $[cAMP]_i$ mediated this stimulation was assessed using a range of protein kinases inhibitors and other signalling molecules. Two different PKA inhibitors, H-89 and RpcAMP, markedly reduced the Fsk-stimulated apical Cl^-/HCO_3^- AE activity. This provides further support for an important role of PKA, as well for a role of CFTR in the regulation of this exchanger, as CFTR activity depends on PKA phosphorylation, and is consistent with the most recent finding by Garnett *et.al*., 2013 (Garnett *et al.*,

2013). It also supports previous work that suggested CFTR regulates pendrin through a direct interaction between the two proteins via a physical interaction between their R and STAS domains, respectively (Dorwart *et al.*, 2008). However, PKA inhibition did not fully block the apical AE activity, and I suspected that another cAMP-dependent pathway might be involved in the regulation of the apical AE activity in Calu-3 cells. My results strongly suggest that this was Epac, since Epac inhibition significantly reduced the activity of the apical AE under Fsk stimulated conditions. This implies that cAMP-stimulated apical AE activity is through a PKA and Epac-dependent mechanism in Calu-3 cells. Future experiments would need to assess the effect of inhibition of both PKA and Epac on AE activity to confirm this. Paradoxically, inhibition of Epac also enhanced the activity of the apical Cl^-/HCO_3^- AE under non cAMP-stimulated conditions, suggesting that Epac has a dual effect on apical AE activity; i.e. inhibitory when cAMP levels are low, but stimulatory when cAMP levels rise in response to cAMP agonists.

Further support for a role of CFTR in regulating the apical AE activity came from CFTR inhibitor studies. Here the CFTR inhibitor, GlyH-101 caused a reduction in apical AE activity. Since GlyH-101 is a CFTR pore blocker (Norimatsu *et al.*, 2012), this suggests that anion transport by CFTR plays an important role in the regulation of apical Cl^-/HCO_3^- AE activity in Calu-3 cells. This could because Cl^- efflux through CFTR provides external Cl^- for the anion exchanger as has been suggested in pancreatic duct cells (Gray *et al.*, 2001). My results are also consistent with the recent finding that the rate of apical AE activity was decreased in CFTR knockdown Calu-3 cells (Garnett *et al.*, 2011). However, the effect of GlyH-101 on the apical AE activity was nearly abolished by application of DIDS to the basolateral perfusate, suggesting that the effect of GlyH-101 was indirect and potentially through a change in activity of a DIDS-sensitive basolateral transporter, which was involved in pH_i regulation. The identity of this basolateral transporter is uncertain but it could be AE2. However, further pharmacological and genetic studies will be necessary to gain a complete profile of this basolateral transporter that regulates apical AE activity under cAMP stimulated conditions.

I also found that intracellular Ca^{2+} plays an important role in the regulation of apical AE activity as BAPTA-AM markedly decreased the activity of the transporter. The effect of BAPTA-AM might be due to a decrease in resting $[Ca^{2+}]_i$ which could potentially block the synergistic interaction between Ca^{2+} and cAMP, that has been shown to be required for maximal stimulation of pancreatic ductal epithelial anion secretion (Lee *et al.*, 2012). Paradoxically, I also found that an increase in $[Ca^{2+}]_i$, by thapsigargin, also significantly decreased the apical AE activity. This effect could potentially be through a PKA-dependent mechanism, because it has been shown that an increase in $[Ca^{2+}]_i$ can inhibit the PKA- dependent signalling pathway (Santana *et al.*, 2002) or even cause direct inhibition of PKA itself (Orie *et al.*, 2009). However, it could also be related to a decrease in the plasma membrane levels of CFTR, since recent work on the effects of cigarette smoke has shown that a sustained increase in cytosolic calcium due to smoke exposure caused internalisation of CFTR (Rasmussen *et al.*, 2014). In addition, an increase in $[Ca^{2+}]_i$ did not abolish the BAPTA-AM induced decrease in the activity of the apical anion exchanger, but instead caused a further, and significant, decrease in apical AE activity, under Fsk stimulated conditions. This further decrease in AE activity might be because thapsigargin caused an increases in $[Ca^{2+}]_i$ even in the presence of BAPTA-AM, to reduce the PKA-dependent apical Cl^-/HCO_3^- AE activity. This effect of thapsigargin is supported by recent results in our laboratory, which demonstrated that thapsigargin produced a small increase in $[Ca^{2+}]_i$ even in the presence of BAPTA-AM in HEK-293Tcells (W. Patel, unpublished observations). The effects of calmodulin, and Ca^{2+}/calmodulin dependent kinases on the apical AE activity were also investigated. Here it was found that the mechanism by which intracellular Ca^{2+} regulates the apical AE activity was not dependent on either calmodulin or Ca^{2+}/calmodulin dependent kinases.

The role of dynamin in the regulation of apical AE activity was also assessed as it has been shown that dynamin is a Ca^{2+}-sensitive protein (Liu *et al.*, 1994). Dynamin inhibition caused a significant decrease in the apical AE activity in Calu-3 cells, potentially via reducing the expression level of CFTR, and thereby reducing the Cl^-/HCO_3^- exchange activity. This effect of dynamin was

unexpected as previous studies have shown that dynamin inhibition reduces the rate of endocytosis, and generally increases surface levels of transporter such as CFTR (Young *et al.*, 2009). It would therefore be of interest to perform cell surface biotinylation in dynamin-treated Calu-3 cells to establish whether dynamin reduces the surface expression of apical CFTR as well as the Cl^-/HCO_3^- AE.

It should be noted, however, that dynamin inhibition blocks the release of newly formed vesicles from the trans face of the Golgi complex to the plasma membrane (Nabi and Le, 2003; Abazeed *et al.*, 2005; Cao *et al.*, 2005), and it can also affect the kinetics of released protein into the plasma membrane (Anantharam *et al.*, 2011). This therefore could provide an explanation for the decrease in apical AE activity. The exact mechanism how this would occur is unclear, but one possible explanation could be via the actin cytoskeleton, as there is a direct interaction between dynamin and the actin cytoskeleton (Gu *et al.*, 2010). However, cytoskeleton disruption did not affect the apical AE activity, suggesting that dynamin regulates AE activity through a cytoskeleton-independent mechanism.

I also provided new evidence that CK2 also regulates apical AE activity, which supports previous work that showed CK2 plays an important role in HCO_3^- secretion in pancreatic duct epithelial cells, under cAMP stimulated condition (Treharne *et al.*, 2009). According to sequence analysis of SLC26A4, there is a wide range of potential CK2 phosphorylation sites. My results showed for the first time that CK2 inhibition caused a marked decrease in the apical AE activity in Calu-3 cells, suggesting that CK2 play a novel role in the regulation of this transporter in Calu-3 cells. However, it will be important to establish whether CK2 inhibition directly affects the apical anion transporter, or whether it works through another protein, such as CFTR.

6.1.2 Basolateral Cl^-/HCO_3^- anion exchanger in Calu-3 cells

My work has been the first to demonstrate that the IC_{50} for DIDS inhibition of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells was about 17µM. However, 500µM DIDS was required to fully inhibit AE activity, suggesting that other DIDS-sensitive transporters might be present in the basolateral membrane of Calu-3 cells. This could be other SLC4 family members, such as SLC4A9 (AE4) which has been recently found as a basolateral Cl^-/HCO_3^- anion exchanger in mouse submandibular gland acinar cells (Pena-Munzenmayer *et al.*, 2015), or even other members of the SLC26 family, such as SLC26A7, which plays an important role in HCO_3^- transport across the basolateral membrane of gastric parietal cells (Petrovic *et al.*, 2003) and intercalated cells of the outer medullary collecting duct (Petrovic *et al.*, 2004). SLC26A7 is expressed in Calu-3 cells (J Garnett PhD thesis, 2010) and is also sensitive to DIDS (Petrovic *et al.*, 2003). However, the IC_{50} for DIDS block of the basolateral AE activity in Calu-3 cells was much lower than the reported IC_{50} for DIDS inhibition of the SLC26A7, which was 126µM (Petrovic *et al.*, 2003). Also, my results showed that basolateral AE had a strict dependency on Cl^- and HCO_3^- but it has been shown that SLC26A7 is impermeable to HCO_3^- (Kim *et al.*, 2005). Thus, my results are consistent with the expression of functional AE2 on the basolateral membrane of Calu-3 cells under resting conditions.

In contrast to previous studies which showed that an increase in $[cAMP]_i$ had no effect on the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells (Huang *et al.*, 2012; Shan *et al.*, 2012; Kim *et al.*, 2014), I have demonstrated that the addition of cAMP agonists Fsk, ADO, dbcAMP or IBMX almost completely inhibited the basolateral Cl^-/HCO_3^- AE activity, which is consistent with the recent study by Garnett *et al*., (2013). In addition, inhibition of the cAMP efflux transporter, most likely MRP4, mimicked the effect of cAMP agonists on basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. The reasons for the different results are not clear but it could be due to differences in cell culture conditions or different batches of Calu-3 cells.

My work has also been the first to investigate the mechanism by which increased cAMP levels inhibit the basolateral AE activity, and I have shown that the downstream targets of cAMP, including PKA, Epac, CNG channel, and mTOR, are not involved in the cAMP-induced inhibition of the basolateral Cl^-/HCO_3^- AE activity. Interestingly, Epac inhibition caused a marked reduction in the basolateral AE activity under resting conditions, suggesting, for the first time, that Epac helps keep the basolateral anion exchanger active under resting conditions. Interestingly, Epac had the opposite effect on the apical anion exchanger where it appeared to inhibit pendrin activity under resting conditions. The net effect of this regulation by Epac would be to reduce the rate of HCO_3^- secretion under non-cAMP stimulated conditions. This is consistent with previous results from our group (Garnett *et al.*, 2011), which showed that under resting conditions Calu-3 cells secrete little HCO_3^- into the luminal compartment. It is also consistent with previous results from pancreatic duct cells which suggested that basolateral AE2 activity is reduced after cAMP stimulation (Ishiguro *et al.*, 2002). Note that I also showed that cGMP was not involved in the regulating basolateral Cl^-/HCO_3^- AE activity under either resting, or cAMP stimulated conditions, similar to the apical anion exchangers.

Intracellular elevation of Ca^{2+} did not affect the activity of the basolateral AE; however, intracellular depletion of Ca^{2+}, by BAPTA-AM, caused a significant decrease in basolateral AE activity under resting conditions. Interestingly, I have shown that intracellular Ca^{2+} regulates the basolateral Cl^-/HCO_3^- AE activity independently of cAMP. On the other hand, the absence of extracellular Ca^{2+} did not affect basolateral AE activity under resting conditions, but it did partially remove the cAMP-induced inhibition of this transporter. This latter effect could be linked to the CaSR and a change in $[cAMP]_i$ production, since cAMP production is decreased in the absence of extracellular Ca^{2+} concentration (Ferreira *et al.*, 1998). A drop in cAMP would thus partially overcome the cAMP-induced inhibition of the basolateral AE activity. I also found that basolateral AE activity was significantly reduced in the absence of extracellular Ca^{2+} in ER Ca^{2+}-store depleted Calu-3 cells, possibly through an increase in $[cAMP]_i$, via store-operated cAMP production (Lefkimmiatis *et al.*, 2009). I demonstrated that Ca^{2+}/CaM dependent kinases were not involved in the

regulation of the basolateral AE activity under cAMP-stimulated conditions, but CaM inhibition itself did significantly reduce the activity of the basolateral AE, suggesting that decreasing $[Ca^{2+}]_i$ potentially inhibits the activity of the basolateral Cl^-/HCO_3^- AE via a CaM-dependent mechanism in Calu-3 cells. However, it was also shown that CaM and cAMP induce their effects on the basolateral AE through a separate regulatory mechanism.

My studies also found that dynamin inhibition markedly reduced basolateral AE activity under resting condition, similar to the effects observed for the apical anion exchanger. However, unlike the effect on pendrin activity, actin cytoskeleton disruption significantly reduced the basolateral Cl^-/HCO_3^- AE activity under resting conditions. However, neither dynamin inhibition nor cytoskeleton disruption had any effect on the ability of cAMP to further reduce the basolateral AE activity in Calu-3 cells, suggesting that cAMP works via a non-dynamin/cytoskeleton mechanism.

I also demonstrated that CK2 plays an important role in the regulation of the basolateral AE activity in Calu-3 cells, possibly through a CaM-dependent mechanism since simultaneous inhibition of CaM and CK2 did not produce a further decrease in basolateral Cl^-/HCO_3^- AE activity, compared to CaM inhibition or CK2 inhibition alone. Furthermore, my work also showed that PP1/2A was important in regulating basolateral AE activity, suggesting that CK2 may signal through a CaM and PP1-dependent mechanism, as CK2 can phosphorylate CaM (Arrigoni *et al.*, 2004) and PP1 (Van Eynde *et al.*, 1994). This is consistent with a recent study that demonstrated CK2 regulates K^+-channel activity through a CaM and PP1-dependent mechanism (Kang *et al.*, 2014).

6.1.3 Regulation of mAE2 in transfected HEK-293T cells

In order to provide further insights into the identity and regulation of the basolateral anion exchanger in Calu-3 cells, I studied the properties and regulation of mAE2 after transient transfection into HEK-293T cells. My work has further investigated the effect of different concentration of DIDS on mAE2 in transfected HEK-293T cells. It was found that only concentrations in excess of 100μM and 200μM DIDS significantly reduced the activity of mAE2 in HEK-293T cells. Consistent with this, it has been shown that high concentration of DIDS significantly inhibited murine AE2 activity in transfected HEK-293 cells, by 68.5% at 300μM DIDS and by 74.2% at 400μM DIDS (Ruetz *et al.*, 1993). However, in contrast to results obtained in Calu-3 cells, elevation of intracellular cAMP did not inhibit mAE2 activity; the most likely explanation would be lack of CFTR expression in these cells (Domingue *et al.*, 2014). Consistent with this, an increase in $[cAMP]_i$ caused a marked decrease in the rate of reacidification produced by transfected mAE2 activity in response to Cl^- free solution in HEK-293T cells cotransfected with mAE2 and CFTR, suggesting that CFTR is involved in the cAMP induced inhibition of the mAE2 activity.

In a similar fashion to Calu-3 cells, mAE2 activity was significantly reduced by a decrease in $[Ca^{2+}]_i$ and CaM inhibition, which further reinforces the finding that intracellular Ca^{2+} plays an important role in the regulation of AE2 activity, potentially through a CaM dependent mechanism. It was also found that Epac inhibition reduced mAE2 activity, and this effect could potentially be through a Ca^{2+}-dependent mechanism, since it has been shown that Epac enhances Ca^{2+} release in cardiac myocytes (Oestreich *et al.*, 2009). However, it would be of interest to measure $[Ca^{2+}]_i$ in ESI-09 treated HEK-293T cells in order to investigate whether Epac inhibition affects intracellular Ca^{2+} concentration. Interestingly, the presence of CFTR abolished the effect of Epac inhibition on the mAE2 activity in HEK-293T cells cotransfected with mAE2 and CFTR, which might be due to the involvement of CFTR in HCO_3^- transport.

My studies have also demonstrated for the first time that CK2 play a crucial role in the regulation of mAE2 activity in HEK-293T cells, in a similar fashion to Calu-3 cells, as both CK2 inhibitors, TBB and CX4945, caused a significant decrease in the mAE2 activity. Interestingly, the TBB-induced inhibition of mAE2 activity was significantly reduced by cotransfection of mAE2 with a TBB-resistant CK2 mutant (DM-CK2), compared to control cells transfected with mAE2 alone. Most interestingly, mAE2 activity was markedly decreased in CK2-knockout HEK-293T cells, both in the magnitude of alkalinisation in response to Cl^- free solution, and the rate of reacidification upon Cl^- readdition, compared to control cells with mAE2. This effect was significantly recovered by cotransfection of CK2-knock out HEK-293T cells with WT-CK2 and mAE2, compared to control co-transfected cells with mAE2 and empty plasmid. This suggests that CK2 plays a novel role in the regulation of the AE2 activity both in the transfected HEK-293T cells and Calu-3 cells. Since inhibition of CaM and PP1/2A significantly reduced the activity of the transfected mAE2, CK2 potentially control mAE2 activity via CaM and PP1 in transfected HEK-293T cells.

6.2 Final conclusion

The present work provides further evidence and insights into how signalling molecules control the CFTR-dependent apical Cl^-/HCO_3^- AE, as well as the basolateral Cl^-/HCO_3^- AE activity, under resting and cAMP-stimulated conditions in Calu-3 cells, and to some extent how the activity of the two anion exchangers are coordinated by the cell.

In the apical membrane of Calu-3 cells, there was no apical AE activity under resting condition. However, elevation of $[cAMP]_i$ markedly increased apical Cl^-/HCO_3^- AE activity in synergism with a normal resting concentration of intracellular Ca^{2+}. The cAMP-induced activation of the apical AE activity was through a PKA/Epac-dependent mechanism, but did not involve Ca^{2+}/CaM-dependent protein kinases. CK2 inhibition did not alter the resting activity of the

apical AE, but markedly reduced the cAMP-stimulated apical AE (Figure 6.1, apical side).

In the basolateral membrane, there was a DIDS-sensitive, Cl^- and HCO_3^--dependent Cl^-/HCO_3^- AE activity under resting condition, which was almost completely inhibited by elevation of $[cAMP]_i$, This cAMP-dependent inhibition was, however, independent of direct downstream targets of cAMP, including PKA, Epac, CNG channels and mTOR kinase. Under resting conditions, Epac was found to be required to maintain the basolateral AE activity. The resting activity of the basolateral AE was Ca^{2+} and CaM-dependent, but did not involve Ca^{2+}/CaM-dependent protein kinases. My work has also suggested for the first time that CK2 play a novel role in regulating the resting activity of the basolateral AE, potentially through a CaM and PP1-dependent mechanism (Figure 6.1, basolateral side).

Overall, based on the fact that basolateral DIDS significantly increased the pH, but not the amount of secreted fluid from Calu-3 cells under resting conditions, and that cAMP agonists inhibited basolateral AE activity, I conclude that the basolateral anion exchanger is not required for cAMP-stimulated HCO_3^- secretion. Indeed inhibiting the exchanger would be predicted to enhance HCO_3^- secretion, potentially by providing more HCO_3^- inside the cells and thereby increasing the driven force for HCO_3^- and fluid secretion across the apical membrane, where stimulation of Calu-3 cells by cAMP agonist caused a marked increase in both the pH and amount of secreted fluid.

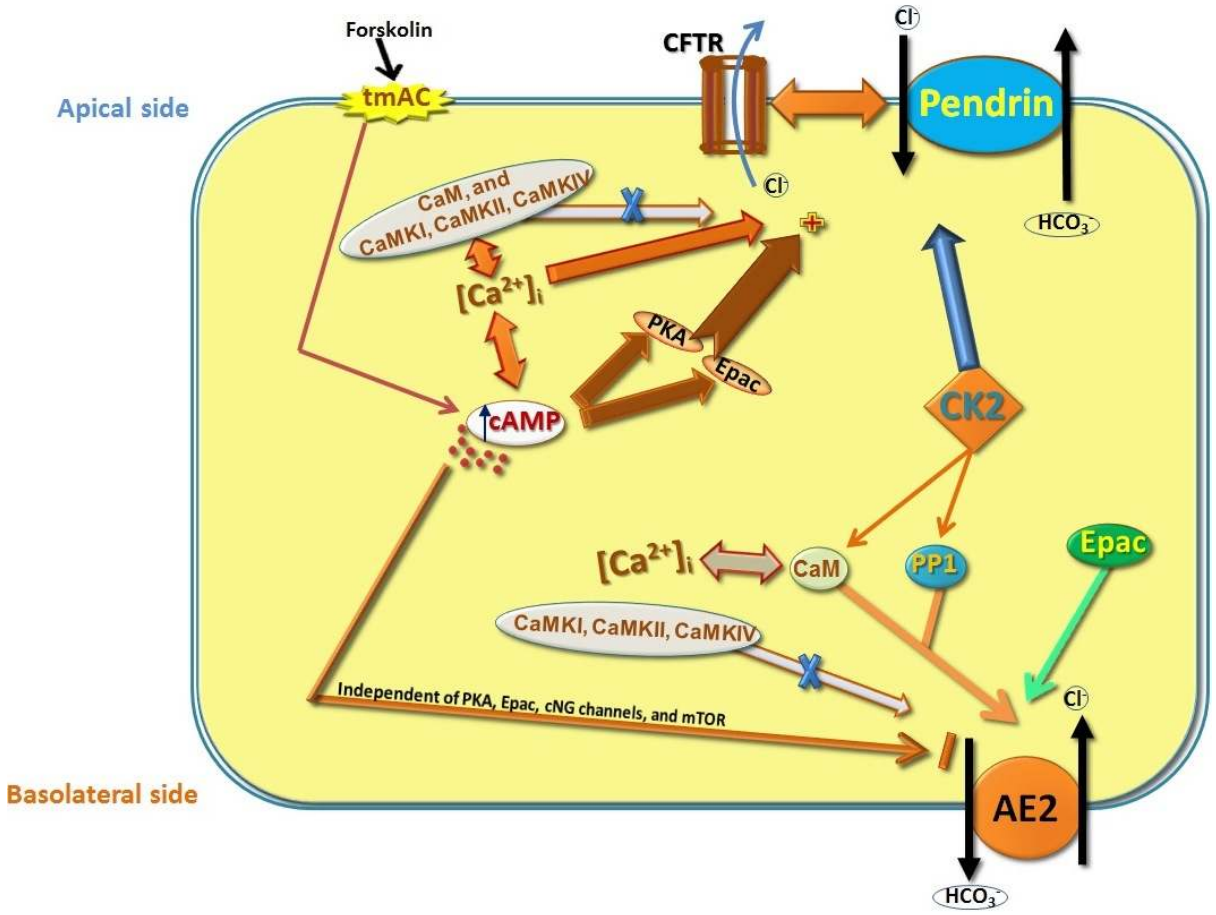


Figure 6. 1: Current model of the regulatory pathways identified in this study which impact on anion secretion in Calu-3 cells. Summary of the potential mechanisms regulating HCO_3^- transport by CFTR-dependent apical Cl^-/HCO_3^- AE, as well as the basolateral AE activity in Calu-3 cells. (+) Indicates stimulatory regulation, (-) Indicates inhibitory regulation, and (X) indicates not involved.

6.3 Future experiments

In addition to the future experiments I have already described previously in chapters 3, 4 and 5, there are also a range of other experiments that could be performed in order to reinforce the conclusions from the current study, and to provide further information into regulation of the apical and basolateral Cl^-/HCO_3^- anion exchangers in airway epithelial cells. Human cell lines are only models of native cells, thus findings from experiments, and interpreting data from an immortalised cell lines, might not reveal what happens physiologically *in vivo*. It has been shown that primary serous and mucous cell cultures derived from human airway glands can be produced, which resemble native serous or mucous cells (Finkbeiner *et al.*, 2010). The study by Finkbeiner *et al*., (2010), showed that both cell cultures produced tight junctions become polarized, and generated a transepithelial electrical resistance (Finkbeiner *et al.*, 2010). They also found that in both cell types, cAMP stimulation increased short-circuit current, via an increase in CFTR-mediated Cl^- secretion , which was inhibited by the CFTR inhibitor, $CFTR_{inh}$-172 (Finkbeiner *et al.*, 2010). Furthermore, the study by Lee and Foskett, (2010), showed that primary serous acinar cells from human and porcine airway submucosal glands play an important role in Cl^- secretion, via CFTR, in response to vasoactive intestinal peptide and other cAMP agonists (Lee and Foskett, 2010), but HCO_3^- transport was not studied in detail. Therefore, it would be beneficial to repeat experiments on primary airway epithelia in order to establish whether native serous cells also express apical or basolateral anion exchangers, using a similar methodology outlined in this thesis. For instance, measurement of intracellular pH in cAMP stimulated native serous cells, in the presence of a CFTR inhibitor, with and without basolateral DIDS, in order to identify the potential role of apical and basolateral Cl^-/HCO_3^- AE in the regulation of pH_i and HCO_3^- secretion.

Since the apical Cl^-/HCO_3^- AE provides a CFTR-independent source of secreted HCO_3^- in airway epithelial cells, and inhibition of the basolateral AE might enable more HCO_3^- to be accumulated inside the cells via the action of the basolateral NBC, my results could have implications for CF, because HCO_3^- secretion is known to be defective in CF airways (Smith and Welsh, 1992).

Thus, it would be important to study if apical and basolateral Cl^-/HCO_3^- anion exchangers are present in native serous cells of submucosal glands from CF tissues or in cultured CF cell lines. In addition, cell lines such as CFSMEo- and 6CFSMEo-, which are deltaF508 CF cell lines that do not express detectable CFTR protein could also be studied (da Paula *et al.*, 2005).

My data also suggests that bilateral Ca^{2+} removal partially removed the cAMP-induced inhibition of the basolateral AE activity in Calu-3 cells, possibly through a CaSR-dependent mechanism. With this in mind, it would be of interest to investigate the expression of the CaSR in Calu-3 cells, as well as to determine the effect of CaSR agonists or antagonists on the basolateral Cl^-/HCO_3^- AE activity to gain further insights into the role of CaSR in the regulation of the basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells.

The results obtained in the present study mostly involved the use of pharmacological agents. Therefore, it would be important to repeat some experiments in genetically modified Calu-3 cells in which the target protein of interest had been knocked down or knocked out. Specifically, CK2 knockout in Calu-3 cells would provide further insights into the role of CK2 in the regulation of apical and basolateral Cl^-/HCO_3^- AE activity in Calu-3 cells. This could involve approaches to silence CK2-gene expression in Calu-3 cells, which could be performed by recently described technology, such as TALEN and CRISPR-Cas9. In CRISPR-Cas9 technology, clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas proteins), endogenous to prokaryotes, are transfected into eukaryotic cells, and then Cas proteins unwind and split the targeted specific DNA sequences, and thereby inhibit gene expression (Mali *et al.*, 2013; Qi *et al.*, 2013). Alternatively, methods that targeted mRNA could also be employed to knock down protein expression, as we have previously described for CFTR and pendrin (Garnett *et al.*, 2011; Garnett *et al.*, 2013).

Appendix

Mouse AE2 protein contains 51 CK2 phosphorylation motifs described in the literature, compared to 42 CK2 phosphorylation motifs in human AE2, and are highlighted in below diagram:

- **Mouse AE2**

MSSAPRRPASGADSLHTPEPESLSPGTPGFPEQEEDELRTLGVERFEEILQEAGSRGGEEPGRS
YGEEDFEYHRQSSHHIHHPLSTHLPPDARRRKTPQGPGRKPRRRPGASPTGETPTIEEGEEDE
EEASEAEGFRAPPQQPSPATTPSAVQFFLQEDEGAERKPERTSPSPPTQTPHQEAAPRASKGA
QTGTLVEEMVAVASATAGGDDGGAAGRPLTKAQPGHRSYNLQERRRIGSMTGVEQALLPR
VPTDESEAQTLATADLDLMKSHRFEDVPGVRRHLVRKNAKGSTQAAREGREPGPTPRARPR
APHKPHEVFVELNELLLDKNQEPQWRETARWIKFEEDVEEETERWGKPHVASLSFRSLLELL
RRTLAHGAVLLDLDQQTLPGVAHQVVEQMVISDQIKAEDRANVLRALLLKHSHPSDEKEFSF
PRNISAGSLGSLLGHHHAQGTESDPHVTEPLIGGVPETRLEVDRERELPPPAPPAGITRSKSKH
ELKLLEKIPENAEATVVLVGCVEFLSRPTMAFVRLREAVELDAVLEVPVPVRFLFLLLGPSSA
NMDYHEIGRSISTLMSDKQFHEAAYLADERDDLLTAINAFLDCSVVLPPSEVQGEELLRSVAH
FQRQMLKKREEQGRLLPPGAGLEPKSAQDKALLQMVEVAGAAEDDPLRRTGRPFGGLIRD
VRRRYPHYLSDFRDALDPQCLAAVIFIYFAALSPAITFGGLLGEKTKDLIGVSELIMSTALQGV
VFCLLGAQPLLVIGFSGPLLVFEEAFFSFCSSNELEYLVGRVWIGFWLVFLALLMVALEGSFL
VRFVSRFTQEIFAFLISLIFIYETFYKLIKIFQEHPLHGCSGSNDSEAGSSSSSNMTWATTILVPD
NSSASGQSGQEKPRGQPNTALLSLVLMAGTFFIAFFLRKFKNSRFFPGRIRRVIGDFGVPIAILI
MVLVDYSIEDTYTQKLSVPSGFSVTAPDKRGWVINPLGEKTPFPVWMMVASLLPAVLVFILIF
METQITTLIISKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVTHANALTVM
SKAVAPGDKPKIQEVKEQRVTGLLVALLVGLSMVIGDLLRQIPLAVLFGIFLYMGVTSLNGI
QFYERLHLLLMPPKHHPDVTYVKKVRTMRMHLFTALQLLCLALLWAVMSTAASLAFPFILI
LTVPLRMVVLTRIFTEREMKCLDANEAEPVFDECEGVDEYNEMPMPV

- **Human AE2**

MSSAPRRPAKGADSFCTPEPESLGPGTPGFPEQEEDELHRTLGVERFEEILQEAGSRGGEEPG
RSYGEEDFEYHRQSSHHIHHPLSTHLPPDARRRKTPQGPGRKPRRRPGASPTGETPTIEEGEE
DEDEASEAEGARALTQPSPVSTPSSVQFFLREDDSADRKAERTSPSSPAPLPHQEATPRASKGA
QAGTQVEEAEAEAVAVASGTAGGDDGGASGRPLPKAQPGHRSYNLQERRRIGSMTGAEQA
LLPRVPTDEIEAQTLATADLDLMKSHRFEDVPGVRRHLVRKNAKGSTQSGREGREPGPTPRA
RPRAPHKPHEVFVELNELLLDKNQEPQWRETARWIKFEEDVEEETERWGKPHVASLSFRSL
LELRRTLAHGAVLLDLDQQTLPGVAHQVVEQMVISDQIKAEDRANVLRALLLKHSHPSDEK
DFSFPRNISAGSLGSLLGHHHGQGAESDPHVTEPLMGGVPETRLEVERERDVPPPAPPAGITR
SKSKHELKLLEKIPENAEATVVLVGCVEFLSRPTMAFVRLREAVELDAVLEVPVPVRFLFLLL
GPSSANMDYHEIGRSISTLMSDKQFHEAAYLADEREDLLTAINAFLDCSVVLPPSEVQGEELL
RSVAHFQRQMLKKREEQGRLLPTGAGLEPKSAQDKALLQMVEAAGAAEDDPLRRTGRPFG
GLIRDVRRRYPHYLSDFRDALDPQCLAAVIFIYFAALSPAITFGGLLGEKTQDLIGVSELIMST
ALQGVVFCLLGAQPLLVIGFSGPLLVFEEAFFSFCSSNHLEYLVGRVWIGFWLVFLALLMVA
LEGSFLVRFVSRFTQEIFAFLISLIFIYETFYKLVKIFQEHPLHGCSASNSSEVDGGENMTWAG
ARPTLGPGNRSLAGQSGQGKPRGQPNTALLSLVLMAGTFFIAFFLRKFKNSRFFPGRIRRVIG
DFGVPIAILIMVLVDYSIEDTYTQKLSVPSGFSVTAPEKRGWVINPLGEKSPFPVWMMVASLL
PAILVFILIFMETQITTLIISKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVT
HANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVALLVGLSIVIGDLLRQIPLAVLFGIFLYM
GVTSLNGIQFYERLHLLLMPPKHHPDVTYVKKVRTLRMHLFTALQLLCLALLWAVMSTAAS
LAFPFILILTVPLRMVVLTRIFTDREMKCLDANEAEPVFDEREGVDEYNEMPMPV

References:

Abazeed, M.E., Blanchette, J.M. and Fuller, R.S. (2005) 'Cell-free transport from the trans-golgi network to late endosome requires factors involved in formation and consumption of clathrin-coated vesicles', *J Biol Chem*, 280(6), pp. 4442-50.

Ahmad, A., Schaack, J.B., White, C.W. and Ahmad, S. (2013) 'Adenosine A2A receptor-dependent proliferation of pulmonary endothelial cells is mediated through calcium mobilization, PI3-kinase and ERK1/2 pathways', *Biochem Biophys Res Commun*, 434(3), pp. 566-71.

Al-Bazzaz, F.J., Hafez, N., Tyagi, S., Gailey, C.A., Toofanfard, M., Alrefai, W.A., Nazir, T.M., Ramaswamy, K. and Dudeja, P.K. (2001) 'Detection of Cl--HCO3- and Na+-H+ exchangers in human airways epithelium', *JOP*, 2(4 Suppl), pp. 285-90.

Almahariq, M., Tsalkova, T., Mei, F.C., Chen, H., Zhou, J., Sastry, S.K., Schwede, F. and Cheng, X. (2013) 'A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion', *Mol Pharmacol*, 83(1), pp. 122-8.

Alper, S.L., Rossmann, H., Wilhelm, S., Stuart-Tilley, A.K., Shmukler, B.E. and Seidler, U. (1999) 'Expression of AE2 anion exchanger in mouse intestine', *Am J Physiol*, 277(2 Pt 1), pp. G321-32.

Anantharam, A., Bittner, M.A., Aikman, R.L., Stuenkel, E.L., Schmid, S.L., Axelrod, D. and Holz, R.W. (2011) 'A new role for the dynamin GTPase in the regulation of fusion pore expansion', *Mol Biol Cell*, 22(11), pp. 1907-18.

Ares, G.R. and Ortiz, P.A. (2012) 'Dynamin2, clathrin, and lipid rafts mediate endocytosis of the apical Na/K/2Cl cotransporter NKCC2 in thick ascending limbs', *J Biol Chem*, 287(45), pp. 37824-34.

Arrigoni, G., Marin, O., Pagano, M.A., Settimo, L., Paolin, B., Meggio, F. and Pinna, L.A. (2004) 'Phosphorylation of calmodulin fragments by protein kinase CK2. Mechanistic aspects and structural consequences', *Biochemistry*, 43(40), pp. 12788-98.

Ballard, S.T. and Inglis, S.K. (2004) 'Liquid secretion properties of airway submucosal glands', *J Physiol*, 556(Pt 1), pp. 1-10.

Ballou, L.M. and Lin, R.Z. (2008) 'Rapamycin and mTOR kinase inhibitors', *J Chem Biol*, 1(1-4), pp. 27-36.

Barnes, P.J. (1995) 'Cyclic nucleotides and phosphodiesterases and airway function', *Eur Respir J*, 8(3), pp. 457-62.

Bauerfeind, R., Takei, K. and De Camilli, P. (1997) 'Amphiphysin I is associated with coated endocytic intermediates and undergoes stimulation-dependent dephosphorylation in nerve terminals', *J Biol Chem*, 272(49), pp. 30984-92.

Bissonnette, M., Tien, X.Y., Niedziela, S.M., Hartmann, S.C., Frawley, B.P., Jr., Roy, H.K., Sitrin, M.D., Perlman, R.L. and Brasitus, T.A. (1994) '1,25(OH)2 vitamin D3 activates PKC-alpha in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca2+]i', *Am J Physiol*, 267(3 Pt 1), pp. G465-75.

Brown, E.M. (2007) 'Clinical lessons from the calcium-sensing receptor', *Nat Clin Pract Endocrinol Metab*, 3(2), pp. 122-33.

Caci, E., Folli, C., Zegarra-Moran, O., Ma, T., Springsteel, M.F., Sammelson, R.E., Nantz, M.H., Kurth, M.J., Verkman, A.S. and Galietta, L.J. (2003) 'CFTR activation in human bronchial epithelial cells by novel benzoflavone and benzimidazolone compounds', *Am J Physiol Lung Cell Mol Physiol*, 285(1), pp. L180-8.

Cao, H., Weller, S., Orth, J.D., Chen, J., Huang, B., Chen, J.L., Stamnes, M. and McNiven, M.A. (2005) 'Actin and Arf1-dependent recruitment of a cortactin-

dynamin complex to the Golgi regulates post-Golgi transport', *Nat Cell Biol*, 7(5), pp. 483-92.

Carreno, S., Engqvist-Goldstein, A.E., Zhang, C.X., McDonald, K.L. and Drubin, D.G. (2004) 'Actin dynamics coupled to clathrin-coated vesicle formation at the trans-Golgi network', *J Cell Biol*, 165(6), pp. 781-8.

Chen, L., Bosworth, C.A., Pico, T., Collawn, J.F., Varga, K., Gao, Z., Clancy, J.P., Fortenberry, J.A., Lancaster, J.R., Jr. and Matalon, S. (2008) 'DETANO and nitrated lipids increase chloride secretion across lung airway cells', *Am J Respir Cell Mol Biol*, 39(2), pp. 150-62.

Chen, Z.S., Lee, K. and Kruh, G.D. (2001) 'Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine', *J Biol Chem*, 276(36), pp. 33747-54.

Chernova, M.N., Stewart, A.K., Jiang, L., Friedman, D.J., Kunes, Y.Z. and Alper, S.L. (2003) 'Structure-function relationships of AE2 regulation by Ca(i)(2+)-sensitive stimulators NH(4+) and hypertonicity', *Am J Physiol Cell Physiol*, 284(5), pp. C1235-46.

Choi, J.Y., Joo, N.S., Krouse, M.E., Wu, J.V., Robbins, R.C., Ianowski, J.P., Hanrahan, J.W. and Wine, J.J. (2007) 'Synergistic airway gland mucus secretion in response to vasoactive intestinal peptide and carbachol is lost in cystic fibrosis', *J Clin Invest*, 117(10), pp. 3118-27.

Clancy, J.P., Ruiz, F.E. and Sorscher, E.J. (1999) 'Adenosine and its nucleotides activate wild-type and R117H CFTR through an A2B receptor-coupled pathway', *Am J Physiol*, 276(2 Pt 1), pp. C361-9.

Cobb, B.R., Fan, L., Kovacs, T.E., Sorscher, E.J. and Clancy, J.P. (2003) 'Adenosine receptors and phosphodiesterase inhibitors stimulate Cl- secretion in Calu-3 cells', *Am J Respir Cell Mol Biol*, 29(3 Pt 1), pp. 410-8.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G., Malone, R.W., Bennett, M.J. and Gruenert, D.C. (2000) 'Transfer and expression of foreign genes in mammalian cells', *Biotechniques*, 29(2), pp. 314-8, 320-2, 324 passim.

Craven, K.B. and Zagotta, W.N. (2006) 'CNG and HCN channels: two peas, one pod', *Annu Rev Physiol*, 68, pp. 375-401.

da Paula, A.C., Ramalho, A.S., Farinha, C.M., Cheung, J., Maurisse, R., Gruenert, D.C., Ousingsawat, J., Kunzelmann, K. and Amaral, M.D. (2005) 'Characterization of novel airway submucosal gland cell models for cystic fibrosis studies', *Cell Physiol Biochem*, 15(6), pp. 251-62.

de Wit, C., von Bismarck, P. and Pohl, U. (1994) 'Synergistic action of vasodilators that increase cGMP and cAMP in the hamster cremaster microcirculation', *Cardiovasc Res*, 28(10), pp. 1513-8.

de Wit, R.J., Hekstra, D., Jastorff, B., Stec, W.J., Baraniak, J., Van Driel, R. and Van Haastert, P.J. (1984) 'Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases', *Eur J Biochem*, 142(2), pp. 255-60.

Dean, M., Rzhetsky, A. and Allikmets, R. (2001) 'The human ATP-binding cassette (ABC) transporter superfamily', *Genome Res*, 11(7), pp. 1156-66.

Delmotte, P. and Sanderson, M.J. (2006) 'Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices', *Am J Respir Cell Mol Biol*, 35(1), pp. 110-7.

Domingue, J.C., Ao, M., Sarathy, J., George, A., Alrefai, W.A., Nelson, D.J. and Rao, M.C. (2014) 'HEK-293 cells expressing the cystic fibrosis transmembrane conductance regulator (CFTR): a model for studying regulation of Cl- transport', *Physiol Rep*, 2(9).

Dorwart, M.R., Shcheynikov, N., Yang, D. and Muallem, S. (2008) 'The solute carrier 26 family of proteins in epithelial ion transport', *Physiology (Bethesda)*, 23, pp. 104-14.

Duszyk, M. (2001) 'Regulation of anion secretion by nitric oxide in human airway epithelial cells', *Am J Physiol Lung Cell Mol Physiol*, 281(2), pp. L450-7.

Egea, G., Serra-Peinado, C., Gavilan, M.P. and Rios, R.M. (2015) 'Cytoskeleton and Golgi-apparatus interactions:a two-way road of function and structure', *Cell Health and cytoskeleton*, 7, pp. 37-54.

Fantidis, P. (2010) 'The role of intracellular 3'5'-cyclic adenosine monophosphate (cAMP) in atherosclerosis', *Curr Vasc Pharmacol*, 8(4), pp. 464-72.

Ferguson, G.D. and Storm, D.R. (2004) 'Why calcium-stimulated adenylyl cyclases?', *Physiology (Bethesda)*, 19, pp. 271-6.

Ferreira, M.C.J., Toussaint, C.H., Martine Imbert-Teboul, C.B., Jean-Marc Verbavatz, Anne-Christine Bellanger and Chabarde`s, a.D. (1998) 'Co-expression of a Ca2+-inhibitable Adenylyl Cyclase and of a Ca2+-sensing Receptor in the Cortical Thick Ascending Limb

Cell of the Rat Kidney', *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 273(12), pp. 15192-15202.

Finkbeiner, W.E., Zlock, L.T., Mehdi, I. and Widdicombe, J.H. (2010) 'Cultures of human tracheal gland cells of mucous or serous phenotype', *In Vitro Cell Dev Biol Anim*, 46(5), pp. 450-6.

Finn, J.T., Grunwald, M.E. and Yau, K.W. (1996) 'Cyclic nucleotide-gated ion channels: an extended family with diverse functions', *Annu Rev Physiol*, 58, pp. 395-426.

Fujinaga, J., Loiselle, F.B. and Casey, J.R. (2003) 'Transport activity of chimaeric AE2-AE3 chloride/bicarbonate anion exchange proteins', *Biochem J*, 371(Pt 3), pp. 687-96.

Garnett, J.P., Hickman, E., Burrows, R., Hegyi, P., Tiszlavicz, L., Cuthbert, A.W., Fong, P. and Gray, M.A. (2011) 'Novel role for pendrin in orchestrating bicarbonate secretion in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing airway serous cells', *J Biol Chem*, 286(47), pp. 41069-82.

Garnett, J.P., Hickman, E., Tunkamnerdthai, O., Cuthbert, A.W. and Gray, M.A. (2013) 'Protein phosphatase 1 coordinates CFTR-dependent airway epithelial HCO3- secretion by reciprocal regulation of apical and basolateral membrane Cl(-)-HCO3- exchangers', *Br J Pharmacol*, 168(8), pp. 1946-60.

Garnier, A., Cote, J., Nadeau, I., Kamen, A. and Massie, B. (1994) 'Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells', *Cytotechnology*, 15(1-3), pp. 145-55.

Gawenis, L.R., Bradford, E.M., Alper, S.L., Prasad, V. and Shull, G.E. (2010) 'AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon', *Am J Physiol Gastrointest Liver Physiol*, 298(4), pp. G493-503.

Graham, F.L. (1987) 'Growth of 293 cells in suspension culture', *J Gen Virol*, 68 (Pt 3), pp. 937-40.

Gray, M.A., O'Reilly, C., Winpenny, J. and Argent, B. (2001) 'Functional interactions of HCO3- with cystic fibrosis transmembrane conductance regulator', *JOP*, 2(4 Suppl), pp. 207-11.

Grubb, B.R., Vick, R.N. and Boucher, R.C. (1994) 'Hyperabsorption of Na+ and raised Ca(2+)-mediated Cl- secretion in nasal epithelia of CF mice', *Am J Physiol*, 266(5 Pt 1), pp. C1478-83.

Gu, C., Yaddanapudi, S., Weins, A., Osborn, T., Reiser, J., Pollak, M., Hartwig, J. and Sever, S. (2010) 'Direct dynamin-actin interactions regulate the actin cytoskeleton', *EMBO J*, 29(21), pp. 3593-606.

Guggino, W.B. and Stanton, B.A. (2006) 'New insights into cystic fibrosis: molecular switches that regulate CFTR', *Nat Rev Mol Cell Biol*, 7(6), pp. 426-36.

Hamilton, K.O., Topp, E., Makagiansar, I., Siahaan, T., Yazdanian, M. and Audus, K.L. (2001) 'Multidrug resistance-associated protein-1 functional activity in Calu-3 cells', *J Pharmacol Exp Ther*, 298(3), pp. 1199-205.

Hartzell, C., Putzier, I. and Arreola, J. (2005) 'Calcium-activated chloride channels', *Annu Rev Physiol*, 67, pp. 719-58.

Hens, J.J., De Wit, M., Ghijsen, W.E., Leenders, A.G., Boddeke, H.W., Kissmehl, R., Wiegant, V.M., Weller, U., Gispen, W.H. and De Graan, P.N. (1998) 'Role of calcineurin in Ca2+-induced release of catecholamines and neuropeptides', *J Neurochem*, 71(5), pp. 1978-86.

Hochbaum, D., Barila, G., Ribeiro-Neto, F. and Altschuler, D.L. (2011) 'Radixin assembles cAMP effectors Epac and PKA into a functional cAMP compartment: role in cAMP-dependent cell proliferation', *J Biol Chem*, 286(1), pp. 859-66.

Hoque, K.M., Woodward, O.M., van Rossum, D.B., Zachos, N.C., Chen, L., Leung, G.P., Guggino, W.B., Guggino, S.E. and Tse, C.M. (2010) 'Epac1 mediates protein kinase A-independent mechanism of forskolin-activated intestinal chloride secretion', *J Gen Physiol*, 135(1), pp. 43-58.

Huang, J., Shan, J., Kim, D., Liao, J., Evagelidis, A., Alper, S.L. and Hanrahan, J.W. (2012) 'Basolateral chloride loading by the anion exchanger type 2: role in fluid secretion by the human airway epithelial cell line Calu-3', *J Physiol*, 590(Pt 21), pp. 5299-316.

Huang, P., Lazarowski, E.R., Tarran, R., Milgram, S.L., Boucher, R.C. and Stutts, M.J. (2001) 'Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells', *Proc Natl Acad Sci U S A*, 98(24), pp. 14120-5.

Humphreys, B.D., Jiang, L., Chernova, M.N. and Alper, S.L. (1994) 'Functional characterization and regulation by pH of murine AE2 anion exchanger expressed in Xenopus oocytes', *Am J Physiol*, 267(5 Pt 1), pp. C1295-307.

Illek, B., Yankaskas, J.R. and Machen, T.E. (1997) 'cAMP and genistein stimulate HCO3- conductance through CFTR in human airway epithelia', *Am J Physiol*, 272(4 Pt 1), pp. L752-61.

Inglis, S.K., Finlay, L., Ramminger, S.J., Richard, K., Ward, M.R., Wilson, S.M. and Olver, R.E. (2002) 'Regulation of intracellular pH in Calu-3 human airway cells', *J Physiol*, 538(Pt 2), pp. 527-39.

Ishiguro, H., Naruse, S., Kitagawa, M., Mabuchi, T., Kondo, T., Hayakawa, T., Case, R.M. and Steward, M.C. (2002) 'Chloride transport in microperfused interlobular ducts isolated from guinea-pig pancreas', *J Physiol*, 539(Pt 1), pp. 175-89.

Jung, J. and Lee, M.G. (2014) 'Role of calcium signaling in epithelial bicarbonate secretion', *Cell Calcium*, 55(6), pp. 376-84.

Kang, S., Xu, M., Cooper, E.C. and Hoshi, N. (2014) 'Channel-anchored protein kinase CK2 and protein phosphatase 1 reciprocally regulate KCNQ2-containing M-channels via phosphorylation of calmodulin', *J Biol Chem*, 289(16), pp. 11536-44.

Kaufman, R.J. (1997) 'DNA transfection to study translational control in mammalian cells', *Methods*, 11(4), pp. 361-70.

Kaupp, U.B. and Seifert, R. (2002) 'Cyclic nucleotide-gated ion channels', *Physiol Rev*, 82(3), pp. 769-824.

Kazi Mirajul Hoque, O.M.W., Damian B. van Rossum, Nicholas C. Zachos, Linxi Chen, George P.H. Leung, William B. Guggino, Sandra E. Guggino, and Chung-Ming Tse (2009) 'Epac1 mediates protein kinase A–independent mechanism of forskolin-activated intestinal chloride secretion', *J. Gen. Physiol.*, 135(1), pp. 43-58.

Keller, D.X., Franks, K.M., Bartol, T.M., Jr. and Sejnowski, T.J. (2008) 'Calmodulin activation by calcium transients in the postsynaptic density of dendritic spines', *PLoS One*, 3(4), p. e2045.

Kerkhoff, E., Simpson, J.C., Leberfinger, C.B., Otto, I.M., Doerks, T., Bork, P., Rapp, U.R., Raabe, T. and Pepperkok, R. (2001) 'The Spir actin organizers are involved in vesicle transport processes', *Curr Biol*, 11(24), pp. 1963-8.

Kessels, M.M., Dong, J., Leibig, W., Westermann, P. and Qualmann, B. (2006) 'Complexes of syndapin II with dynamin II promote vesicle formation at the trans-Golgi network', *J Cell Sci*, 119(Pt 8), pp. 1504-16.

Kessels, M.M. and Qualmann, B. (2004) 'The syndapin protein family: linking membrane trafficking with the cytoskeleton', *J Cell Sci*, 117(Pt 15), pp. 3077-86.
Kim, C.H., Oh, Y. and Lee, T.H. (1997) 'Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells', *Gene*, 199(1-2), pp. 293-301.

Kim, D., Kim, J., Burghardt, B., Best, L. and Steward, M.C. (2014) 'Role of anion exchangers in Cl- and HCO3- secretion by the human airway epithelial cell line Calu-3', *Am J Physiol Cell Physiol*, 307(2), pp. C208-19.

Kim, H.W., Ha, S.H., Lee, M.N., Huston, E., Kim, D.H., Jang, S.K., Suh, P.G., Houslay, M.D. and Ryu, S.H. (2010) 'Cyclic AMP controls mTOR through

regulation of the dynamic interaction between Rheb and phosphodiesterase 4D', *Mol Cell Biol*, 30(22), pp. 5406-20.

Kim, J. and Hwan Kim, S. (2013) 'CK2 inhibitor CX-4945 blocks TGF-beta1-induced epithelial-to-mesenchymal transition in A549 human lung adenocarcinoma cells', *PLoS One*, 8(9), p. e74342.

Kim, K.H., Shcheynikov, N., Wang, Y. and Muallem, S. (2005) 'SLC26A7 is a Cl- channel regulated by intracellular pH', *J Biol Chem*, 280(8), pp. 6463-70.

Ko, S.B., Shcheynikov, N., Choi, J.Y., Luo, X., Ishibashi, K., Thomas, P.J., Kim, J.Y., Kim, K.H., Lee, M.G., Naruse, S. and Muallem, S. (2002) 'A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis', *EMBO J*, 21(21), pp. 5662-72.

Ko, S.B., Zeng, W., Dorwart, M.R., Luo, X., Kim, K.H., Millen, L., Goto, H., Naruse, S., Soyombo, A., Thomas, P.J. and Muallem, S. (2004) 'Gating of CFTR by the STAS domain of SLC26 transporters', *Nat Cell Biol*, 6(4), pp. 343-50.

Krouse, M.E., Talbott, J.F., Lee, M.M., Joo, N.S. and Wine, J.J. (2004) 'Acid and base secretion in the Calu-3 model of human serous cells', *Am J Physiol Lung Cell Mol Physiol*, 287(6), pp. L1274-83.

Lai, L. and Tan, T.M. (2002) 'Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues', *Biochem J*, 361(Pt 3), pp. 497-503.

Lai, M.M., Hong, J.J., Ruggiero, A.M., Burnett, P.E., Slepnev, V.I., De Camilli, P. and Snyder, S.H. (1999) 'The calcineurin-dynamin 1 complex as a calcium sensor for synaptic vesicle endocytosis', *J Biol Chem*, 274(37), pp. 25963-6.

Lamyel, F., Warnken-Uhlich, M., Seemann, W.K., Mohr, K., Kostenis, E., Ahmedat, A.S., Smit, M., Gosens, R., Meurs, H., Miller-Larsson, A. and Racke, K. (2011) 'The beta2-subtype of adrenoceptors mediates inhibition of pro-

fibrotic events in human lung fibroblasts', *Naunyn Schmiedebergs Arch Pharmacol*, 384(2), pp. 133-45.

Lee, B.S., Gunn, R.B. and Kopito, R.R. (1991) 'Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line', *J Biol Chem*, 266(18), pp. 11448-54.

Lee, H.J., Yoo, J.E., Namkung, W., Cho, H.J., Kim, K., Kang, J.W., Yoon, J.H. and Choi, J.Y. (2015) 'Thick airway surface liquid volume and weak mucin expression in pendrin-deficient human airway epithelia', *Physiol Rep*, 3(8).

Lee, M.C., Penland, C.M., Widdicombe, J.H. and Wine, J.J. (1998) 'Evidence that Calu-3 human airway cells secrete bicarbonate', *Am J Physiol*, 274(3 Pt 1), pp. L450-3.

Lee, M.G., Ohana, E., Park, H.W., Yang, D. and Muallem, S. (2012) 'Molecular mechanism of pancreatic and salivary gland fluid and HCO3 secretion', *Physiol Rev*, 92(1), pp. 39-74.

Lee, R.J. and Foskett, J.K. (2010) 'cAMP-activated Ca2+ signaling is required for CFTR-mediated serous cell fluid secretion in porcine and human airways', *J Clin Invest*, 120(9), pp. 3137-48.

Lefkimmiatis, K., Srikanthan, M., Maiellaro, I., Moyer, M.P., Curci, S. and Hofer, A.M. (2009) 'Store-operated cyclic AMP signalling mediated by STIM1', *Nat Cell Biol*, 11(4), pp. 433-42.

Li, C., Krishnamurthy, P.C., Penmatsa, H., Marrs, K.L., Wang, X.Q., Zaccolo, M., Jalink, K., Li, M., Nelson, D.J., Schuetz, J.D. and Naren, A.P. (2007) 'Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia', *Cell*, 131(5), pp. 940-51.

Liedtke, C.M., Cody, D. and Cole, T.S. (2001) 'Differential regulation of Cl-transport proteins by PKC in Calu-3 cells', *Am J Physiol Lung Cell Mol Physiol*, 280(4), pp. L739-47.

Liu, J.P., Powell, K.A., Sudhof, T.C. and Robinson, P.J. (1994) 'Dynamin I is a Ca(2+)-sensitive phospholipid-binding protein with very high affinity for protein kinase C', *J Biol Chem*, 269(33), pp. 21043-50.

Loffing, J., Moyer, B.D., Reynolds, D., Shmukler, B.E., Alper, S.L. and Stanton, B.A. (2000) 'Functional and molecular characterization of an anion exchanger in airway serous epithelial cells', *Am J Physiol Cell Physiol*, 279(4), pp. C1016-23.

Lukacs, G.L., Segal, G., Kartner, N., Grinstein, S. and Zhang, F. (1997) 'Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation', *Biochem J*, 328 (Pt 2), pp. 353-61.

Luz, S., Kongsuphol, P., Mendes, A.I., Romeiras, F., Sousa, M., Schreiber, R., Matos, P., Jordan, P., Mehta, A., Amaral, M.D., Kunzelmann, K. and Farinha, C.M. (2011) 'Contribution of casein kinase 2 and spleen tyrosine kinase to CFTR trafficking and protein kinase A-induced activity', *Mol Cell Biol*, 31(22), pp. 4392-404.

Luz, S.F. (2008) *Novel insight into CFTR phosphorylation : implications for its processing, trafficking and function*. Msc thesis. Universidade de Lisboa.

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C. and Kirchhausen, T. (2006) 'Dynasore, a cell-permeable inhibitor of dynamin', *Dev Cell*, 10(6), pp. 839-50.

Magno, A.L., Ward, B.K. and Ratajczak, T. (2011) 'The calcium-sensing receptor: a molecular perspective', *Endocr Rev*, 32(1), pp. 3-30.

Maiellaro, I., Lefkimmiatis, K., Moyer, M.P., Curci, S. and Hofer, A.M. (2012) 'Termination and activation of store-operated cyclic AMP production', *J Cell Mol Med*, 16(11), pp. 2715-25.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G.M. (2013) 'RNA-guided human genome engineering via Cas9', *Science*, 339(6121), pp. 823-6.

Marks, B. and McMahon, H.T. (1998) 'Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals', *Curr Biol*, 8(13), pp. 740-9.

Mayerhofer, A., Fohr, K.J., Sterzik, K. and Gratzl, M. (1992) 'Carbachol increases intracellular free calcium concentrations in human granulosa-lutein cells', *J Endocrinol*, 135(1), pp. 153-9.

Mehta, A. (2008) 'Cystic fibrosis as a bowel cancer syndrome and the potential role of CK2', *Mol Cell Biochem*, 316(1-2), pp. 169-75.

Milara, J., Mata, M., Serrano, A., Peiro, T., Morcillo, E.J. and Cortijo, J. (2010) 'Extracellular calcium-sensing receptor mediates human bronchial epithelial wound repair', *Biochem Pharmacol*, 80(2), pp. 236-46.

Monterisi, S., Favia, M., Guerra, L., Cardone, R.A., Marzulli, D., Reshkin, S.J., Casavola, V. and Zaccolo, M. (2012) 'CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity', *J Cell Sci*, 125(Pt 5), pp. 1106-17.

Moran, O. and Zegarra-Moran, O. (2005) 'A quantitative description of the activation and inhibition of CFTR by potentiators: Genistein', *FEBS Lett*, 579(18), pp. 3979-83.

Mount, D.B. and Romero, M.F. (2004) 'The SLC26 gene family of multifunctional anion exchangers', *Pflugers Arch*, 447(5), pp. 710-21.

Nabi, I.R. and Le, P.U. (2003) 'Caveolae/raft-dependent endocytosis', *J Cell Biol*, 161(4), pp. 673-7.

Namkung, W., Finkbeiner, W.E. and Verkman, A.S. (2010) 'CFTR-adenylyl cyclase I association responsible for UTP activation of CFTR in well-differentiated primary human bronchial cell cultures', *Mol Biol Cell*, 21(15), pp. 2639-48.

Namkung, W., Lee, J.A., Ahn, W., Han, W., Kwon, S.W., Ahn, D.S., Kim, K.H. and Lee, M.G. (2003) 'Ca2+ activates cystic fibrosis transmembrane conductance regulator- and Cl- -dependent HCO3 transport in pancreatic duct cells', *J Biol Chem*, 278(1), pp. 200-7.

Nguyen, H.V., Stuart-Tilley, A., Alper, S.L. and Melvin, J.E. (2004) 'Cl(-)/HCO(3)(-) exchange is acetazolamide sensitive and activated by a muscarinic receptor-induced [Ca(2+)](i) increase in salivary acinar cells', *Am J Physiol Gastrointest Liver Physiol*, 286(2), pp. G312-20.

Norimatsu, Y., Ivetac, A., Alexander, C., O'Donnell, N., Frye, L., Sansom, M.S. and Dawson, D.C. (2012) 'Locating a plausible binding site for an open-channel blocker, GlyH-101, in the pore of the cystic fibrosis transmembrane conductance regulator', *Mol Pharmacol*, 82(6), pp. 1042-55.

O'Day, D.H. (2003) 'CaMBOT: profiling and characterizing calmodulin-binding proteins', *Cell Signal*, 15(4), pp. 347-54.

Oestreich, E.A., Malik, S., Goonasekera, S.A., Blaxall, B.C., Kelley, G.G., Dirksen, R.T. and Smrcka, A.V. (2009) 'Epac and phospholipase Cepsilon regulate Ca2+ release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase II', *J Biol Chem*, 284(3), pp. 1514-22.

Orie, N.N., Thomas, A.M., Perrino, B.A., Tinker, A. and Clapp, L.H. (2009) 'Ca2+/calcineurin regulation of cloned vascular K ATP channels: crosstalk with the protein kinase A pathway', *Br J Pharmacol*, 157(4), pp. 554-64.

Paradiso, A.M., Cheng, E.H. and Boucher, R.C. (1991) 'Effects of bradykinin on intracellular calcium regulation in human ciliated airway epithelium', *Am J Physiol*, 261(2 Pt 1), pp. L63-9.

Pena-Munzenmayer, G., Catalan, M.A., Kondo, Y., Jaramillo, Y., Liu, F., Shull, G.E. and Melvin, J.E. (2015) 'Ae4 (Slc4a9) Anion Exchanger Drives Cl- Uptake-dependent Fluid Secretion by Mouse Submandibular Gland Acinar Cells', *J Biol Chem*, 290(17), pp. 10677-88.

Petrovic, S., Barone, S., Xu, J., Conforti, L., Ma, L., Kujala, M., Kere, J. and Soleimani, M. (2004) 'SLC26A7: a basolateral Cl-/HCO3- exchanger specific to intercalated cells of the outer medullary collecting duct', *Am J Physiol Renal Physiol*, 286(1), pp. F161-9.

Petrovic, S., Ju, X., Barone, S., Seidler, U., Alper, S.L., Lohi, H., Kere, J. and Soleimani, M. (2003) 'Identification of a basolateral Cl-/HCO3- exchanger specific to gastric parietal cells', *Am J Physiol Gastrointest Liver Physiol*, 284(6), pp. G1093-103.

Pezzulo, A.A., Tang, X.X., Hoegger, M.J., Alaiwa, M.H., Ramachandran, S., Moninger, T.O., Karp, P.H., Wohlford-Lenane, C.L., Haagsman, H.P., van Eijk, M., Banfi, B., Horswill, A.R., Stoltz, D.A., McCray, P.B., Jr., Welsh, M.J. and Zabner, J. (2012) 'Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung', *Nature*, 487(7405), pp. 109-13.

Pierre, F., Chua, P.C., O'Brien, S.E., Siddiqui-Jain, A., Bourbon, P., Haddach, M., Michaux, J., Nagasawa, J., Schwaebe, M.K., Stefan, E., Vialettes, A., Whitten, J.P., Chen, T.K., Darjania, L., Stansfield, R., Bliesath, J., Drygin, D., Ho, C., Omori, M., Proffitt, C., Streiner, N., Rice, W.G., Ryckman, D.M. and Anderes, K. (2011) 'Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer', *Mol Cell Biochem*, 356(1-2), pp. 37-43.

Poulsen, J.H., Fischer, H., Illek, B. and Machen, T.E. (1994) 'Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator', *Proc Natl Acad Sci U S A*, 91(12), pp. 5340-4.

Praefcke, G.J. and McMahon, H.T. (2004) 'The dynamin superfamily: universal membrane tubulation and fission molecules?', *Nat Rev Mol Cell Biol*, 5(2), pp. 133-47.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. and Lim, W.A. (2013) 'Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression', *Cell*, 152(5), pp. 1173-83.

Qiu, W., Laheri, A., Leung, S. and Guggino, S.E. (2000a) 'Hormones increase mRNA of cyclic-nucleotide-gated cation channels in airway epithelia', *Pflugers Arch*, 441(1), pp. 69-77.

Qiu, W., Lee, B., Lancaster, M., Xu, W., Leung, S. and Guggino, S.E. (2000b) 'Cyclic nucleotide-gated cation channels mediate sodium and calcium influx in rat colon', *Am J Physiol Cell Physiol*, 278(2), pp. C336-43.

Quadroni, M., James, P. and Carafoli, E. (1994) 'Isolation of phosphorylated calmodulin from rat liver and identification of the in vivo phosphorylation sites', *J Biol Chem*, 269(23), pp. 16116-22.

Racz, G.Z., Kittel, A., Riccardi, D., Case, R.M., Elliott, A.C. and Varga, G. (2002) 'Extracellular calcium sensing receptor in human pancreatic cells', *Gut*, 51(5), pp. 705-11.

Rasmussen, J.E., Sheridan, J.T., Polk, W., Davies, C.M. and Tarran, R. (2014) 'Cigarette smoke-induced Ca2+ release leads to cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction', *J Biol Chem*, 289(11), pp. 7671-81.

Romero, M.F., Fulton, C.M. and Boron, W.F. (2004) 'The SLC4 family of HCO 3 - transporters', *Pflugers Arch*, 447(5), pp. 495-509.

Rossmann, H., Bachmann, O., Wang, Z., Shull, G.E., Obermaier, B., Stuart-Tilley, A., Alper, S.L. and Seidler, U. (2001) 'Differential expression and regulation of AE2 anion exchanger subtypes in rabbit parietal and mucous cells', *J Physiol*, 534(Pt 3), pp. 837-48.

Ruetz, S., Lindsey, A.E., Ward, C.L. and Kopito, R.R. (1993) 'Functional activation of plasma membrane anion exchangers occurs in a pre-Golgi compartment', *J Cell Biol*, 121(1), pp. 37-48.

Salonikidis, P.S., Zeug, A., Kobe, F., Ponimaskin, E. and Richter, D.W. (2008) 'Quantitative measurement of cAMP concentration using an exchange protein directly activated by a cAMP-based FRET-sensor', *Biophys J*, 95(11), pp. 5412-23.

Santana, L.F., Chase, E.G., Votaw, V.S., Nelson, M.T. and Greven, R. (2002) 'Functional coupling of calcineurin and protein kinase A in mouse ventricular myocytes', *J Physiol*, 544(Pt 1), pp. 57-69.

Sarno, S., Ruzzene, M., Frascella, P., Pagano, M.A., Meggio, F., Zambon, A., Mazzorana, M., Di Maira, G., Lucchini, V. and Pinna, L.A. (2005) 'Development and exploitation of CK2 inhibitors', *Mol Cell Biochem*, 274(1-2), pp. 69-76.

Schmidt, M., Dekker, F.J. and Maarsingh, H. (2013) 'Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions', *Pharmacol Rev*, 65(2), pp. 670-709.

Senatore, A., Boone, A.N. and Spafford, J.D. (2011) 'Optimized transfection strategy for expression and electrophysiological recording of recombinant voltage-gated ion channels in HEK-293T cells', *J Vis Exp*, (47).

Shan, J., Liao, J., Huang, J., Robert, R., Palmer, M.L., Fahrenkrug, S.C., O'Grady, S.M. and Hanrahan, J.W. (2012) 'Bicarbonate-dependent chloride

transport drives fluid secretion by the human airway epithelial cell line Calu-3', *J Physiol*, 590(Pt 21), pp. 5273-97.

Shoji, K., Ohashi, K., Sampei, K., Oikawa, M. and Mizuno, K. (2012) 'Cytochalasin D acts as an inhibitor of the actin-cofilin interaction', *Biochem Biophys Res Commun*, 424(1), pp. 52-7.

Smith, J.J. and Welsh, M.J. (1992) 'cAMP stimulates bicarbonate secretion across normal, but not cystic fibrosis airway epithelia', *J Clin Invest*, 89(4), pp. 1148-53.

Soderling, T.R. (1999) 'The Ca-calmodulin-dependent protein kinase cascade', *Trends Biochem Sci*, 24(6), pp. 232-6.

Sterling, D., Alvarez, B.V. and Casey, J.R. (2002) 'The extracellular component of a transport metabolon. Extracellular loop 4 of the human AE1 Cl-/HCO3- exchanger binds carbonic anhydrase IV', *J Biol Chem*, 277(28), pp. 25239-46.

Stewart, A.K., Chernova, M.N., Shmukler, B.E., Wilhelm, S. and Alper, S.L. (2002) 'Regulation of AE2-mediated Cl- transport by intracellular or by extracellular pH requires highly conserved amino acid residues of the AE2 NH2-terminal cytoplasmic domain', *J Gen Physiol*, 120(5), pp. 707-22.

Stewart, A.K., Kurschat, C.E., Vaughan-Jones, R.D., Shmukler, B.E. and Alper, S.L. (2007) 'Acute regulation of mouse AE2 anion exchanger requires isoform-specific amino acid residues from most of the transmembrane domain', *J Physiol*, 584(Pt 1), pp. 59-73.

Sun, F., Hug, M.J., Lewarchik, C.M., Yun, C.H., Bradbury, N.A. and Frizzell, R.A. (2000) 'E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells', *J Biol Chem*, 275(38), pp. 29539-46.

Swulius, M.T. and Waxham, M.N. (2008) 'Ca(2+)/calmodulin-dependent protein kinases', *Cell Mol Life Sci*, 65(17), pp. 2637-57.

Szkotak, A.J., Man, S.F. and Duszyk, M. (2003) 'The role of the basolateral outwardly rectifying chloride channel in human airway epithelial anion secretion', *Am J Respir Cell Mol Biol*, 29(6), pp. 710-20.

Tarran, R., Trout, L., Donaldson, S.H. and Boucher, R.C. (2006) 'Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia', *J Gen Physiol*, 127(5), pp. 591-604.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) 'Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase', *Proc Natl Acad Sci U S A*, 87(7), pp. 2466-70.

Tian, Y., Kongsuphol, P., Hug, M., Ousingsawat, J., Witzgall, R., Schreiber, R. and Kunzelmann, K. (2011) 'Calmodulin-dependent activation of the epithelial calcium-dependent chloride channel TMEM16A', *FASEB J*, 25(3), pp. 1058-68.

Tian, Y. and Laychock, S.G. (2001) 'Protein kinase C and calcium regulation of adenylyl cyclase in isolated rat pancreatic islets', *Diabetes*, 50(11), pp. 2505-13.

Tokumitsu, H., Wayman, G.A., Muramatsu, M. and Soderling, T.R. (1997) 'Calcium/calmodulin-dependent protein kinase kinase: identification of regulatory domains', *Biochemistry*, 36(42), pp. 12823-7.

Treharne, K.J., Xu, Z., Chen, J.H., Best, O.G., Cassidy, D.M., Gruenert, D.C., Hegyi, P., Gray, M.A., Sheppard, D.N., Kunzelmann, K. and Mehta, A. (2009) 'Inhibition of protein kinase CK2 closes the CFTR Cl channel, but has no effect on the cystic fibrosis mutant deltaF508-CFTR', *Cell Physiol Biochem*, 24(5-6), pp. 347-60.

Tsalkova, T., Mei, F.C. and Cheng, X. (2012) 'A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists', *PLoS One*, 7(1), p. e30441.

van Aubel, R.A., Smeets, P.H., Peters, J.G., Bindels, R.J. and Russel, F.G. (2002) 'The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP', *J Am Soc Nephrol*, 13(3), pp. 595-603.

Van Eynde, A., Beullens, M., Stalmans, W. and Bollen, M. (1994) 'Full activation of a nuclear species of protein phosphatase-1 by phosphorylation with protein kinase A and casein kinase-2', *Biochem J*, 297 (Pt 3), pp. 447-9.

Ward, D. (2004) 'Calcium receptor-mediated intracellular signalling', *Cell Calcium*, 35(3), p. 217.28.

Willoughby, D. and Cooper, D.M. (2007) 'Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains', *Physiol Rev*, 87(3), pp. 965-1010.

Wyatt, T.A., Forget, M.A., Adams, J.M. and Sisson, J.H. (2005) 'Both cAMP and cGMP are required for maximal ciliary beat stimulation in a cell-free model of bovine ciliary axonemes', *Am J Physiol Lung Cell Mol Physiol*, 288(3), pp. L546-51.

Yacoub, D., Theoret, J.F., Villeneuve, L., Abou-Saleh, H., Mourad, W., Allen, B.G. and Merhi, Y. (2006) 'Essential role of protein kinase C delta in platelet signaling, alpha IIb beta 3 activation, and thromboxane A2 release', *J Biol Chem*, 281(40), pp. 30024-35.

Young, A., Gentzsch, M., Abban, C.Y., Jia, Y., Meneses, P.I., Bridges, R.J. and Bradbury, N.A. (2009) 'Dynasore inhibits removal of wild-type and DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) from the plasma membrane', *Biochem J*, 421(3), pp. 377-85.

Zufall, F., Firestein, S. and Shepherd, G.M. (1994) 'Cyclic nucleotide-gated ion channels and sensory transduction in olfactory receptor neurons', *Annu Rev Biophys Biomol Struct*, 23, pp. 577-607.

Abazeed, M.E., Blanchette, J.M. and Fuller, R.S. (2005) 'Cell-free transport from the trans-golgi network to late endosome requires factors involved in formation and consumption of clathrin-coated vesicles', J Biol Chem, 280(6), pp. 4442-50.

Ahmad, A., Schaack, J.B., White, C.W. and Ahmad, S. (2013) 'Adenosine A2A receptor-dependent proliferation of pulmonary endothelial cells is mediated through calcium mobilization, PI3-kinase and ERK1/2 pathways', Biochem Biophys Res Commun, 434(3), pp. 566-71.

Ahuja, M., Jha, A., Maleth, J., Park, S. and Muallem, S. (2014) 'cAMP and Ca(2)(+) signaling in secretory epithelia: crosstalk and synergism', Cell Calcium, 55(6), pp. 385-93.

Al-Bazzaz, F.J., Hafez, N., Tyagi, S., Gailey, C.A., Toofanfard, M., Alrefai, W.A., Nazir, T.M., Ramaswamy, K. and Dudeja, P.K. (2001) 'Detection of Cl--HCO3- and Na+-H+ exchangers in human airways epithelium', JOP, 2(4 Suppl), pp. 285-90.

Alesutan, I., Daryadel, A., Mohebbi, N., Pelzl, L., Leibrock, C., Voelkl, J., Bourgeois, S., Dossena, S., Nofziger, C., Paulmichl, M., Wagner, C.A. and Lang, F. (2011) 'Impact of bicarbonate, ammonium chloride, and acetazolamide on hepatic and renal SLC26A4 expression', Cell Physiol Biochem, 28(3), pp. 553-8.

Allen, A. and Flemstrom, G. (2005) 'Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin', Am J Physiol Cell Physiol, 288(1), pp. C1-19.

Allen, A., Flemstrom, G., Garner, A. and Kivilaakso, E. (1993) 'Gastroduodenal mucosal protection', Physiol Rev, 73(4), pp. 823-57.

Allen, D.B., Maguire, J.J., Mahdavian, M., Wicke, C., Marcocci, L., Scheuenstuhl, H., Chang, M., Le, A.X., Hopf, H.W. and Hunt, T.K. (1997)

'Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms', Arch Surg, 132(9), pp. 991-6.

Almahariq, M., Tsalkova, T., Mei, F.C., Chen, H., Zhou, J., Sastry, S.K., Schwede, F. and Cheng, X. (2013) 'A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion', Mol Pharmacol, 83(1), pp. 122-8.

Almstahl, A. and Wikstrom, M. (2003) 'Electrolytes in stimulated whole saliva in individuals with hyposalivation of different origins', Arch Oral Biol, 48(5), pp. 337-44.

Alper, S.L. (2002) 'Genetic diseases of acid-base transporters', Annu Rev Physiol, 64, pp. 899-923.

Alper, S.L., Rossmann, H., Wilhelm, S., Stuart-Tilley, A.K., Shmukler, B.E. and Seidler, U. (1999) 'Expression of AE2 anion exchanger in mouse intestine', Am J Physiol, 277(2 Pt 1), pp. G321-32.

Alper, S.L., Stuart-Tilley, A.K., Biemesderfer, D., Shmukler, B.E. and Brown, D. (1997) 'Immunolocalization of AE2 anion exchanger in rat kidney', Am J Physiol, 273(4 Pt 2), pp. F601-14.

Alvarez-Leefmans, F. and Delpire., E. (2009) Physiology and Pathology of chloride transporters and channels in the nervous system. 1st edn.

Amlal, H., Petrovic, S., Xu, J., Wang, Z., Sun, X., Barone, S. and Soleimani, M. (2010) 'Deletion of the anion exchanger Slc26a4 (pendrin) decreases apical Cl(-)/HCO3(-) exchanger activity and impairs bicarbonate secretion in kidney collecting duct', Am J Physiol Cell Physiol, 299(1), pp. C33-41.

Anagnostopoulou, P., Riederer, B., Duerr, J., Michel, S., Binia, A., Agrawal, R., Liu, X., Kalitzki, K., Xiao, F., Chen, M., Schatterny, J., Hartmann, D., Thum, T., Kabesch, M., Soleimani, M., Seidler, U. and Mall, M.A. (2012) 'SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation', J Clin Invest, 122(10), pp. 3629-34.

Anantharam, A., Bittner, M.A., Aikman, R.L., Stuenkel, E.L., Schmid, S.L., Axelrod, D. and Holz, R.W. (2011) 'A new role for the dynamin GTPase in the regulation of fusion pore expansion', Mol Biol Cell, 22(11), pp. 1907-18.

Aravind, L. and Koonin, E.V. (2000) 'The STAS domain - a link between anion transporters and antisigma-factor antagonists', Curr Biol, 10(2), pp. R53-5.

Ares, G.R. and Ortiz, P.A. (2012) 'Dynamin2, clathrin, and lipid rafts mediate endocytosis of the apical Na/K/2Cl cotransporter NKCC2 in thick ascending limbs', J Biol Chem, 287(45), pp. 37824-34.

Argent, B.E., Gray, M.A., Steward, M.C. and and Case, R.M. (2012) Physiology of the Gastronintestinal Tract. Fifth edn. Oxford.

Arrigoni, G., Marin, O., Pagano, M.A., Settimo, L., Paolin, B., Meggio, F. and Pinna, L.A. (2004) 'Phosphorylation of calmodulin fragments by protein kinase CK2. Mechanistic aspects and structural consequences', Biochemistry, 43(40), pp. 12788-98.

Bachhuber, T., Almaca, J., Aldehni, F., Mehta, A., Amaral, M.D., Schreiber, R. and Kunzelmann, K. (2008) 'Regulation of the epithelial Na+ channel by the protein kinase CK2', J Biol Chem, 283(19), pp. 13225-32.

Baker, J.M., Hudson, R.P., Kanelis, V., Choy, W.Y., Thibodeau, P.H., Thomas, P.J. and Forman-Kay, J.D. (2007) 'CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices', Nat Struct Mol Biol, 14(8), pp. 738-45.

Ballard, S.T. and Inglis, S.K. (2004) 'Liquid secretion properties of airway submucosal glands', J Physiol, 556(Pt 1), pp. 1-10.

Ballou, L.M. and Lin, R.Z. (2008) 'Rapamycin and mTOR kinase inhibitors', J Chem Biol, 1(1-4), pp. 27-36.

Bals, R., Wang, X., Wu, Z., Freeman, T., Bafna, V., Zasloff, M. and Wilson, J.M. (1998) 'Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung', J Clin Invest, 102(5), pp. 874-80.

Barkley, R.A., Chakravarti, A., Cooper, R.S., Ellison, R.C., Hunt, S.C., Province, M.A., Turner, S.T., Weder, A.B., Boerwinkle, E. and Family Blood Pressure, P. (2004) 'Positional identification of hypertension susceptibility genes on chromosome 2', Hypertension, 43(2), pp. 477-82.

Barnes, P.J. (1995) 'Cyclic nucleotides and phosphodiesterases and airway function', Eur Respir J, 8(3), pp. 457-62.

Bauerfeind, R., Takei, K. and De Camilli, P. (1997) 'Amphiphysin I is associated with coated endocytic intermediates and undergoes stimulation-dependent dephosphorylation in nerve terminals', J Biol Chem, 272(49), pp. 30984-92.

Berger, H.A., Travis, S.M. and Welsh, M.J. (1993) 'Regulation of the Cystic-Fibrosis Transmembrane Conductance Regulator Cl- Channel by Specific Protein-Kinases and Protein Phosphatases', Journal of Biological Chemistry, 268(3), pp. 2037-2047.

Bertrand, C.A., Zhang, R., Pilewski, J.M. and Frizzell, R.A. (2009) 'SLC26A9 is a constitutively active, CFTR-regulated anion conductance in human bronchial epithelia', J Gen Physiol, 133(4), pp. 421-38.

Bevensee, M.O., Schmitt, B.M., Choi, I., Romero, M.F. and Boron, W.F. (2000) 'An electrogenic Na(+)-HCO(-)(3) cotransporter (NBC) with a novel COOH-terminus, cloned from rat brain', Am J Physiol Cell Physiol, 278(6), pp. C1200-11.

Bidart, J.M., Lacroix, L., Evain-Brion, D., Caillou, B., Lazar, V., Frydman, R., Bellet, D., Filetti, S. and Schlumberger, M. (2000) 'Expression of Na+/I-symporter and Pendred syndrome genes in trophoblast cells', J Clin Endocrinol Metab, 85(11), pp. 4367-72.

Bissig, M., Hagenbuch, B., Stieger, B., Koller, T. and Meier, P.J. (1994) 'Functional expression cloning of the canalicular sulfate transport system of rat hepatocytes', J Biol Chem, 269(4), pp. 3017-21.

Bissonnette, M., Tien, X.Y., Niedziela, S.M., Hartmann, S.C., Frawley, B.P., Jr., Roy, H.K., Sitrin, M.D., Perlman, R.L. and Brasitus, T.A. (1994) '1,25(OH)2

vitamin D3 activates PKC-alpha in Caco-2 cells: a mechanism to limit secosteroid-induced rise in [Ca2+]i', Am J Physiol, 267(3 Pt 1), pp. G465-75.

Boron, W.F. (2001) 'Sodium-coupled bicarbonate transporters', JOP, 2(4 Suppl), pp. 176-81.

Borowitz, D. (2015) 'CFTR, bicarbonate, and the pathophysiology of cystic fibrosis', Pediatr Pulmonol, 50 Suppl 40, pp. 2S4-S30.

Boucher, R.C. (1999) 'Molecular insights into the physiology of the 'thin film' of airway surface liquid', J Physiol, 516 (Pt 3), pp. 631-8.

Boucher, R.C. (2002) 'An overview of the pathogenesis of cystic fibrosis lung disease', Adv Drug Deliv Rev, 54(11), pp. 1359-71.

Boucher, R.C. (2007a) 'Airway surface dehydration in cystic fibrosis: pathogenesis and therapy', Annu Rev Med, 58, pp. 157-70.

Boucher, R.C. (2007b) 'Cystic fibrosis: a disease of vulnerability to airway surface dehydration', Trends Mol Med, 13(6), pp. 231-40.

Brosius, F.C., 3rd, Pisoni, R.L., Cao, X., Deshmukh, G., Yannoukakos, D., Stuart-Tilley, A.K., Haller, C. and Alper, S.L. (1997) 'AE anion exchanger mRNA and protein expression in vascular smooth muscle cells, aorta, and renal microvessels', Am J Physiol, 273(6 Pt 2), pp. F1039-47.

Brown, E.M. (2007) 'Clinical lessons from the calcium-sensing receptor', Nat Clin Pract Endocrinol Metab, 3(2), pp. 122-33.

Bruce, L.J., Cope, D.L., Jones, G.K., Schofield, A.E., Burley, M., Povey, S., Unwin, R.J., Wrong, O. and Tanner, M.J. (1997) 'Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene', J Clin Invest, 100(7), pp. 1693-707.

Button, B., Cai, L.H., Ehre, C., Kesimer, M., Hill, D.B., Sheehan, J.K., Boucher, R.C. and Rubinstein, M. (2012) 'A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia', Science, 337(6097), pp. 937-41.

Caci, E., Folli, C., Zegarra-Moran, O., Ma, T., Springsteel, M.F., Sammelson, R.E., Nantz, M.H., Kurth, M.J., Verkman, A.S. and Galietta, L.J. (2003) 'CFTR activation in human bronchial epithelial cells by novel benzoflavone and benzimidazolone compounds', Am J Physiol Lung Cell Mol Physiol, 285(1), pp. L180-8.

Cao, H., Weller, S., Orth, J.D., Chen, J., Huang, B., Chen, J.L., Stamnes, M. and McNiven, M.A. (2005) 'Actin and Arf1-dependent recruitment of a cortactin-dynamin complex to the Golgi regulates post-Golgi transport', Nat Cell Biol, 7(5), pp. 483-92.

Carden DL, M.M., and George RB (2000) 'Functional anatomy of the respiratory system', Chest Medicine, pp. 1-25.

Carreno, S., Engqvist-Goldstein, A.E., Zhang, C.X., McDonald, K.L. and Drubin, D.G. (2004) 'Actin dynamics coupled to clathrin-coated vesicle formation at the trans-Golgi network', J Cell Biol, 165(6), pp. 781-8.

Chen, E.Y., Yang, N., Quinton, P.M. and Chin, W.C. (2010) 'A new role for bicarbonate in mucus formation', Am J Physiol Lung Cell Mol Physiol, 299(4), pp. L542-9.

Chen, L., Bosworth, C.A., Pico, T., Collawn, J.F., Varga, K., Gao, Z., Clancy, J.P., Fortenberry, J.A., Lancaster, J.R., Jr. and Matalon, S. (2008) 'DETANO and nitrated lipids increase chloride secretion across lung airway cells', Am J Respir Cell Mol Biol, 39(2), pp. 150-62.

Chen, Z.S., Lee, K. and Kruh, G.D. (2001) 'Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine', J Biol Chem, 276(36), pp. 33747-54.

Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R. and Smith, A.E. (1990) 'Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis', Cell, 63(4), pp. 827-34.

Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J. and Smith, A.E. (1991) 'Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel', Cell, 66(5), pp. 1027-36.

Chernova, M.N., Stewart, A.K., Jiang, L., Friedman, D.J., Kunes, Y.Z. and Alper, S.L. (2003) 'Structure-function relationships of AE2 regulation by Ca(i)(2+)-sensitive stimulators NH(4+) and hypertonicity', Am J Physiol Cell Physiol, 284(5), pp. C1235-46.

Choi, J.Y., Joo, N.S., Krouse, M.E., Wu, J.V., Robbins, R.C., Ianowski, J.P., Hanrahan, J.W. and Wine, J.J. (2007) 'Synergistic airway gland mucus secretion in response to vasoactive intestinal peptide and carbachol is lost in cystic fibrosis', J Clin Invest, 117(10), pp. 3118-27.

Choi, J.Y., Muallem, D., Kiselyov, K., Lee, M.G., Thomas, P.J. and Muallem, S. (2001) 'Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis', Nature, 410(6824), pp. 94-7.

Clancy, J.P., Ruiz, F.E. and Sorscher, E.J. (1999) 'Adenosine and its nucleotides activate wild-type and R117H CFTR through an A2B receptor-coupled pathway', Am J Physiol, 276(2 Pt 1), pp. C361-9.

Clary-Meinesz, C., Mouroux, J., Cosson, J., Huitorel, P. and Blaive, B. (1998) 'Influence of external pH on ciliary beat frequency in human bronchi and bronchioles', Eur Respir J, 11(2), pp. 330-3.

Coakley, R.D., Grubb, B.R., Paradiso, A.M., Gatzy, J.T., Johnson, L.G., Kreda, S.M., O'Neal, W.K. and Boucher, R.C. (2003) 'Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium', Proc Natl Acad Sci U S A, 100(26), pp. 16083-8.

Cobb, B.R., Fan, L., Kovacs, T.E., Sorscher, E.J. and Clancy, J.P. (2003) 'Adenosine receptors and phosphodiesterase inhibitors stimulate Cl- secretion in Calu-3 cells', Am J Respir Cell Mol Biol, 29(3 Pt 1), pp. 410-8.

Cobb, B.R., Ruiz, F., King, C.M., Fortenberry, J., Greer, H., Kovacs, T., Sorscher, E.J. and Clancy, J.P. (2002) 'A(2) adenosine receptors regulate CFTR

through PKA and PLA(2)', Am J Physiol Lung Cell Mol Physiol, 282(1), pp. L12-25.

Cole, A.M., Dewan, P. and Ganz, T. (1999) 'Innate antimicrobial activity of nasal secretions', Infect Immun, 67(7), pp. 3267-75.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G., Malone, R.W., Bennett, M.J. and Gruenert, D.C. (2000) 'Transfer and expression of foreign genes in mammalian cells', Biotechniques, 29(2), pp. 314-8, 320-2, 324 passim.

Concepcion, A.R., Lopez, M., Ardura-Fabregat, A. and Medina, J.F. (2013) 'Role of AE2 for pHi regulation in biliary epithelial cells', Front Physiol, 4, p. 413.

Cordat, E. and Casey, J.R. (2009) 'Bicarbonate transport in cell physiology and disease', Biochem J, 417(2), pp. 423-39.

Craven, K.B. and Zagotta, W.N. (2006) 'CNG and HCN channels: two peas, one pod', Annu Rev Physiol, 68, pp. 375-401.

da Paula, A.C., Ramalho, A.S., Farinha, C.M., Cheung, J., Maurisse, R., Gruenert, D.C., Ousingsawat, J., Kunzelmann, K. and Amaral, M.D. (2005) 'Characterization of novel airway submucosal gland cell models for cystic fibrosis studies', Cell Physiol Biochem, 15(6), pp. 251-62.

Davis, P.B. (2006) 'Cystic fibrosis since 1938', Am J Respir Crit Care Med, 173(5), pp. 475-82.

de Wit, C., von Bismarck, P. and Pohl, U. (1994) 'Synergistic action of vasodilators that increase cGMP and cAMP in the hamster cremaster microcirculation', Cardiovasc Res, 28(10), pp. 1513-8.

de Wit, R.J., Hekstra, D., Jastorff, B., Stec, W.J., Baraniak, J., Van Driel, R. and Van Haastert, P.J. (1984) 'Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases', Eur J Biochem, 142(2), pp. 255-60.

Dean, M., Rzhetsky, A. and Allikmets, R. (2001) 'The human ATP-binding cassette (ABC) transporter superfamily', Genome Res, 11(7), pp. 1156-66.

Delmotte, P. and Sanderson, M.J. (2006) 'Ciliary beat frequency is maintained at a maximal rate in the small airways of mouse lung slices', Am J Respir Cell Mol Biol, 35(1), pp. 110-7.

Demirci, F.Y., Chang, M.H., Mah, T.S., Romero, M.F. and Gorin, M.B. (2006) 'Proximal renal tubular acidosis and ocular pathology: a novel missense mutation in the gene (SLC4A4) for sodium bicarbonate cotransporter protein (NBCe1)', Mol Vis, 12, pp. 324-30.

Derand, R., Montoni, A., Bulteau-Pignoux, L., Janet, T., Moreau, B., Muller, J.M. and Becq, F. (2004) 'Activation of VPAC1 receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion', Br J Pharmacol, 141(4), pp. 698-708.

Devor, D.C., Singh, A.K., Lambert, L.C., DeLuca, A., Frizzell, R.A. and Bridges, R.J. (1999) 'Bicarbonate and chloride secretion in Calu-3 human airway epithelial cells', J Gen Physiol, 113(5), pp. 743-60.

Di Valentin E, C.C., Garbacki N, Hennuy B, Gueders M, Noel A, Foidart JM, Grooten J, Colige A, Piette J, Cataldo D (2009) 'New asthma biomarkers: Lessons from murine models of acute and chronic asthma', Am J Physiol Lung Cell Mol Physiol, 296:, pp. 185-197.

Dirami, T., Rode, B., Jollivet, M., Da Silva, N., Escalier, D., Gaitch, N., Norez, C., Tuffery, P., Wolf, J.P., Becq, F., Ray, P.F., Dulioust, E., Gacon, G., Bienvenu, T. and Toure, A. (2013) 'Missense mutations in SLC26A8, encoding a sperm-specific activator of CFTR, are associated with human asthenozoospermia', Am J Hum Genet, 92(5), pp. 760-6.

Domingue, J.C., Ao, M., Sarathy, J., George, A., Alrefai, W.A., Nelson, D.J. and Rao, M.C. (2014) 'HEK-293 cells expressing the cystic fibrosis transmembrane conductance regulator (CFTR): a model for studying regulation of Cl- transport', Physiol Rep, 2(9).

Dorwart, M.R., Shcheynikov, N., Yang, D. and Muallem, S. (2008) 'The solute carrier 26 family of proteins in epithelial ion transport', Physiology (Bethesda), 23, pp. 104-14.

Dror, A.A., Politi, Y., Shahin, H., Lenz, D.R., Dossena, S., Nofziger, C., Fuchs, H., Hrabe de Angelis, M., Paulmichl, M., Weiner, S. and Avraham, K.B. (2010) 'Calcium oxalate stone formation in the inner ear as a result of an Slc26a4 mutation', J Biol Chem, 285(28), pp. 21724-35.

Dubin, R.F., Robinson, S.K. and Widdicombe, J.H. (2004) 'Secretion of lactoferrin and lysozyme by cultures of human airway epithelium', Am J Physiol Lung Cell Mol Physiol, 286(4), pp. L750-5.

Dudeja, P.K., Hafez, N., Tyagi, S., Gailey, C.A., Toofanfard, M., Alrefai, W.A., Nazir, T.M., Ramaswamy, K. and Al-Bazzaz, F.J. (1999) 'Expression of the Na+/H+ and Cl-/HCO-3 exchanger isoforms in proximal and distal human airways', Am J Physiol, 276(6 Pt 1), pp. L971-8.

Durie, P.R. (1989) 'The pathophysiology of the pancreatic defect in cystic fibrosis', Acta Paediatr Scand Suppl, 363, pp. 41-4.

Duszyk, M. (2001a) 'CFTR and lysozyme secretion in human airway epithelial cells', Pflugers Arch, 443 Suppl 1, pp. S45-9.

Duszyk, M. (2001b) 'Regulation of anion secretion by nitric oxide in human airway epithelial cells', Am J Physiol Lung Cell Mol Physiol, 281(2), pp. L450-7.

Egea, G., Serra-Peinado, C., Gavilan, M.P. and Rios, R.M. (2015) 'Cytoskeleton and Golgi-apparatus interactions: a two-way road of function and structure', Cell Health and cytoskeleton, 7, pp. 37-54.

Engelhardt, J.F., Yankaskas, J.R., Ernst, S.A., Yang, Y., Marino, C.R., Boucher, R.C., Cohn, J.A. and Wilson, J.M. (1992) 'Submucosal glands are the predominant site of CFTR expression in the human bronchus', Nat Genet, 2(3), pp. 240-8.

Estivill, X., Ortigosa, L., Perez-Frias, J., Dapena, J., Ferrer, J., Pena, L., Llevadot, R., Gimenez, J., Nunes, V. and et al. (1995) 'Clinical characteristics of 16 cystic fibrosis patients with the missense mutation R334W, a pancreatic insufficiency mutation with variable age of onset and interfamilial clinical differences', Hum Genet, 95(3), pp. 331-6.

Everett, L.A., Glaser, B., Beck, J.C., Idol, J.R., Buchs, A., Heyman, M., Adawi, F., Hazani, E., Nassir, E., Baxevanis, A.D., Sheffield, V.C. and Green, E.D. (1997) 'Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS)', Nat Genet, 17(4), pp. 411-22.

Fantidis, P. (2010) 'The role of intracellular 3'5'-cyclic adenosine monophosphate (cAMP) in atherosclerosis', Curr Vasc Pharmacol, 8(4), pp. 464-72.

Ferguson, G.D. and Storm, D.R. (2004) 'Why calcium-stimulated adenylyl cyclases?', Physiology (Bethesda), 19, pp. 271-6.

Ferreira, M.C.J., Toussaint, C.H., Martine Imbert-Teboul, C.B., Jean-Marc Verbavatz, Anne-Christine Bellanger and Chabarde`s, a.D. (1998) 'Co-expression of a Ca2+-inhibitable Adenylyl Cyclase and of a Ca2+-sensing Receptor in the Cortical Thick Ascending Limb Cell of the Rat Kidney', THE JOURNAL OF BIOLOGICAL CHEMISTRY, 273(12), pp. 15192-15202.

Finkbeiner, W.E., Zlock, L.T., Mehdi, I. and Widdicombe, J.H. (2010) 'Cultures of human tracheal gland cells of mucous or serous phenotype', In Vitro Cell Dev Biol Anim, 46(5), pp. 450-6.

Finn, J.T., Grunwald, M.E. and Yau, K.W. (1996) 'Cyclic nucleotide-gated ion channels: an extended family with diverse functions', Annu Rev Physiol, 58, pp. 395-426.

Flemstrom, G. and Isenberg, J.I. (2001) 'Gastroduodenal mucosal alkaline secretion and mucosal protection', News Physiol Sci, 16, pp. 23-8.

Fowler, W.S. (1948) 'Respiratory dead space', Fed Proc, 7(1 Pt 1), p. 35.

Fujinaga, J., Loiselle, F.B. and Casey, J.R. (2003) 'Transport activity of chimaeric AE2-AE3 chloride/bicarbonate anion exchange proteins', Biochem J, 371(Pt 3), pp. 687-96.

Gadsby, D.C. and Nairn, A.C. (1999) 'Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis', Physiol Rev, 79(1 Suppl), pp. S77-S107.

Garcia, M.A., Yang, N. and Quinton, P.M. (2009) 'Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion', J Clin Invest, 119(9), pp. 2613-22.

Garnett, J.P., Hickman, E., Burrows, R., Hegyi, P., Tiszlavicz, L., Cuthbert, A.W., Fong, P. and Gray, M.A. (2011) 'Novel role for pendrin in orchestrating bicarbonate secretion in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing airway serous cells', J Biol Chem, 286(47), pp. 41069-82.

Garnett, J.P., Hickman, E., Tunkamnerdthai, O., Cuthbert, A.W. and Gray, M.A. (2013) 'Protein phosphatase 1 coordinates CFTR-dependent airway epithelial HCO3- secretion by reciprocal regulation of apical and basolateral membrane Cl(-)-HCO3- exchangers', Br J Pharmacol, 168(8), pp. 1946-60.

Garnier, A., Cote, J., Nadeau, I., Kamen, A. and Massie, B. (1994) 'Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells', Cytotechnology, 15(1-3), pp. 145-55.

Gawenis, L.R., Bradford, E.M., Alper, S.L., Prasad, V. and Shull, G.E. (2010) 'AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon', Am J Physiol Gastrointest Liver Physiol, 298(4), pp. G493-503.

Gelman, M.S., Kannegaard, E.S. and Kopito, R.R. (2002) 'A principal role for the proteasome in endoplasmic reticulum-associated degradation of misfolded intracellular cystic fibrosis transmembrane conductance regulator', J Biol Chem, 277(14), pp. 11709-14.

Goco, R.V., Kress, M.B. and Brantigan, O.C. (1963) 'Comparison of mucus glands in the tracheobronchial tree of man and animals', Ann N Y Acad Sci, 106, pp. 555-71.

Goodman, B.E. and Percy, W.H. (2005) 'CFTR in cystic fibrosis and cholera: from membrane transport to clinical practice', Adv Physiol Educ, 29(2), pp. 75-82.

Graham, F.L. (1987) 'Growth of 293 cells in suspension culture', J Gen Virol, 68 (Pt 3), pp. 937-40.

Gray, M.A., Greenwell, J.R. and Argent, B.E. (1988) 'Secretin-regulated chloride channel on the apical plasma membrane of pancreatic duct cells', J Membr Biol, 105(2), pp. 131-42.

Gray, M.A., O'Reilly, C., Winpenny, J. and Argent, B. (2001) 'Functional interactions of HCO3- with cystic fibrosis transmembrane conductance regulator', JOP, 2(4 Suppl), pp. 207-11.

Gray, M.A., Plant, S. and Argent, B.E. (1993) 'cAMP-regulated whole cell chloride currents in pancreatic duct cells', Am J Physiol, 264(3 Pt 1), pp. C591-602.

Groneberg, D.A., Hartmann, P., Dinh, Q.T. and Fischer, A. (2001) 'Expression and distribution of vasoactive intestinal polypeptide receptor VPAC(2) mRNA in human airways', Lab Invest, 81(5), pp. 749-55.

Groneberg, D.A., Rabe, K.F. and Fischer, A. (2006) 'Novel concepts of neuropeptide-based drug therapy: vasoactive intestinal polypeptide and its receptors', Eur J Pharmacol, 533(1-3), pp. 182-94.

Grubb, B.R., Vick, R.N. and Boucher, R.C. (1994) 'Hyperabsorption of Na+ and raised Ca(2+)-mediated Cl- secretion in nasal epithelia of CF mice', Am J Physiol, 266(5 Pt 1), pp. C1478-83.

Gu, C., Yaddanapudi, S., Weins, A., Osborn, T., Reiser, J., Pollak, M., Hartwig, J. and Sever, S. (2010) 'Direct dynamin-actin interactions regulate the actin cytoskeleton', EMBO J, 29(21), pp. 3593-606.

Guggino, W.B. and Stanton, B.A. (2006) 'New insights into cystic fibrosis: molecular switches that regulate CFTR', Nat Rev Mol Cell Biol, 7(6), pp. 426-36.

Gustafsson, J.K., Ermund, A., Ambort, D., Johansson, M.E., Nilsson, H.E., Thorell, K., Hebert, H., Sjovall, H. and Hansson, G.C. (2012) 'Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype', J Exp Med, 209(7), pp. 1263-72.

Hamilton, K.O., Topp, E., Makagiansar, I., Siahaan, T., Yazdanian, M. and Audus, K.L. (2001) 'Multidrug resistance-associated protein-1 functional activity in Calu-3 cells', J Pharmacol Exp Ther, 298(3), pp. 1199-205.

Hartzell, C., Putzier, I. and Arreola, J. (2005) 'Calcium-activated chloride channels', Annu Rev Physiol, 67, pp. 719-58.

Hastbacka, J., de la Chapelle, A., Mahtani, M.M., Clines, G., Reeve-Daly, M.P., Daly, M., Hamilton, B.A., Kusumi, K., Trivedi, B., Weaver, A. and et al. (1994) 'The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping', Cell, 78(6), pp. 1073-87.

Haws, C., Finkbeiner, W.E., Widdicombe, J.H. and Wine, J.J. (1994) 'CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl- conductance', Am J Physiol, 266(5 Pt 1), pp. L502-12.

Hens, J.J., De Wit, M., Ghijsen, W.E., Leenders, A.G., Boddeke, H.W., Kissmehl, R., Wiegant, V.M., Weller, U., Gispen, W.H. and De Graan, P.N. (1998) 'Role of calcineurin in Ca2+-induced release of catecholamines and neuropeptides', J Neurochem, 71(5), pp. 1978-86.

Hentchel-Franks, K., Lozano, D., Eubanks-Tarn, V., Cobb, B., Fan, L., Oster, R., Sorscher, E. and Clancy, J.P. (2004) 'Activation of airway cl- secretion in human subjects by adenosine', Am J Respir Cell Mol Biol, 31(2), pp. 140-6.

Hess, K.C., Jones, B.H., Marquez, B., Chen, Y., Ord, T.S., Kamenetsky, M., Miyamoto, C., Zippin, J.H., Kopf, G.S., Suarez, S.S., Levin, L.R., Williams, C.J., Buck, J. and Moss, S.B. (2005) 'The "soluble" adenylyl cyclase in sperm

mediates multiple signaling events required for fertilization', Dev Cell, 9(2), pp. 249-59.

Hochbaum, D., Barila, G., Ribeiro-Neto, F. and Altschuler, D.L. (2011) 'Radixin assembles cAMP effectors Epac and PKA into a functional cAMP compartment: role in cAMP-dependent cell proliferation', J Biol Chem, 286(1), pp. 859-66.

Hoglund, P., Haila, S., Socha, J., Tomaszewski, L., Saarialho-Kere, U., Karjalainen-Lindsberg, M.L., Airola, K., Holmberg, C., de la Chapelle, A. and Kere, J. (1996) 'Mutations of the Down-regulated in adenoma (DRA) gene cause congenital chloride diarrhoea', Nat Genet, 14(3), pp. 316-9.

Hoque, K.M., Woodward, O.M., van Rossum, D.B., Zachos, N.C., Chen, L., Leung, G.P., Guggino, W.B., Guggino, S.E. and Tse, C.M. (2010) 'Epac1 mediates protein kinase A-independent mechanism of forskolin-activated intestinal chloride secretion', J Gen Physiol, 135(1), pp. 43-58.

Huang, J., Shan, J., Kim, D., Liao, J., Evagelidis, A., Alper, S.L. and Hanrahan, J.W. (2012) 'Basolateral chloride loading by the anion exchanger type 2: role in fluid secretion by the human airway epithelial cell line Calu-3', J Physiol, 590(Pt 21), pp. 5299-316.

Huang, P., Lazarowski, E.R., Tarran, R., Milgram, S.L., Boucher, R.C. and Stutts, M.J. (2001) 'Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells', Proc Natl Acad Sci U S A, 98(24), pp. 14120-5.

Hug, M.J., Clarke, L.L. and Gray, M.A. (2011) 'How to measure CFTR-dependent bicarbonate transport: from single channels to the intact epithelium', Methods Mol Biol, 741, pp. 489-509.

Hug, M.J., Tamada, T. and Bridges, R.J. (2003) 'CFTR and bicarbonate secretion by [correction of to] epithelial cells', News Physiol Sci, 18, pp. 38-42.

Humphreys, B.D., Jiang, L., Chernova, M.N. and Alper, S.L. (1994) 'Functional characterization and regulation by pH of murine AE2 anion exchanger expressed in Xenopus oocytes', Am J Physiol, 267(5 Pt 1), pp. C1295-307.

Hunt, S.C., Xin, Y., Wu, L.L., Cawthon, R.M., Coon, H., Hasstedt, S.J. and Hopkins, P.N. (2006) 'Sodium bicarbonate cotransporter polymorphisms are associated with baseline and 10-year follow-up blood pressures', Hypertension, 47(3), pp. 532-6.

Hunter, M.J. (1977) 'Human erythrocyte anion permeabilities measured under conditions of net charge transfer', J Physiol, 268(1), pp. 35-49.

Hwang, T.C. and Sheppard, D.N. (2009) 'Gating of the CFTR Cl- channel by ATP-driven nucleotide-binding domain dimerisation', J Physiol, 587(Pt 10), pp. 2151-61.

Ianowski, J.P., Choi, J.Y., Wine, J.J. and Hanrahan, J.W. (2008) 'Substance P stimulates CFTR-dependent fluid secretion by mouse tracheal submucosal glands', Pflugers Arch, 457(2), pp. 529-37.

Illek, B., Yankaskas, J.R. and Machen, T.E. (1997) 'cAMP and genistein stimulate HCO3- conductance through CFTR in human airway epithelia', Am J Physiol, 272(4 Pt 1), pp. L752-61.

Inglis, S.K., Finlay, L., Ramminger, S.J., Richard, K., Ward, M.R., Wilson, S.M. and Olver, R.E. (2002) 'Regulation of intracellular pH in Calu-3 human airway cells', J Physiol, 538(Pt 2), pp. 527-39.

Inglis, S.K. and Wilson, S.M. (2005) 'Cystic fibrosis and airway submucosal glands', Pediatr Pulmonol, 40(4), pp. 279-84.

Ishiguro, H., Naruse, S., Kitagawa, M., Mabuchi, T., Kondo, T., Hayakawa, T., Case, R.M. and Steward, M.C. (2002) 'Chloride transport in microperfused interlobular ducts isolated from guinea-pig pancreas', J Physiol, 539(Pt 1), pp. 175-89.

Ishiguro, H., Naruse, S., San Roman, J.I., Case, M. and Steward, M.C. (2001) 'Pancreatic ductal bicarbonate secretion: past, present and future', JOP, 2(4 Suppl), pp. 192-7.

Ishiguro, H., Steward, M.C., Naruse, S., Ko, S.B., Goto, H., Case, R.M., Kondo, T. and Yamamoto, A. (2009) 'CFTR functions as a bicarbonate channel in pancreatic duct cells', J Gen Physiol, 133(3), pp. 315-26.

Ishiguro, H., Steward, M.C., Wilson, R.W. and Case, R.M. (1996) 'Bicarbonate secretion in interlobular ducts from guinea-pig pancreas', J Physiol, 495 (Pt 1), pp. 179-91.

Ishiguro, H., Yamamoto, A., Nakakuki, M., Yi, L.J., Ishiguro, M., Yamaguchi, M., Kondo, S. and Mochimaru, Y. (2012) 'Physiology and Pathophysiology of Bicarbonate Secretion by Pancreatic Duct Epithelium', Nagoya Journal of Medical Science, 74(1-2), pp. 1-18.

Jacquot, J., Puchelle, E., Hinnrasky, J., Fuchey, C., Bettinger, C., Spilmont, C., Bonnet, N., Dieterle, A., Dreyer, D., Pavirani, A. and et al. (1993) 'Localization of the cystic fibrosis transmembrane conductance regulator in airway secretory glands', Eur Respir J, 6(2), pp. 169-76.

James-Kracke, M.R. (1992) 'Quick and accurate method to convert BCECF fluorescence to pHi: calibration in three different types of cell preparations', J Cell Physiol, 151(3), pp. 596-603.

Jiang, Z., Asplin, J.R., Evan, A.P., Rajendran, V.M., Velazquez, H., Nottoli, T.P., Binder, H.J. and Aronson, P.S. (2006) 'Calcium oxalate urolithiasis in mice lacking anion transporter Slc26a6', Nat Genet, 38(4), pp. 474-8.

Jiang, Z., Grichtchenko, II, Boron, W.F. and Aronson, P.S. (2002) 'Specificity of anion exchange mediated by mouse Slc26a6', J Biol Chem, 277(37), pp. 33963-7.

Joo, N.S., Saenz, Y., Krouse, M.E. and Wine, J.J. (2002) 'Mucus secretion from single submucosal glands of pig. Stimulation by carbachol and vasoactive intestinal peptide', J Biol Chem, 277(31), pp. 28167-75.

Joo, N.S., Wu, J.V., Krouse, M.E., Saenz, Y. and Wine, J.J. (2001) 'Optical method for quantifying rates of mucus secretion from single submucosal glands', Am J Physiol Lung Cell Mol Physiol, 281(2), pp. L458-68.

Jung, J. and Lee, M.G. (2014) 'Role of calcium signaling in epithelial bicarbonate secretion', Cell Calcium, 55(6), pp. 376-84.

Kang, S., Xu, M., Cooper, E.C. and Hoshi, N. (2014) 'Channel-anchored protein kinase CK2 and protein phosphatase 1 reciprocally regulate KCNQ2-containing M-channels via phosphorylation of calmodulin', J Biol Chem, 289(16), pp. 11536-44.

Karniski, L.P., Lotscher, M., Fucentese, M., Hilfiker, H., Biber, J. and Murer, H. (1998) 'Immunolocalization of sat-1 sulfate/oxalate/bicarbonate anion exchanger in the rat kidney', Am J Physiol, 275(1 Pt 2), pp. F79-87.

Kaufman, R.J. (1997) 'DNA transfection to study translational control in mammalian cells', Methods, 11(4), pp. 361-70.

Kaupp, U.B. and Seifert, R. (2002) 'Cyclic nucleotide-gated ion channels', Physiol Rev, 82(3), pp. 769-824.

Kazi Mirajul Hoque, O.M.W., Damian B. van Rossum, Nicholas C. Zachos, Linxi Chen, George P.H. Leung, William B. Guggino, Sandra E. Guggino, and Chung-Ming Tse (2009) 'Epac1 mediates protein kinase A–independent mechanism of forskolin-activated intestinal chloride secretion', J. Gen. Physiol., 135(1), pp. 43-58.

Keller, D.X., Franks, K.M., Bartol, T.M., Jr. and Sejnowski, T.J. (2008) 'Calmodulin activation by calcium transients in the postsynaptic density of dendritic spines', PLoS One, 3(4), p. e2045.

Kellum, J.A. (2000) 'Determinants of blood pH in health and disease', Crit Care, 4(1), pp. 6-14.

Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989) 'Identification of the cystic fibrosis gene: genetic analysis', Science, 245(4922), pp. 1073-80.

Kerkhoff, E., Simpson, J.C., Leberfinger, C.B., Otto, I.M., Doerks, T., Bork, P., Rapp, U.R., Raabe, T. and Pepperkok, R. (2001) 'The Spir actin organizers are involved in vesicle transport processes', Curr Biol, 11(24), pp. 1963-8.

Kessels, M.M., Dong, J., Leibig, W., Westermann, P. and Qualmann, B. (2006) 'Complexes of syndapin II with dynamin II promote vesicle formation at the trans-Golgi network', J Cell Sci, 119(Pt 8), pp. 1504-16.

Kessels, M.M. and Qualmann, B. (2004) 'The syndapin protein family: linking membrane trafficking with the cytoskeleton', J Cell Sci, 117(Pt 15), pp. 3077-86.

Kim, C.H., Oh, Y. and Lee, T.H. (1997) 'Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells', Gene, 199(1-2), pp. 293-301.

Kim, D., Kim, J., Burghardt, B., Best, L. and Steward, M.C. (2014) 'Role of anion exchangers in Cl- and HCO3- secretion by the human airway epithelial cell line Calu-3', Am J Physiol Cell Physiol, 307(2), pp. C208-19.

Kim, H.W., Ha, S.H., Lee, M.N., Huston, E., Kim, D.H., Jang, S.K., Suh, P.G., Houslay, M.D. and Ryu, S.H. (2010) 'Cyclic AMP controls mTOR through regulation of the dynamic interaction between Rheb and phosphodiesterase 4D', Mol Cell Biol, 30(22), pp. 5406-20.

Kim, J. and Hwan Kim, S. (2013) 'CK2 inhibitor CX-4945 blocks TGF-beta1-induced epithelial-to-mesenchymal transition in A549 human lung adenocarcinoma cells', PLoS One, 8(9), p. e74342.

Kim, K.H., Shcheynikov, N., Wang, Y. and Muallem, S. (2005) 'SLC26A7 is a Cl- channel regulated by intracellular pH', J Biol Chem, 280(8), pp. 6463-70.

Knowles, M.R. and Boucher, R.C. (2002) 'Mucus clearance as a primary innate defense mechanism for mammalian airways', J Clin Invest, 109(5), pp. 571-7.

Ko, S.B., Shcheynikov, N., Choi, J.Y., Luo, X., Ishibashi, K., Thomas, P.J., Kim, J.Y., Kim, K.H., Lee, M.G., Naruse, S. and Muallem, S. (2002) 'A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis', EMBO J, 21(21), pp. 5662-72.

Ko, S.B., Zeng, W., Dorwart, M.R., Luo, X., Kim, K.H., Millen, L., Goto, H., Naruse, S., Soyombo, A., Thomas, P.J. and Muallem, S. (2004) 'Gating of CFTR by the STAS domain of SLC26 transporters', Nat Cell Biol, 6(4), pp. 343-50.

Kobayashi, S., Morgans, C.W., Casey, J.R. and Kopito, R.R. (1994) 'AE3 anion exchanger isoforms in the vertebrate retina: developmental regulation and differential expression in neurons and glia', J Neurosci, 14(10), pp. 6266-79.

Kongsuphol, P., Hieke, B., Ousingsawat, J., Almaca, J., Viollet, B., Schreiber, R. and Kunzelmann, K. (2009) 'Regulation of Cl- secretion by AMPK in vivo', Pflugers Archiv-European Journal of Physiology, 457(5), pp. 1071-1078.

Krouse, M.E., Talbott, J.F., Lee, M.M., Joo, N.S. and Wine, J.J. (2004) 'Acid and base secretion in the Calu-3 model of human serous cells', Am J Physiol Lung Cell Mol Physiol, 287(6), pp. L1274-83.

Kudrycki, K.E., Newman, P.R. and Shull, G.E. (1990) 'cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl-/HCO3- exchanger', J Biol Chem, 265(1), pp. 462-71.

Kurtz, I. (2014) 'NBCe1 as a model carrier for understanding the structure-function properties of Na(+) -coupled SLC4 transporters in health and disease', Pflugers Arch, 466(8), pp. 1501-16.

Lacroix, L., Mian, C., Caillou, B., Talbot, M., Filetti, S., Schlumberger, M. and Bidart, J.M. (2001) 'Na(+)/I(-) symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues', Eur J Endocrinol, 144(3), pp. 297-302.

Lai, L. and Tan, T.M. (2002) 'Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues', Biochem J, 361(Pt 3), pp. 497-503.

Lai, M.M., Hong, J.J., Ruggiero, A.M., Burnett, P.E., Slepnev, V.I., De Camilli, P. and Snyder, S.H. (1999) 'The calcineurin-dynamin 1 complex as a calcium sensor for synaptic vesicle endocytosis', J Biol Chem, 274(37), pp. 25963-6.

Lamprecht, G., Heil, A., Baisch, S., Lin-Wu, E., Yun, C.C., Kalbacher, H., Gregor, M. and Seidler, U. (2002) 'The down regulated in adenoma (dra) gene product binds to the second PDZ domain of the NHE3 kinase A regulatory protein (E3KARP), potentially linking intestinal Cl-/HCO3- exchange to Na+/H+ exchange', Biochemistry, 41(41), pp. 12336-42.

Lamyel, F., Warnken-Uhlich, M., Seemann, W.K., Mohr, K., Kostenis, E., Ahmedat, A.S., Smit, M., Gosens, R., Meurs, H., Miller-Larsson, A. and Racke, K. (2011) 'The beta2-subtype of adrenoceptors mediates inhibition of pro-fibrotic events in human lung fibroblasts', Naunyn Schmiedebergs Arch Pharmacol, 384(2), pp. 133-45.

Landolt-Marticorena, C., Casey, J.R. and Reithmeier, R.A. (1995) 'Transmembrane helix-helix interactions and accessibility of H2DIDS on labelled band 3, the erythrocyte anion exchange protein', Mol Membr Biol, 12(2), pp. 173-82.

Lee, B.S., Gunn, R.B. and Kopito, R.R. (1991) 'Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line', J Biol Chem, 266(18), pp. 11448-54.

Lee, H.J., Yoo, J.E., Namkung, W., Cho, H.J., Kim, K., Kang, J.W., Yoon, J.H. and Choi, J.Y. (2015) 'Thick airway surface liquid volume and weak mucin expression in pendrin-deficient human airway epithelia', Physiol Rep, 3(8).

Lee, M.C., Penland, C.M., Widdicombe, J.H. and Wine, J.J. (1998) 'Evidence that Calu-3 human airway cells secrete bicarbonate', Am J Physiol, 274(3 Pt 1), pp. L450-3.

Lee, M.G., Ohana, E., Park, H.W., Yang, D. and Muallem, S. (2012) 'Molecular mechanism of pancreatic and salivary gland fluid and HCO3 secretion', Physiol Rev, 92(1), pp. 39-74.

Lee, R.J. and Foskett, J.K. (2010) 'cAMP-activated Ca2+ signaling is required for CFTR-mediated serous cell fluid secretion in porcine and human airways', J Clin Invest, 120(9), pp. 3137-48.

Lefkimmiatis, K., Srikanthan, M., Maiellaro, I., Moyer, M.P., Curci, S. and Hofer, A.M. (2009) 'Store-operated cyclic AMP signalling mediated by STIM1', Nat Cell Biol, 11(4), pp. 433-42.

Li, C., Krishnamurthy, P.C., Penmatsa, H., Marrs, K.L., Wang, X.Q., Zaccolo, M., Jalink, K., Li, M., Nelson, D.J., Schuetz, J.D. and Naren, A.P. (2007)

'Spatiotemporal coupling of cAMP transporter to CFTR chloride channel function in the gut epithelia', Cell, 131(5), pp. 940-51.

Li, J., Xia, F. and Reithmeier, R.A. (2014) 'N-glycosylation and topology of the human SLC26 family of anion transport membrane proteins', Am J Physiol Cell Physiol, 306(10), pp. C943-60.

Liedtke, C.M., Cody, D. and Cole, T.S. (2001) 'Differential regulation of Cl- transport proteins by PKC in Calu-3 cells', Am J Physiol Lung Cell Mol Physiol, 280(4), pp. L739-47.

Litchfield, D.W. (2003) 'Protein kinase CK2: structure, regulation and role in cellular decisions of life and death', Biochem J, 369(Pt 1), pp. 1-15.

Liu, J.P., Powell, K.A., Sudhof, T.C. and Robinson, P.J. (1994) 'Dynamin I is a Ca(2+)-sensitive phospholipid-binding protein with very high affinity for protein kinase C', J Biol Chem, 269(33), pp. 21043-50.

Liu, X.Z., Ouyang, X.M., Xia, X.J., Zheng, J., Pandya, A., Li, F., Du, L.L., Welch, K.O., Petit, C., Smith, R.J., Webb, B.T., Yan, D., Arnos, K.S., Corey, D., Dallos, P., Nance, W.E. and Chen, Z.Y. (2003) 'Prestin, a cochlear motor protein, is defective in non-syndromic hearing loss', Hum Mol Genet, 12(10), pp. 1155-62.

Loffing, J., Moyer, B.D., Reynolds, D., Shmukler, B.E., Alper, S.L. and Stanton, B.A. (2000) 'Functional and molecular characterization of an anion exchanger in airway serous epithelial cells', Am J Physiol Cell Physiol, 279(4), pp. C1016-23.

Loriol, C., Dulong, S., Avella, M., Gabillat, N., Boulukos, K., Borgese, F. and Ehrenfeld, J. (2008) 'Characterization of SLC26A9, facilitation of Cl(-) transport by bicarbonate', Cell Physiol Biochem, 22(1-4), pp. 15-30.

Lukacs, G.L., Segal, G., Kartner, N., Grinstein, S. and Zhang, F. (1997) 'Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation', Biochem J, 328 (Pt 2), pp. 353-61.

Luz, S., Kongsuphol, P., Mendes, A.I., Romeiras, F., Sousa, M., Schreiber, R., Matos, P., Jordan, P., Mehta, A., Amaral, M.D., Kunzelmann, K. and Farinha, C.M. (2011) 'Contribution of casein kinase 2 and spleen tyrosine kinase to CFTR trafficking and protein kinase A-induced activity', Mol Cell Biol, 31(22), pp. 4392-404.

Luz, S.F. (2008) Novel insight into CFTR phosphorylation : implications

for its processing, trafficking and function. Msc thesis. Universidade de Lisboa

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C. and Kirchhausen, T. (2006) 'Dynasore, a cell-permeable inhibitor of dynamin', Dev Cell, 10(6), pp. 839-50.

Magno, A.L., Ward, B.K. and Ratajczak, T. (2011) 'The calcium-sensing receptor: a molecular perspective', Endocr Rev, 32(1), pp. 3-30.

Maiellaro, I., Lefkimmiatis, K., Moyer, M.P., Curci, S. and Hofer, A.M. (2012) 'Termination and activation of store-operated cyclic AMP production', J Cell Mol Med, 16(11), pp. 2715-25.

Maleth, J. and Hegyi, P. (2014) 'Calcium signaling in pancreatic ductal epithelial cells: an old friend and a nasty enemy', Cell Calcium, 55(6), pp. 337-45.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G.M. (2013) 'RNA-guided human genome engineering via Cas9', Science, 339(6121), pp. 823-6.

Mall, M., Bleich, M., Kuehr, J., Brandis, M., Greger, R. and Kunzelmann, K. (1999) 'CFTR-mediated inhibition of epithelial Na+ conductance in human colon is defective in cystic fibrosis', Am J Physiol, 277(3 Pt 1), pp. G709-16.

Mall, M., Grubb, B.R., Harkema, J.R., O'Neal, W.K. and Boucher, R.C. (2004) 'Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice', Nat Med, 10(5), pp. 487-93.

Marks, B. and McMahon, H.T. (1998) 'Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals', Curr Biol, 8(13), pp. 740-9.

Martinez-Anso, E., Castillo, J.E., Diez, J., Medina, J.F. and Prieto, J. (1994) 'Immunohistochemical detection of chloride/bicarbonate anion exchangers in human liver', Hepatology, 19(6), pp. 1400-6.

Mateu, E., Calafell, F., Ramos, M.D., Casals, T. and Bertranpetit, J. (2002) Can a place of origin of the main cystic fibrosis mutations be identified? 2001/11/20 edn.

Matsui, H., Grubb, B.R., Tarran, R., Randell, S.H., Gatzy, J.T., Davis, C.W. and Boucher, R.C. (1998) 'Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease', Cell, 95(7), pp. 1005-15.

Matsui, H., Verghese, M.W., Kesimer, M., Schwab, U.E., Randell, S.H., Sheehan, J.K., Grubb, B.R. and Boucher, R.C. (2005) 'Reduced three-dimensional motility in dehydrated airway mucus prevents neutrophil capture and killing bacteria on airway epithelial surfaces', J Immunol, 175(2), pp. 1090-9.

Mayerhofer, A., Fohr, K.J., Sterzik, K. and Gratzl, M. (1992) 'Carbachol increases intracellular free calcium concentrations in human granulosa-lutein cells', J Endocrinol, 135(1), pp. 153-9.

McKenna, R. and Frost, S.C. (2014) 'Overview of the carbonic anhydrase family', Subcell Biochem, 75, pp. 3-5.

Medina, J.F., Martinez, A., Vazquez, J.J. and Prieto, J. (1997) 'Decreased anion exchanger 2 immunoreactivity in the liver of patients with primary biliary cirrhosis', Hepatology, 25(1), pp. 12-7.

Medina, J.F., Recalde, S., Prieto, J., Lecanda, J., Saez, E., Funk, C.D., Vecino, P., van Roon, M.A., Ottenhoff, R., Bosma, P.J., Bakker, C.T. and Elferink, R.P. (2003) 'Anion exchanger 2 is essential for spermiogenesis in mice', Proc Natl Acad Sci U S A, 100(26), pp. 15847-52.

Mehta, A. (2008) 'Cystic fibrosis as a bowel cancer syndrome and the potential role of CK2', Mol Cell Biochem, 316(1-2), pp. 169-75.

Meyers, R.L. and Orlando, R.C. (1992) 'In vivo bicarbonate secretion by human esophagus', Gastroenterology, 103(4), pp. 1174-8.

Meyrick, B., Sturgess, J.M. and Reid, L. (1969) 'A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland', Thorax, 24(6), pp. 729-36.

Milara, J., Mata, M., Serrano, A., Peiro, T., Morcillo, E.J. and Cortijo, J. (2010) 'Extracellular calcium-sensing receptor mediates human bronchial epithelial wound repair', Biochem Pharmacol, 80(2), pp. 236-46.

Monterisi, S., Favia, M., Guerra, L., Cardone, R.A., Marzulli, D., Reshkin, S.J., Casavola, V. and Zaccolo, M. (2012) 'CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity', J Cell Sci, 125(Pt 5), pp. 1106-17.

Moran, O. and Zegarra-Moran, O. (2005) 'A quantitative description of the activation and inhibition of CFTR by potentiators: Genistein', FEBS Lett, 579(18), pp. 3979-83.

Morgans, M.E. and Trotter, W.R. (1958) 'Association of congenital deafness with goitre; the nature of the thyroid defect', Lancet, 1(7021), pp. 607-9.

Moseley, R.H., Hoglund, P., Wu, G.D., Silberg, D.G., Haila, S., de la Chapelle, A., Holmberg, C. and Kere, J. (1999) 'Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea', Am J Physiol, 276(1 Pt 1), pp. G185-92.

Mount, D.B. and Romero, M.F. (2004) 'The SLC26 gene family of multifunctional anion exchangers', Pflugers Arch, 447(5), pp. 710-21.

Muchekehu, R.W. and Quinton, P.M. (2010) 'A new role for bicarbonate secretion in cervico-uterine mucus release', J Physiol, 588(Pt 13), pp. 2329-42.

Nabi, I.R. and Le, P.U. (2003) 'Caveolae/raft-dependent endocytosis', J Cell Biol, 161(4), pp. 673-7.

Nadel, J.A. (1983) 'Neural control of airway submucosal gland secretion', Eur J Respir Dis Suppl, 128 (Pt 1), pp. 322-6.

Nadel, J.A., Davis, B. and Phipps, R.J. (1979) 'Control of mucus secretion and ion transport in airways', Annu Rev Physiol, 41, pp. 369-81.

Nagaki, M., Ishihara, H., Shimura, S., Sasaki, T., Takishima, T. and Shirato, K. (1994) 'Tachykinins induce a [Ca2+]i rise in the acinar cells of feline tracheal submucosal gland', Respir Physiol, 98(1), pp. 111-20.

Nagel, G., Hwang, T.C., Nastiuk, K.L., Nairn, A.C. and Gadsby, D.C. (1992) 'The protein kinase A-regulated cardiac Cl- channel resembles the cystic fibrosis transmembrane conductance regulator', Nature, 360(6399), pp. 81-4.

Nakagami, Y., Favoreto, S., Jr., Zhen, G., Park, S.W., Nguyenvu, L.T., Kuperman, D.A., Dolganov, G.M., Huang, X., Boushey, H.A., Avila, P.C. and Erle, D.J. (2008) 'The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model', J Immunol, 181(3), pp. 2203-10.

Namkung, W., Finkbeiner, W.E. and Verkman, A.S. (2010) 'CFTR-adenylyl cyclase I association responsible for UTP activation of CFTR in well-differentiated primary human bronchial cell cultures', Mol Biol Cell, 21(15), pp. 2639-48.

Namkung, W., Lee, J.A., Ahn, W., Han, W., Kwon, S.W., Ahn, D.S., Kim, K.H. and Lee, M.G. (2003) 'Ca2+ activates cystic fibrosis transmembrane conductance regulator- and Cl- -dependent HCO3 transport in pancreatic duct cells', J Biol Chem, 278(1), pp. 200-7.

Naren, A.P. and Kirk, K.L. (2000) 'CFTR Chloride Channels: Binding Partners and Regulatory Networks', News Physiol Sci, 15, pp. 57-61.

Nguyen, H.V., Stuart-Tilley, A., Alper, S.L. and Melvin, J.E. (2004) 'Cl(-)/HCO(3)(-) exchange is acetazolamide sensitive and activated by a muscarinic receptor-induced [Ca(2+)](i) increase in salivary acinar cells', Am J Physiol Gastrointest Liver Physiol, 286(2), pp. G312-20.

Norimatsu, Y., Ivetac, A., Alexander, C., O'Donnell, N., Frye, L., Sansom, M.S. and Dawson, D.C. (2012) 'Locating a plausible binding site for an open-channel

blocker, GlyH-101, in the pore of the cystic fibrosis transmembrane conductance regulator', Mol Pharmacol, 82(6), pp. 1042-55.

Novak, I. and Greger, R. (1988a) 'Electrophysiological study of transport systems in isolated perfused pancreatic ducts: properties of the basolateral membrane', Pflugers Arch, 411(1), pp. 58-68.

Novak, I. and Greger, R. (1988b) 'Properties of the luminal membrane of isolated perfused rat pancreatic ducts. Effect of cyclic AMP and blockers of chloride transport', Pflugers Arch, 411(5), pp. 546-53.

Nozik-Grayck, E., Piantadosi, C.A., van Adelsberg, J., Alper, S.L. and Huang, Y.C. (1997) 'Protection of perfused lung from oxidant injury by inhibitors of anion exchange', Am J Physiol, 273(2 Pt 1), pp. L296-304.

O'Day, D.H. (2003) 'CaMBOT: profiling and characterizing calmodulin-binding proteins', Cell Signal, 15(4), pp. 347-54.

Oestreich, E.A., Malik, S., Goonasekera, S.A., Blaxall, B.C., Kelley, G.G., Dirksen, R.T. and Smrcka, A.V. (2009) 'Epac and phospholipase Cepsilon regulate Ca2+ release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase II', J Biol Chem, 284(3), pp. 1514-22.

Ohana, E., Yang, D., Shcheynikov, N. and Muallem, S. (2009) 'Diverse transport modes by the solute carrier 26 family of anion transporters', J Physiol, 587(Pt 10), pp. 2179-85.

Orie, N.N., Thomas, A.M., Perrino, B.A., Tinker, A. and Clapp, L.H. (2009) 'Ca2+/calcineurin regulation of cloned vascular K ATP channels: crosstalk with the protein kinase A pathway', Br J Pharmacol, 157(4), pp. 554-64.

Pagano, M.A., Arrigoni, G., Marin, O., Sarno, S., Meggio, F., Treharne, K.J., Mehta, A. and Pinna, L.A. (2008) 'Modulation of protein kinase CK2 activity by fragments of CFTR encompassing F508 may reflect functional links with cystic fibrosis pathogenesis', Biochemistry, 47(30), pp. 7925-36.

Paradiso, A.M., Cheng, E.H. and Boucher, R.C. (1991) 'Effects of bradykinin on intracellular calcium regulation in human ciliated airway epithelium', Am J Physiol, 261(2 Pt 1), pp. L63-9.

Paradiso, A.M., Coakley, R.D. and Boucher, R.C. (2003) 'Polarized distribution of HCO3- transport in human normal and cystic fibrosis nasal epithelia', J Physiol, 548(Pt 1), pp. 203-18.

Pedemonte, N., Caci, E., Sondo, E., Caputo, A., Rhoden, K., Pfeffer, U., Di Candia, M., Bandettini, R., Ravazzolo, R., Zegarra-Moran, O. and Galietta, L.J. (2007) 'Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels', J Immunol, 178(8), pp. 5144-53.

Pena-Munzenmayer, G., Catalan, M.A., Kondo, Y., Jaramillo, Y., Liu, F., Shull, G.E. and Melvin, J.E. (2015) 'Ae4 (Slc4a9) Anion Exchanger Drives Cl- Uptake-dependent Fluid Secretion by Mouse Submandibular Gland Acinar Cells', J Biol Chem, 290(17), pp. 10677-88.

Petrovic, S., Barone, S., Xu, J., Conforti, L., Ma, L., Kujala, M., Kere, J. and Soleimani, M. (2004) 'SLC26A7: a basolateral Cl-/HCO3- exchanger specific to intercalated cells of the outer medullary collecting duct', Am J Physiol Renal Physiol, 286(1), pp. F161-9.

Petrovic, S., Ju, X., Barone, S., Seidler, U., Alper, S.L., Lohi, H., Kere, J. and Soleimani, M. (2003) 'Identification of a basolateral Cl-/HCO3- exchanger specific to gastric parietal cells', Am J Physiol Gastrointest Liver Physiol, 284(6), pp. G1093-103.

Pezzulo, A.A., Tang, X.X., Hoegger, M.J., Abou Alaiwa, M.H., Ramachandran, S., Moninger, T.O., Karp, P.H., Wohlford-Lenane, C.L., Haagsman, H.P., van Eijk, M., Banfi, B., Horswill, A.R., Stoltz, D.A., McCray, P.B., Welsh, M.J. and Zabner, J. (2012a) 'Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung', Nature, 487(7405), pp. 109-+.

Pezzulo, A.A., Tang, X.X., Hoegger, M.J., Alaiwa, M.H., Ramachandran, S., Moninger, T.O., Karp, P.H., Wohlford-Lenane, C.L., Haagsman, H.P., van Eijk,

M., Banfi, B., Horswill, A.R., Stoltz, D.A., McCray, P.B., Jr., Welsh, M.J. and Zabner, J. (2012b) 'Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung', Nature, 487(7405), pp. 109-13.

Phillips, J.E., Hey, J.A. and Corboz, M.R. (2003) 'Tachykinin NK3 and NK1 receptor activation elicits secretion from porcine airway submucosal glands', Br J Pharmacol, 138(1), pp. 254-60.

Pierre, F., Chua, P.C., O'Brien, S.E., Siddiqui-Jain, A., Bourbon, P., Haddach, M., Michaux, J., Nagasawa, J., Schwaebe, M.K., Stefan, E., Vialettes, A., Whitten, J.P., Chen, T.K., Darjania, L., Stansfield, R., Bliesath, J., Drygin, D., Ho, C., Omori, M., Proffitt, C., Streiner, N., Rice, W.G., Ryckman, D.M. and Anderes, K. (2011) 'Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer', Mol Cell Biochem, 356(1-2), pp. 37-43.

Poulsen, J.H., Fischer, H., Illek, B. and Machen, T.E. (1994) 'Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator', Proc Natl Acad Sci U S A, 91(12), pp. 5340-4.

Praefcke, G.J. and McMahon, H.T. (2004) 'The dynamin superfamily: universal membrane tubulation and fission molecules?', Nat Rev Mol Cell Biol, 5(2), pp. 133-47.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. and Lim, W.A. (2013) 'Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression', Cell, 152(5), pp. 1173-83.

Qiu, W., Laheri, A., Leung, S. and Guggino, S.E. (2000a) 'Hormones increase mRNA of cyclic-nucleotide-gated cation channels in airway epithelia', Pflugers Arch, 441(1), pp. 69-77.

Qiu, W., Lee, B., Lancaster, M., Xu, W., Leung, S. and Guggino, S.E. (2000b) 'Cyclic nucleotide-gated cation channels mediate sodium and calcium influx in rat colon', Am J Physiol Cell Physiol, 278(2), pp. C336-43.

Quadroni, M., James, P. and Carafoli, E. (1994) 'Isolation of phosphorylated calmodulin from rat liver and identification of the in vivo phosphorylation sites', J Biol Chem, 269(23), pp. 16116-22.

Quinton, P.M. (2001) 'The neglected ion: HCO3', Nat Med, 7(3), pp. 292-3.

Quinton, P.M. (2008) 'Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis', Lancet, 372(9636), pp. 415-7.

Quinton, P.M. (2010) 'Role of epithelial HCO3(-) transport in mucin secretion: lessons from cystic fibrosis', Am J Physiol Cell Physiol, 299(6), pp. C1222-33.

Racz, G.Z., Kittel, A., Riccardi, D., Case, R.M., Elliott, A.C. and Varga, G. (2002) 'Extracellular calcium sensing receptor in human pancreatic cells', Gut, 51(5), pp. 705-11.

Raghuram, V., Mak, D.O. and Foskett, J.K. (2001) 'Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction', Proc Natl Acad Sci U S A, 98(3), pp. 1300-5.

Rakonczay, Z., Jr., Hegyi, P., Hasegawa, M., Inoue, M., You, J., Iida, A., Ignath, I., Alton, E.W., Griesenbach, U., Ovari, G., Vag, J., Da Paula, A.C., Crawford, R.M., Varga, G., Amaral, M.D., Mehta, A., Lonovics, J., Argent, B.E. and Gray, M.A. (2008) 'CFTR gene transfer to human cystic fibrosis pancreatic duct cells using a Sendai virus vector', J Cell Physiol, 214(2), pp. 442-55.

Randell, S.H. and Boucher, R.C. (2006) 'Effective mucus clearance is essential for respiratory health', Am J Respir Cell Mol Biol, 35(1), pp. 20-8.

Rasmussen, J.E., Sheridan, J.T., Polk, W., Davies, C.M. and Tarran, R. (2014) 'Cigarette smoke-induced Ca2+ release leads to cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction', J Biol Chem, 289(11), pp. 7671-81.

Ratjen, F., Scott C. Bell, Steven M. Rowe, Christopher H. Goss, Quittner, A.L. and Bush6, a.A. (2015) 'Cystic fibrosis', Nature Reviews Disease Primers 1.

Raviv, U., Giasson, S., Kampf, N., Gohy, J.F., Jerome, R. and Klein, J. (2003) 'Lubrication by charged polymers', Nature, 425(6954), pp. 163-5.

Reid, L. (1960) 'Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis', Thorax, 15, pp. 132-41.

Ridley, C., Kouvatsos, N., Raynal, B.D., Howard, M., Collins, R.F., Desseyn, J.L., Jowitt, T.A., Baldock, C., Davis, C.W., Hardingham, T.E. and Thornton, D.J. (2014) 'Assembly of the respiratory mucin MUC5B: a new model for a gel-forming mucin', J Biol Chem, 289(23), pp. 16409-20.

Rillema, J.A. and Hill, M.A. (2003) 'Pendrin transporter carries out iodide uptake into MCF-7 human mammary cancer cells', Exp Biol Med (Maywood), 228(9), pp. 1078-82.

Riordan, J.R. (2008) 'CFTR function and prospects for therapy', Annu Rev Biochem, 77, pp. 701-26.

Romero, M.F., Chen, A.P., Parker, M.D. and Boron, W.F. (2013) 'The SLC4 family of bicarbonate (HCO(3)(-)) transporters', Mol Aspects Med, 34(2-3), pp. 159-82.

Romero, M.F., Fulton, C.M. and Boron, W.F. (2004) 'The SLC4 family of HCO 3 - transporters', Pflugers Arch, 447(5), pp. 495-509.

Roos, A. and Boron, W.F. (1981) 'Intracellular pH', Physiol Rev, 61(2), pp. 296-434.

Rossmann, H., Bachmann, O., Wang, Z., Shull, G.E., Obermaier, B., Stuart-Tilley, A., Alper, S.L. and Seidler, U. (2001) 'Differential expression and regulation of AE2 anion exchanger subtypes in rabbit parietal and mucous cells', J Physiol, 534(Pt 3), pp. 837-48.

Rowntree, R.K. and Harris, A. (2003) 'The phenotypic consequences of CFTR mutations', Ann Hum Genet, 67(Pt 5), pp. 471-85.

Royaux, I.E., Suzuki, K., Mori, A., Katoh, R., Everett, L.A., Kohn, L.D. and Green, E.D. (2000) 'Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells', Endocrinology, 141(2), pp. 839-45.

Royaux, I.E., Wall, S.M., Karniski, L.P., Everett, L.A., Suzuki, K., Knepper, M.A. and Green, E.D. (2001) 'Pendrin, encoded by the Pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion', Proc Natl Acad Sci U S A, 98(7), pp. 4221-6.

Ruetz, S., Lindsey, A.E., Ward, C.L. and Kopito, R.R. (1993) 'Functional activation of plasma membrane anion exchangers occurs in a pre-Golgi compartment', J Cell Biol, 121(1), pp. 37-48.

Salinas, D., Haggie, P.M., Thiagarajah, J.R., Song, Y., Rosbe, K., Finkbeiner, W.E., Nielson, D.W. and Verkman, A.S. (2005) 'Submucosal gland dysfunction as a primary defect in cystic fibrosis', FASEB J, 19(3), pp. 431-3.

Salonikidis, P.S., Zeug, A., Kobe, F., Ponimaskin, E. and Richter, D.W. (2008) 'Quantitative measurement of cAMP concentration using an exchange protein directly activated by a cAMP-based FRET-sensor', Biophys J, 95(11), pp. 5412-23.

Salvi, M., Sarno, S., Marin, O., Meggio, F., Itarte, E. and Pinna, L.A. (2006) 'Discrimination between the activity of protein kinase CK2 holoenzyme and its catalytic subunits', FEBS Lett, 580(16), pp. 3948-52.

Sander, T., Toliat, M.R., Heils, A., Leschik, G., Becker, C., Ruschendorf, F., Rohde, K., Mundlos, S. and Nurnberg, P. (2002) 'Association of the 867Asp variant of the human anion exchanger 3 gene with common subtypes of idiopathic generalized epilepsy', Epilepsy Res, 51(3), pp. 249-55.

Santana, L.F., Chase, E.G., Votaw, V.S., Nelson, M.T. and Greven, R. (2002) 'Functional coupling of calcineurin and protein kinase A in mouse ventricular myocytes', J Physiol, 544(Pt 1), pp. 57-69.

Sarno, S., Ruzzene, M., Frascella, P., Pagano, M.A., Meggio, F., Zambon, A., Mazzorana, M., Di Maira, G., Lucchini, V. and Pinna, L.A. (2005) 'Development and exploitation of CK2 inhibitors', Mol Cell Biochem, 274(1-2), pp. 69-76.

Satoh H, S.M., Shukunami C, Iyama K, Negoro T, Hiraki and Yadav, D. (1998) 'Functional analysis of diastrophic dysplasia sulfate transporter. Its involvement

in growth regulation of chondrocytes mediated by sulfated proteoglycans.', J Biol Chem 273, pp. 12307-12315.

Schmidt, M., Dekker, F.J. and Maarsingh, H. (2013) 'Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions', Pharmacol Rev, 65(2), pp. 670-709.

Schofield, A.E., Reardon, D.M. and Tanner, M.J. (1992) 'Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells', Nature, 355(6363), pp. 836-8.

Scott, D.A. and Karniski, L.P. (2000) 'Human pendrin expressed in Xenopus laevis oocytes mediates chloride/formate exchange', Am J Physiol Cell Physiol, 278(1), pp. C207-11.

Scott DA, K.L. ((2000)) 'Human pendrin expressed in Xenopus laevis oocytes mediates chloride/formate exchange.', Am J Physiol 278, pp. C207-21.

Scott, D.A., Wang, R., Kreman, T.M., Sheffield, V.C. and Karniski, L.P. (1999) 'The Pendred syndrome gene encodes a chloride-iodide transport protein', Nat Genet, 21(4), pp. 440-3.

Scratcherd, T. and Case, R.M. (1973) 'The secretion of electrolytes by the pancreas', Am J Clin Nutr, 26((3)), pp. 326-39.

Sehgal, A., Presente, A. and Engelhardt, J.F. (1996) 'Developmental expression patterns of CFTR in ferret tracheal surface airway and submucosal gland epithelia', Am J Respir Cell Mol Biol, 15(1), pp. 122-31.

Seidler, U., Bachmann, O., Jacob, P., Christiani, S., Blumenstein, I. and Rossmann, H. (2001) 'Na+/HCO3- cotransport in normal and cystic fibrosis intestine', JOP, 2(4 Suppl), pp. 247-56.

Seidler, U., Blumenstein, I., Kretz, A., Viellard-Baron, D., Rossmann, H., Colledge, W.H., Evans, M., Ratcliff, R. and Gregor, M. (1997) 'A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca(2+)-dependent HCO3- secretion', J Physiol, 505 (Pt 2), pp. 411-23.

Senatore, A., Boone, A.N. and Spafford, J.D. (2011) 'Optimized transfection strategy for expression and electrophysiological recording of recombinant voltage-gated ion channels in HEK-293T cells', J Vis Exp, (47).

Shan, J., Huang, J., Liao, J., Robert, R. and Hanrahan, J.W. (2011) 'Anion secretion by a model epithelium: more lessons from Calu-3', Acta Physiol (Oxf), 202(3), pp. 523-31.

Shan, J., Liao, J., Huang, J., Robert, R., Palmer, M.L., Fahrenkrug, S.C., O'Grady, S.M. and Hanrahan, J.W. (2012) 'Bicarbonate-dependent chloride transport drives fluid secretion by the human airway epithelial cell line Calu-3', J Physiol, 590(Pt 21), pp. 5273-97.

Shcheynikov, N., Yang, D., Wang, Y., Zeng, W., Karniski, L.P., So, I., Wall, S.M. and Muallem, S. (2008) 'The Slc26a4 transporter functions as an electroneutral Cl-/I-/HCO3- exchanger: role of Slc26a4 and Slc26a6 in I- and HCO3- secretion and in regulation of CFTR in the parotid duct', J Physiol, 586(16), pp. 3813-24.

Shen, B.Q., Finkbeiner, W.E., Wine, J.J., Mrsny, R.J. and Widdicombe, J.H. (1994) 'Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion', Am J Physiol, 266(5 Pt 1), pp. L493-501.

Shoji, K., Ohashi, K., Sampei, K., Oikawa, M. and Mizuno, K. (2012) 'Cytochalasin D acts as an inhibitor of the actin-cofilin interaction', Biochem Biophys Res Commun, 424(1), pp. 52-7.

Singh, P.K., Jia, H.P., Wiles, K., Hesselberth, J., Liu, L., Conway, B.A., Greenberg, E.P., Valore, E.V., Welsh, M.J., Ganz, T., Tack, B.F. and McCray, P.B., Jr. (1998) 'Production of beta-defensins by human airway epithelia', Proc Natl Acad Sci U S A, 95(25), pp. 14961-6.

Smith, J.J. and Welsh, M.J. (1992) 'cAMP stimulates bicarbonate secretion across normal, but not cystic fibrosis airway epithelia', J Clin Invest, 89(4), pp. 1148-53.

Soderling, T.R. (1999) 'The Ca-calmodulin-dependent protein kinase cascade', Trends Biochem Sci, 24(6), pp. 232-6.

Soleimani, M., Greeley, T., Petrovic, S., Wang, Z., Amlal, H., Kopp, P. and Burnham, C.E. (2001) 'Pendrin: an apical Cl-/OH-/HCO3- exchanger in the kidney cortex', Am J Physiol Renal Physiol, 280(2), pp. F356-64.

Soleimani M, G.T., Petrovic S, Wang Z, Amlal H, Kopp and P, B.C. ((2001)) ' Pendrin: an apical Cl /OH /HCO3 exchanger in the kidney cortex', Am J Physiol, 280, pp. 356-364.

Sterling, D., Alvarez, B.V. and Casey, J.R. (2002) 'The extracellular component of a transport metabolon. Extracellular loop 4 of the human AE1 Cl-/HCO3- exchanger binds carbonic anhydrase IV', J Biol Chem, 277(28), pp. 25239-46.

Steward, M.C., Ishiguro, H. and Case, R.M. (2005) 'Mechanisms of bicarbonate secretion in the pancreatic duct', Annu Rev Physiol, 67, pp. 377-409.

Stewart, A.K., Chernova, M.N., Shmukler, B.E., Wilhelm, S. and Alper, S.L. (2002) 'Regulation of AE2-mediated Cl- transport by intracellular or by extracellular pH requires highly conserved amino acid residues of the AE2 NH2-terminal cytoplasmic domain', J Gen Physiol, 120(5), pp. 707-22.

Stewart, A.K., Kurschat, C.E., Vaughan-Jones, R.D., Shmukler, B.E. and Alper, S.L. (2007) 'Acute regulation of mouse AE2 anion exchanger requires isoform-specific amino acid residues from most of the transmembrane domain', J Physiol, 584(Pt 1), pp. 59-73.

Stewart, A.K., Shmukler, B.E., Vandorpe, D.H., Reimold, F., Heneghan, J.F., Nakakuki, M., Akhavein, A., Ko, S., Ishiguro, H. and Alper, S.L. (2011) 'SLC26 anion exchangers of guinea pig pancreatic duct: molecular cloning and functional characterization', Am J Physiol Cell Physiol, 301(2), pp. C289-303.

Stewart, A.K., Yamamoto, A., Nakakuki, M., Kondo, T., Alper, S.L. and Ishiguro, H. (2009) 'Functional coupling of apical Cl-/HCO3- exchange with CFTR in stimulated HCO3- secretion by guinea pig interlobular pancreatic duct', Am J Physiol Gastrointest Liver Physiol, 296(6), pp. G1307-17.

Stuart-Tilley, A., Sardet, C., Pouyssegur, J., Schwartz, M.A., Brown, D. and Alper, S.L. (1994) 'Immunolocalization of anion exchanger AE2 and cation

exchanger NHE-1 in distinct adjacent cells of gastric mucosa', Am J Physiol, 266(2 Pt 1), pp. C559-68.

Stuenkel, E.L., Machen, T.E. and Williams, J.A. (1988) 'pH regulatory mechanisms in rat pancreatic ductal cells', Am J Physiol, 254(6 Pt 1), pp. G925-30.

Sun, F., Hug, M.J., Lewarchik, C.M., Yun, C.H., Bradbury, N.A. and Frizzell, R.A. (2000) 'E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells', J Biol Chem, 275(38), pp. 29539-46.

Superti-Furga, A., Hastbacka, J., Wilcox, W.R., Cohn, D.H., van der Harten, H.J., Rossi, A., Blau, N., Rimoin, D.L., Steinmann, B., Lander, E.S. and Gitzelmann, R. (1996) 'Achondrogenesis type IB is caused by mutations in the diastrophic dysplasia sulphate transporter gene', Nat Genet, 12(1), pp. 100-2.

Suzuki, K., Royaux, I.E., Everett, L.A., Mori-Aoki, A., Suzuki, S., Nakamura, K., Sakai, T., Katoh, R., Toda, S., Green, E.D. and Kohn, L.D. (2002) 'Expression of PDS/Pds, the Pendred syndrome gene, in endometrium', J Clin Endocrinol Metab, 87(2), p. 938.

Swulius, M.T. and Waxham, M.N. (2008) 'Ca(2+)/calmodulin-dependent protein kinases', Cell Mol Life Sci, 65(17), pp. 2637-57.

Szkotak, A.J., Man, S.F. and Duszyk, M. (2003) 'The role of the basolateral outwardly rectifying chloride channel in human airway epithelial anion secretion', Am J Respir Cell Mol Biol, 29(6), pp. 710-20.

Tang, H.Y., Xia, A., Oghalai, J.S., Pereira, F.A. and Alford, R.L. (2005) 'High frequency of the IVS2-2A>G DNA sequence variation in SLC26A5, encoding the cochlear motor protein prestin, precludes its involvement in hereditary hearing loss', BMC Med Genet, 6, p. 30.

Tarran, R., Trout, L., Donaldson, S.H. and Boucher, R.C. (2006) 'Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia', J Gen Physiol, 127(5), pp. 591-604.

Taylor, C.J. and Aswani, N. (2002) 'The pancreas in cystic fibrosis', Paediatr Respir Rev, 3(1), pp. 77-81.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) 'Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase', Proc Natl Acad Sci U S A, 87(7), pp. 2466-70.

Thiagarajah, J.R., Song, Y., Haggie, P.M. and Verkman, A.S. (2004) 'A small molecule CFTR inhibitor produces cystic fibrosis-like submucosal gland fluid secretions in normal airways', FASEB J, 18(7), pp. 875-7.

Tian, Y., Kongsuphol, P., Hug, M., Ousingsawat, J., Witzgall, R., Schreiber, R. and Kunzelmann, K. (2011) 'Calmodulin-dependent activation of the epithelial calcium-dependent chloride channel TMEM16A', FASEB J, 25(3), pp. 1058-68.

Tian, Y. and Laychock, S.G. (2001) 'Protein kinase C and calcium regulation of adenylyl cyclase in isolated rat pancreatic islets', Diabetes, 50(11), pp. 2505-13.

Tokumitsu, H., Wayman, G.A., Muramatsu, M. and Soderling, T.R. (1997) 'Calcium/calmodulin-dependent protein kinase kinase: identification of regulatory domains', Biochemistry, 36(42), pp. 12823-7.

Tom-Moy, M., Basbaum, C.B. and Nadel, J.A. (1983) 'Localization and release of lysozyme from ferret trachea: effects of adrenergic and cholinergic drugs', Cell Tissue Res, 228(3), pp. 549-62.

Toure, A., Lhuillier, P., Gossen, J.A., Kuil, C.W., Lhote, D., Jegou, B., Escalier, D. and Gacon, G. (2007) 'The testis anion transporter 1 (Slc26a8) is required for sperm terminal differentiation and male fertility in the mouse', Hum Mol Genet, 16(15), pp. 1783-93.

Treharne, K.J., Xu, Z., Chen, J.H., Best, O.G., Cassidy, D.M., Gruenert, D.C., Hegyi, P., Gray, M.A., Sheppard, D.N., Kunzelmann, K. and Mehta, A. (2009) 'Inhibition of protein kinase CK2 closes the CFTR Cl channel, but has no effect on the cystic fibrosis mutant deltaF508-CFTR', Cell Physiol Biochem, 24(5-6), pp. 347-60.

Tsalkova, T., Mei, F.C. and Cheng, X. (2012) 'A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists', PLoS One, 7(1), p. e30441.

Tu, J., Inthavong, K. and Ahmadi, G. (2013) Computational Fluid and Particle Dynamics in the Human Respiratory System.

Biological and Medical Physics, Biomedical Engineering. New York.

Turi, J.L., Jaspers, I., Dailey, L.A., Madden, M.C., Brighton, L.E., Carter, J.D., Nozik-Grayck, E., Piantadosi, C.A. and Ghio, A.J. (2002) 'Oxidative stress activates anion exchange protein 2 and AP-1 in airway epithelial cells', Am J Physiol Lung Cell Mol Physiol, 283(4), pp. L791-8.

Turi, S., Friedman, A., Bereczki, C., Papp, F., Kovacs, J., Karg, E. and Nemeth, I. (2003) 'Oxidative stress in juvenile essential hypertension', J Hypertens, 21(1), pp. 145-52.

Usui, T., Seki, G., Amano, S., Oshika, T., Miyata, K., Kunimi, M., Taniguchi, S., Uwatoko, S., Fujita, T. and Araie, M. (1999) 'Functional and molecular evidence for Na(+)-HCO3- cotransporter in human corneal endothelial cells', Pflugers Arch, 438(4), pp. 458-62.

van Aubel, R.A., Smeets, P.H., Peters, J.G., Bindels, R.J. and Russel, F.G. (2002) 'The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP', J Am Soc Nephrol, 13(3), pp. 595-603.

Van Eynde, A., Beullens, M., Stalmans, W. and Bollen, M. (1994) 'Full activation of a nuclear species of protein phosphatase-1 by phosphorylation with protein kinase A and casein kinase-2', Biochem J, 297 (Pt 3), pp. 447-9.

Venerando, A., Cesaro, L., Marin, O., Donella-Deana, A. and Pinna, L.A. (2014) 'A "SYDE" effect of hierarchical phosphorylation: possible relevance to the cystic fibrosis basic defect', Cell Mol Life Sci, 71(12), pp. 2193-6.

Vergani, P., Lockless, S.W., Nairn, A.C. and Gadsby, D.C. (2005) 'CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains', Nature, 433(7028), pp. 876-80.

Verkman, A.S., Song, Y. and Thiagarajah, J.R. (2003) 'Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease', Am J Physiol Cell Physiol, 284(1), pp. C2-15.

Verlander, J.W., Madsen, K.M., Low, P.S., Allen, D.P. and Tisher, C.C. (1988) 'Immunocytochemical localization of band 3 protein in the rat collecting duct', Am J Physiol, 255(1 Pt 2), pp. F115-25.

Vince, J.W. and Reithmeier, R.A. (2000) 'Identification of the carbonic anhydrase II binding site in the Cl(-)/HCO(3)(-) anion exchanger AE1', Biochemistry, 39(18), pp. 5527-33.

Wall, S.M. (2006) 'The renal physiology of pendrin (SLC26A4) and its role in hypertension', Novartis Found Symp, 273, pp. 231-9; discussion 239-43, 261-4.

Wang, H. and Brautigan, D.L. (2006) 'Peptide microarray analysis of substrate specificity of the transmembrane Ser/Thr kinase KPI-2 reveals reactivity with cystic fibrosis transmembrane conductance regulator and phosphorylase', Mol Cell Proteomics, 5(11), pp. 2124-30.

Wang, S., Yue, H., Derin, R.B., Guggino, W.B. and Li, M. (2000) 'Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity', Cell, 103(1), pp. 169-79.

Wang, Y., Soyombo, A.A., Shcheynikov, N., Zeng, W., Dorwart, M., Marino, C.R., Thomas, P.J. and Muallem, S. (2006) 'Slc26a6 regulates CFTR activity in vivo to determine pancreatic duct HCO3- secretion: relevance to cystic fibrosis', EMBO J, 25(21), pp. 5049-57.

Wang, Z., Petrovic, S., Mann, E. and Soleimani, M. (2002) 'Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine', Am J Physiol Gastrointest Liver Physiol, 282(3), pp. G573-9.

Wangemann, P., Nakaya, K., Wu, T., Maganti, R.J., Itza, E.M., Sanneman, J.D., Harbidge, D.G., Billings, S. and Marcus, D.C. (2007) 'Loss of cochlear HCO3- secretion causes deafness via endolymphatic acidification and inhibition of Ca2+ reabsorption in a Pendred syndrome mouse model', Am J Physiol Renal Physiol, 292(5), pp. F1345-53.

Wanner, A., Salathe, M. and O'Riordan, T.G. (1996) 'Mucociliary clearance in the airways', American Journal of Respiratory and Critical Care Medicine, 154, pp. 1868–1902.

Ward, C.L. and Kopito, R.R. (1994) 'Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins', J Biol Chem, 269(41), pp. 25710-8.

Ward, C.L., Omura, S. and Kopito, R.R. (1995) 'Degradation of CFTR by the ubiquitin-proteasome pathway', Cell, 83(1), pp. 121-7.

Ward, D. (2004) 'Calcium receptor-mediated intracellular signalling', Cell Calcium, 35(3), p. 217.28.

Welsh, M.J. and Smith, A.E. (1995) 'Cystic fibrosis', Sci Am, 273(6), pp. 52-9.

Welsh, M.J. and Smith, J.J. (2001) 'cAMP stimulation of HCO3- secretion across airway epithelia', JOP, 2(4 Suppl), pp. 291-3.

Welsh, M.J., Tsui, L. -C., Boat, T. F., & Beaudet, A. L. ((1995)), pp. (pp. 3799–3876).

Widdicombe, J.H. (2002a) 'Regulation of the depth and composition of airway surface liquid', J Anat, 201(4), pp. 313-8.

Widdicombe, J.H. (2002b) 'Volume of airway surface liquid in health and disease', Am J Respir Crit Care Med, 165(11), p. 1566.

Widdicombe, J.H. and Wine, J.J. (2015) 'Airway Gland Structure and Function', Physiol Rev, 95(4), pp. 1241-319.

Willoughby, D. and Cooper, D.M. (2007) 'Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains', Physiol Rev, 87(3), pp. 965-1010.

Willumsen, N.J. and Boucher, R.C. (1989) 'Shunt resistance and ion permeabilities in normal and cystic fibrosis airway epithelia', Am J Physiol, 256(5 Pt 1), pp. C1054-63.

Wine, J.J. (2007) 'Parasympathetic control of airway submucosal glands: central reflexes and the airway intrinsic nervous system', Auton Neurosci, 133(1), pp. 35-54.

Wine, J.J. and Joo, N.S. (2004) 'Submucosal glands and airway defense', Proc Am Thorac Soc, 1(1), pp. 47-53.

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K.C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J.R., Randell, S., Boucher, R.C. and Doring, G. (2002) 'Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients', J Clin Invest, 109(3), pp. 317-25.

Wyatt, T.A., Forget, M.A., Adams, J.M. and Sisson, J.H. (2005) 'Both cAMP and cGMP are required for maximal ciliary beat stimulation in a cell-free model of bovine ciliary axonemes', Am J Physiol Lung Cell Mol Physiol, 288(3), pp. L546-51.

Xiao, F., Li, J., Singh, A.K., Riederer, B., Wang, J., Sultan, A., Park, H., Lee, M.G., Lamprecht, G., Scholte, B.J., De Jonge, H.R. and Seidler, U. (2012) 'Rescue of epithelial HCO3- secretion in murine intestine by apical membrane expression of the cystic fibrosis transmembrane conductance regulator mutant F508del', J Physiol, 590(Pt 21), pp. 5317-34.

Xie, Q., Welch, R., Mercado, A., Romero, M.F. and Mount, D.B. (2002) 'Molecular characterization of the murine Slc26a6 anion exchanger: functional comparison with Slc26a1', Am J Physiol Renal Physiol, 283(4), pp. F826-38.

Xingshen Sun, H.S., John T. Fisher, Ziying Yan,Xiaoming Liu, Hyung-Ju Cho, Nam Soo Joo,Yulong Zhang, Weihong Zhou, Yaling Yi, Joann M. Kinyon, Diana C. Lei-Butters, Michelle A. Griffin, Paul Naumann, Meihui Luo, Jill

Ascher, Kai Wang, Timothy Frana, Jeffrey J. Wine, David K. Meyerholz, and John F. Engelhardt (2010) 'Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis ', American Society for Clinical Investigation, 120 ((9):), pp. 3149–3160

Xu, W.M., Chen, J., Chen, H., Diao, R.Y., Fok, K.L., Dong, J.D., Sun, T.T., Chen, W.Y., Yu, M.K., Zhang, X.H., Tsang, L.L., Lau, A., Shi, Q.X., Shi, Q.H., Huang, P.B. and Chan, H.C. (2011) 'Defective CFTR-dependent CREB activation results in impaired spermatogenesis and azoospermia', PLoS One, 6(5), p. e19120.

Yacoub, D., Theoret, J.F., Villeneuve, L., Abou-Saleh, H., Mourad, W., Allen, B.G. and Merhi, Y. (2006) 'Essential role of protein kinase C delta in platelet signaling, alpha IIb beta 3 activation, and thromboxane A2 release', J Biol Chem, 281(40), pp. 30024-35.

Yang, D., Shcheynikov, N., Zeng, W., Ohana, E., So, I., Ando, H., Mizutani, A., Mikoshiba, K. and Muallem, S. (2009) 'IRBIT coordinates epithelial fluid and HCO3- secretion by stimulating the transporters pNBC1 and CFTR in the murine pancreatic duct', J Clin Invest, 119(1), pp. 193-202.

Yannoukakos, D., Stuart-Tilley, A., Fernandez, H.A., Fey, P., Duyk, G. and Alper, S.L. (1994) 'Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart', Circ Res, 75(4), pp. 603-14.

Young, A., Gentzsch, M., Abban, C.Y., Jia, Y., Meneses, P.I., Bridges, R.J. and Bradbury, N.A. (2009) 'Dynasore inhibits removal of wild-type and DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) from the plasma membrane', Biochem J, 421(3), pp. 377-85.

Zabner, J., Smith, J.J., Karp, P.H., Widdicombe, J.H. and Welsh, M.J. (1998) 'Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro', Mol Cell, 2(3), pp. 397-403.

Zhao, C., Wang, I. and Lehrer, R.I. (1996) 'Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells', FEBS Lett, 396(2-3), pp. 319-22.

Zhou, J.J., Li, M.S., Qi, J. and Linsdell, P. (2010) 'Regulation of conductance by the number of fixed positive charges in the intracellular vestibule of the CFTR chloride channel pore', J Gen Physiol, 135(3), pp. 229-45.

Zufall, F., Firestein, S. and Shepherd, G.M. (1994) 'Cyclic nucleotide-gated ion channels and sensory transduction in olfactory receptor neurons', Annu Rev Biophys Biomol Struct, 23, pp. 577-607.