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Aberrant CFTR-dependent HCO_3^- transport inmutations associated with cystic fibrosis

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Abstract

Cystic fibrosis (CF) is a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). Initially, Cl^- conductance in the sweat duct was discovered to be impaired in CF[1], a finding that has been extended to all CFTR-expressing cells[2–4]. Subsequent cloning of the gene[5,6] showed that CFTR functions as a cyclic-AMP-regulated Cl^- channel[7]; and some CF-causing mutations inhibit CFTR Cl^- channel activity[2–4,8]. The identification of additional CF-causing mutants with normal Cl^- channel activity indicates, however, that other CFTR-dependent processes contribute to the disease. Indeed, CFTR regulates other transporters[3,4], including Cl^--coupled HCO_3^- transport[9,10]. Alkaline fluids are secreted by normal tissues, whereas acidic fluids are secreted by mutant CFTR-expressing tissues[11], indicating the importance of this activity. HCO_3^- and pH affect mucin viscosity[12,13] and bacterial binding[14,15]. We have examined Cl^--coupled HCO_3^- transport by CFTR mutants that retain substantial or normal Cl^- channel activity. Here we show that mutants reported to be associated with CF with pancreatic insufficiency do not support HCO_3^- transport, and those associated with pancreatic sufficiency show reduced HCO_3^- transport. Our findings demonstrate the importance of HCO_3^- transport in the function of secretory epithelia and in CF.

We compared the activity of wild-type CFTR to that of 17 disease-causing mutants. Several criteria were used for selection of mutants and the methods to evaluate Cl^- and HCO_3^- transport. All mutants selected code for properly processed proteins[16–21]. The macroscopic Cl^- current and single-channel activity of the mutants have been shown to be significant, normal or even elevated[16–21]. The mutants are from all cytoplasmic domains of CFTR and have been reported to be associated with CF with pancreatic sufficiency or pancreatic insufficiency. Table 1 in the Supplementary Information lists the mutants and the reported clinical status of the patients. Two of the mutations, I148T and R117H, are relatively common, and thus the clinical data are solid. Initially we analysed these mutations to establish the relationship between the CF phenotype and HCO_3^- transport.

CFTR-dependent HCO_3^- transport is dependent on Cl^- transport and is not affected by the membrane potential[9,10], suggesting that it is an electroneutral process that can only be

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followed by measuring intracellular HCO_3^- concentration. Hence, changes in HCO_3^- concentration were evaluated from changes in intracellular pH (pH_i) (see refs 9, 10). To evaluate Cl^- transport under the same conditions, we monitored intracellular chloride concentrations ($[Cl^-]_i$) with *N*-6(6-methoxyquinolyl) acetoethyl ester (MQAE). To validate the ability of this procedure to accurately report Cl^- channel activity, we determined the correlation between expression efficiency (green fluorescent protein (GFP) fluorescence), Cl^- current and changes in $[Cl^-]_i$ in cells transfected with CFTR, CFTR(I148T) and CFTR(R117H). Figure 1a–h shows that similar results are reported by measurement of Cl^- current and $[Cl^-]_i$ for CFTR and the mutants. Therefore, the Cl^- transport capacity of all other CFTR mutants was evaluated from changes in $[Cl^-]_i$.

Figure 1e–l shows the experimental protocols used to measure the effect of the I148T and R117H mutations on Cl^- and HCO_3^- transport. The I148T mutant is normally processed and mediates macroscopic Cl^- current (Fig. 1d; ref. 16), single-channel properties[16] and Cl^- fluxes (Fig. 1h) indistinguishable from those of wild-type CFTR, but is associated with CF with pancreatic insufficiency. Replacing external Cl^- with NO_3^- in non-stimulated cells caused a slow Cl^- efflux in cells expressing CFTR or the I148T mutant. Stimulation of CFTR-expressing cells with forskolin resulted in a change in $[Cl^-]_i$ at a rate of 0.36±0.04 (n = 6) mM s^{-1}. Similar rates were measured for the I148T, G178R, A1067T, G1244E, S1255P and G1349D mutants (see Fig. 3 for location of these mutations in CFTR), all of which are associated with CF with pancreatic insufficiency. The rates of changes in $[Cl^-]_i$ measured here, and the macroscopic Cl^- currents from previous studies are listed in Table 1 in the Supplementary Information. In all cases, the presence of forskolin-stimulated Cl^- fluxes confirmed that the CFTR mutants matured and travelled properly to the plasma membrane.

The similarity of Cl^- transport by CFTR and the mutants implies that aberrant Cl^- transport cannot alone account for these severe forms of CF. Stimulation of CFTR markedly increases Cl^--coupled HCO_3^- transport (Fig. 1i) by an unknown mechanism. Several studies have reported that CFTR functions as both a Cl^- and a HCO_3^- channel[22–24], which might account for the fluxes reported here. In a continuing work, however, we confirmed that Cl^- efflux is obligatory for CFTR-dependent HCO_3^- influx, and that depolarizing the cells does not inhibit the transport by CFTR and the mutants. Therefore, we consider the CFTR-dependent transport as Cl^--coupled HCO_3^- transport. Remarkably, all mutations associated with CF with pancreatic insufficiency tested here, without exception, eliminate the ability of CFTR to support HCO_3^- transport. Results for the I148T mutant are shown in Fig. 1, and the averaged results for all mutants are listed in Table 1 in the Supplementary Information.

We next analysed Cl^- and HCO_3^- transport in CFTR mutants associated with pancreatic sufficiency. The results obtained with the R117H mutation are illustrated in Fig. 1. In sharp contrast with the results obtained with the mutants associated with CF with pancreatic insufficiency, and in agreement with previous reports[25], the R117H mutation reduced Cl^- current and the MQAE response by about 70%. By contrast, the R117H mutation reduced the ability of CFTR to support HCO_3^- transport by only 37%. The E193K, D648V, H949Yand R1070Q mutants, all associated with CF with pancreatic sufficiency, had no effect on Cl^- transport but reduced HCO_3^- transport by 50–65%. Two sets of particularly interesting mutants are G551D and G551S and H620Q and A800G. G551S, which is associated with CF with pancreatic sufficiency, had no effect on Cl^- transport but reduced HCO_3^- transport by 59% (Fig. 2). As reported previously[17,18,26], G551D reduced Cl^- transport to 53% of the control value. By contrast, this mutation, which is associated with CF with pancreatic insufficiency, completely inhibited CFTR-dependent HCO_3^- transport (Fig. 2b, d).

When expressed in oocytes, the H620Q and A800G mutants increased the macroscopic Cl^- current about threefold and the channel open probability by 150–180% (ref. 19). We confirmed these findings, as Cl^- transport by these mutants is about 2.4-fold higher than that by CFTR (Fig. 2e, f). Despite these markedly enhanced Cl^- fluxes, the A800G mutation, which causes CF with pancreatic sufficiency, only stimulated HCO_3^- transport to 75% of the wild-type CFTR value. The H620Q mutant, found in one CF patient with pancreatic insufficiency, only stimulated HCO_3^- transport about 13% as effectively as did CFTR. The enhanced Cl^- transport by the two mutants in heterologous systems seems to be caused by increased expression of the protein in the plasma membrane[19]. If this enhanced expression is not maintained *in vivo*, the mutations will further compromise HCO_3^- transport to exacerbate the pancreatic phenotypes. In this respect, the R1070Q mutation, in the few documented cases, has been found to be associated with CF with either pancreatic sufficiency or pancreatic insufficiency. Our results of substantial HCO_3^- transport by this mutant would predict a phenotype of CF with pancreatic sufficiency.

To account for variable Cl^- transport and possible variable expression of the proteins in the plasma membrane, we normalized the capacity of the CFTR mutants to transport HCO_3^- with respect to their capacity to transport Cl^-. Figure 3 shows the correlation between the reported pancreatic status of the patients and the HCO_3^-:Cl^- transport ratio. When the ratio measured with CFTR is taken as 1, all mutants associated with CF with pancreatic insufficiency show an HCO_3^-:Cl^- transport ratio of less than 0.1. By comparison, all mutations associated with CF with pancreatic sufficiency show an HCO_3^-:Cl^- transport ratio of between 0.31 and 0.46. Notably, although a few of these mutants exhibit altered channel gating or reduced processing, in all of the mutants CFTR-dependent HCO_3^- transport is affected more than CFTR-mediated Cl^- transport. In this regard, although the R117H mutation markedly reduces Cl^- channel activity and Cl^- transport (Fig. 1), it is associated with a mild form of CF, probably because it supports substantial HCO_3^- transport (Fig. 1). Consequently, the CFTR-dependent HCO_3^-:Cl^- transport ratio seems to correlate well with the reported pancreatic function of CF patients.

Cl^- transport across the apical membrane of secretory epithelia is the rate-limiting step in fluid and electrolyte transport[1–4,8], high-lighting the importance of the Cl^- channel function of CFTR. The existence of CF-causing CFTR mutations that support normal or even elevated Cl^- transport indicates, however, that factors other than abnormal Cl^- transport can also lead to CF. An alternative view of the role of CFTR in epithelial transport was introduced with the finding that CFTR can affect the transport of other ions[27–30], including HCO_3^- (refs 9, 10, 22–24). The aberrant HCO_3^- transport by the CF-causing mutations examined here indicates that HCO_3^- transport by CFTR-expressing epithelia is critical for normal tissue physiology, and that impaired HCO_3^- transport is sufficient to derange pancreatic function even in the presence of Cl^- channel activity. Acidic fluid secretion by CFTR-expressing tissues in the disease state may lead to precipitation of mucins and plugging of ductal systems[12,13], and facilitate bacterial infection through binding to the precipitated mucins[14,15]. In the special case of the pancreas, acidic pH would lead to premature activation of digestive enzymes, destruction of the pancreas and pancreatic insufficiency[8]. Thus, the aberrant transport model can account for diverse pathologies observed in the disease. Our findings suggest that simple correction of Cl^- transport is not likely to ameliorate the symptoms of CF. Enhancing HCO_3^- transport by epithelial cells, even in the absence of CFTR, or increasing the HCO_3^- content on the apical surface of affected tissues, should be considered as additional means of reducing the debilitating effects of CF.

Methods

Site-directed mutagenesis

The pCMVNot 6.2 plasmid carrying the human wild-type CFTR gene was a gift from J. Rommens (Hospital for Sick Children, Toronto, Canada). Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). All mutations were verified by sequencing of four separate clones from each mutant. The activity of all four clones was measured to verify that all have similar activity.

Expression of CFTR and cell transfection

All wild-type and CFTR mutants were expressed in HEK293 cells by transient transfection using the lipofectamine reagent. To control for expression of CFTR and identify the CFTR-expressing cells, we co-transfected the cells with a GFP-expressing plasmid (Life Technologies). In previous work, we showed that there is good agreement between expression of GFP and CFTR[9]. GFP fluorescence was measured before current recording or loading the cells with 2′,7′-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF) or after loading with MQAE. Hence, all experiments were performed with cells expressing comparable amount of GFP.

Cl^- currents, $[Cl^-]_i$ and pH_i

HCO_3^- and Cl^- transport were measured with matched pairs of cells transfected in the same dish. In brief, cells grown on two cover slips in each dish were transfected with the same solution and maintained in culture together until use 48–72 h after transfection. On the day of experiment, cells on one of the cover slips were loaded with BCECF (15 min at room temperature) and used immediately to measure HCO_3^- transport. At the same time, cells on the matching cover slip were loaded with MQAE by 1.5–2 h at 37 °C in culture medium containing 5mM MQAE and then used to measure $[Cl^-]_i$.

The procedures and solutions used to measure the CFTR-mediated, whole-cell Cl^- current in the HEK 293 cells transfected with wild-type CFTR and the CFTR mutants were identical to those described for measurement of CFTR-mediated Cl^- current in NIH 3T3 cells[9]. Measurement of pH_i and Cl_i^- with BCECF and MQAE techniques, respectively, in single HEK293 cells, calibration of fluorescent signals and composition of solutions have been described[9,10].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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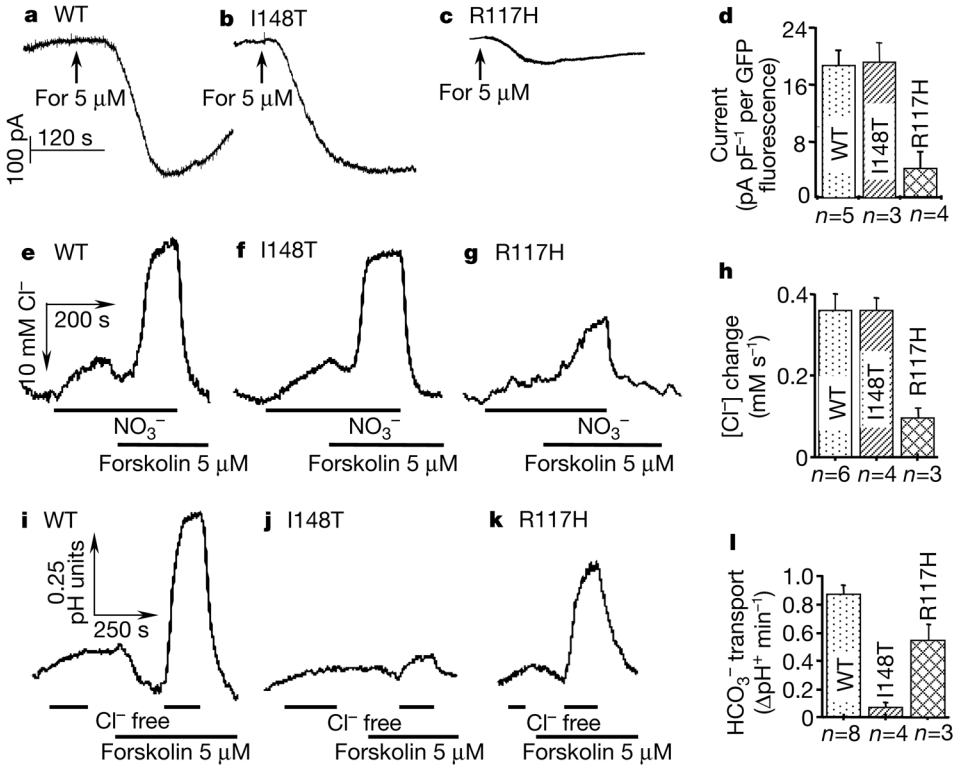

Figure 1.

cAMP-stimulated Cl^- and HCO_3^- transport by wild-type (WT) CFTR and the CFTR mutants I148T and R117H. The whole-cell Cl^- current of HEK293 cells transfected with the indicated constructs was determined (**a–c**). For, forskolin. The current was normalized with respect to membrane capacitance and GFP fluorescence before averaging (**d**). Transfected cells were also loaded with MQAE (**e–h**) or BCECF (**i–l**) for measurements of $[Cl^-]_i$ and pH_i, respectively. Cells loaded with MQAE were exposed to a solution in which Cl^- was replaced with NO_3^- and then stimulated with 5 μM forskolin. For pH measurements, Cl^- was replaced with gluconate. After calibration, initial rates of changes in $[Cl^-]_i$ (**h**) and pH_i (**l**) were averaged.

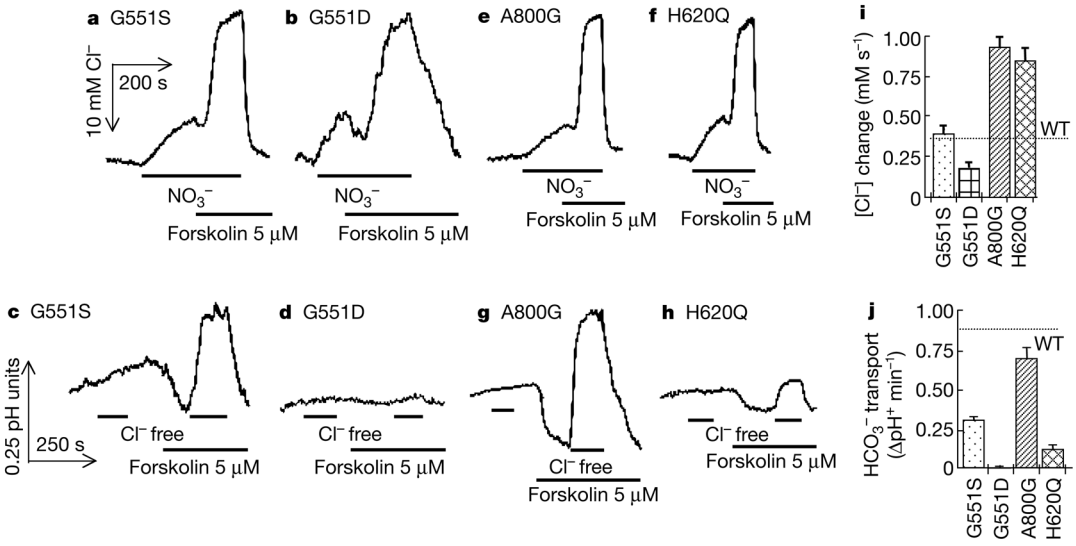

Figure 2.

cAMP-stimulated Cl^- and HCO_3^- transport by CFTR mutants associated with a severe or a mild form of CF. $[Cl^-]_i$ (**a**, **b**, **e**, **f**) and HCO_3^- (**c**, **d**, **g**, **h**) transport were measured as in Fig. 1. Summaries of averaged rates are given for Cl^- (**i**) and for HCO_3^- (**j**).

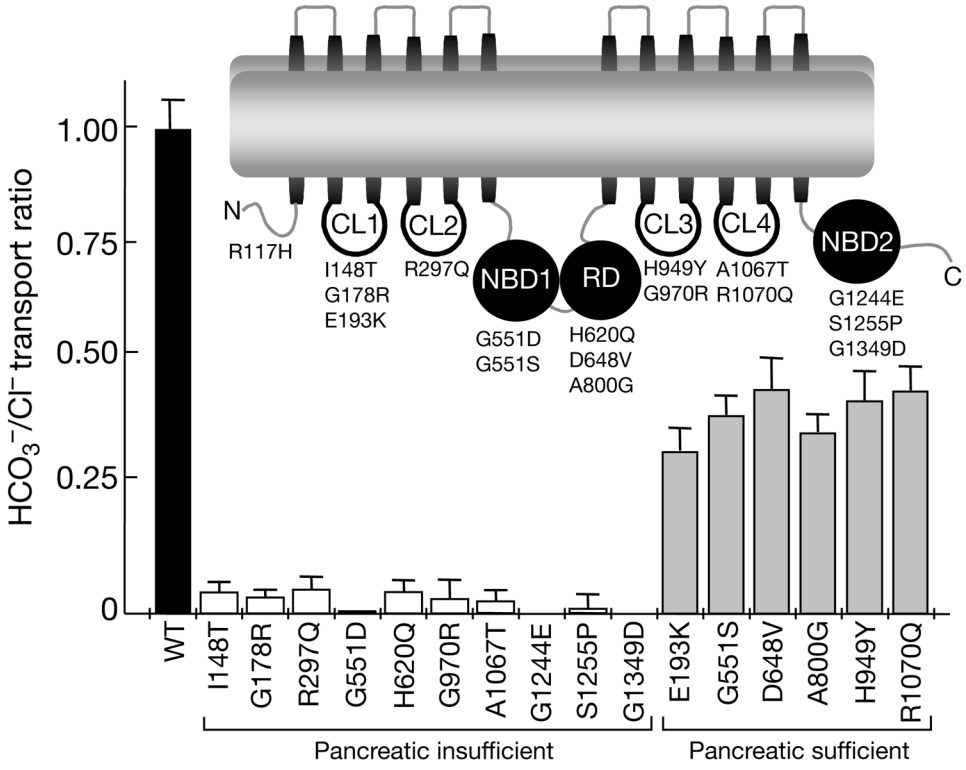

Figure 3.

The HCO_3^-:Cl^- transport ratio of CFTR mutants associated with CF. The HCO_3^-:Cl^- transport ratios were calculated from the averaged rates summarized in Table 1 of the Supplementary Information. The ratio measured in wild-type CFTR was set to 1. Inset illustrates the different cytoplasmic domains of CFTR. CL1, cytoplasmic loop 1; CL2, cytoplasmic loop 2; NBD1, nucleotide-binding domain 1; RD, regulatory domain; CL3, cytoplasmic loop 3; CL4, cytoplasmic loop 4; NBD2, nucleotide-binding domain 2.