Cyclic AMP Oscillations in Insulin-Secreting Cells

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Abstract
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Cyclic AMP is an intracellular messenger that regulates numerous processes in various types of cells. In pancreatic β-cells, cAMP potentiates the secretion of insulin by promoting Ca^{2+} signals and by amplifying Ca^{2+}-triggered exocytosis. Whereas Ca^{2+} signals have been extensively characterized, little is known about the kinetics of cAMP signals. To enable measurements of the cAMP concentration beneath the plasma membrane ($[cAMP]_{pm}$) of individual cells, a translocation biosensor was created based on fluorescent-protein-tagged subunits of protein kinase A (PKA). Evanescent wave microscopy imaging of biosensor-expressing clonal β-cells revealed that the insulinotropic hormones glucagon and GLP-1 triggered pronounced oscillations in $[cAMP]_{pm}$. Simultaneous measurements of the intracellular Ca^{2+} concentration showed that cAMP and Ca^{2+} oscillations were synchronized and interdependent. $[cAMP]_{pm}$ oscillations were also triggered in clonal and primary mouse β-cells by an elevation of the glucose concentration from 3 to 11 mM. These oscillations were preceded and enhanced by elevations of Ca^{2+}. However, conditions raising cytoplasmic ATP could trigger cAMP elevations also without accompanying Ca^{2+} changes, indicating that adenylyl cyclase activity may be directly controlled by the substrate concentration. Experiments with 3-isobutylmethylxanthine (IBMX) and various family-selective phosphodiesterase (PDE) inhibitors indicated that $[cAMP]_{pm}$ oscillations are generated by periodic formation of the messenger by adenylyl cyclases. PDE1 and PDE3 as well as IBMX-insensitive mechanisms shape $[cAMP]_{pm}$, but no single PDE isoform was required for glucose generation of $[cAMP]_{pm}$ oscillations. Recordings of single-cell insulin secretion kinetics with a fluorescent biosensor that reports formation of the phospholipid PIP3 in the plasma membrane in response to autocrine insulin receptor activation showed that $[cAMP]_{pm}$ oscillations were paralleled by pulsatile insulin release. Whereas adenylyl cyclase inhibition suppressed both $[cAMP]_{pm}$ oscillations and pulsatile insulin release, elevation of $[cAMP]_{pm}$ enhanced secretion. Investigation of the effects of different temporal patterns of $[cAMP]_{pm}$ showed that brief $[cAMP]_{pm}$ elevation is sufficient to trigger cytoplasmic responses, whereas sustained elevation is required to induce translocation of the PKA catalytic subunit into the nucleus. In conclusion, these studies demonstrate for the first time in mammalian cells that $[cAMP]_{pm}$ oscillates in response to physiological stimuli. The glucose-induced $[cAMP]_{pm}$ oscillations are generated by periodic cAMP production mediated by interplay between ATP and Ca^{2+} in the sub-membrane space, and may contribute to both triggering and amplifying pathways of insulin secretion. Apart from regulating the precise kinetics of insulin exocytosis, temporal encoding of cAMP signals might constitute a basis for differential regulation of downstream cellular targets.

Keywords: Cyclic AMP, Ca2+, oscillations, evanescent wave microscopy, β-cell, GLP-1, insulin secretion, phosphodiesterase

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Till min älskade mamma, Gunilla Sågetorp
Likt en inte sinande brunn…

Fylld med glädje, engagemang och kärlek

Till Helene Dansk
– Aldrig utan dig!!

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I   Oleg Dyachok, Yegor Isakov, **Jenny Sågetorp** & Anders Tengholm: Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. *Nature* 439, 349-352 (2006).

II   Oleg Dyachok, Olof Idevall-Hagren, **Jenny Sågetorp**, Geng Tian, Anne Wuttke, Cécile Arrieumerlou**,** Göran Akusjärvi, Erik Gylfe & Anders Tengholm: Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metabolism* 8, 26-37 (2008).

III   **Jenny Sågetorp** & Anders Tengholm: Phosphodiesterase shaping of sub-membrane cyclic AMP oscillations in insulin-secreting cells. *Manuscript.*

Contents

Abbreviations

AC	Adenylyl cyclase
ATP	Adenosine-5'-triphosphate
$[Ca^{2+}]_i$	Cytoplasmic Ca^{2+} concentration
$[Ca^{2+}]_{pm}$	Ca^{2+} concentration beneath the plasma membrane
cAMP	3',5'-cyclic adenosine monophosphate
$[cAMP]_{pm}$	cAMP concentration beneath the plasma membrane
CFP	Enhanced cyan fluorescent protein
CNG	Cyclic nucleotide-gated channel
DDA	2',5'-dideoxyadenosine
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
FlAsH	Fluorescein arsenical helix binder
FRET	Förster resonance energy transfer
GIP	Gastric inhibitory peptide/ glucose-dependent-insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GFP	Enhanced green fluorescent protein
IBMX	3-isobutyl-1-methylxanthine
IP_3	Inositol-1,4,5-trisphosphate
K_{ATP} channel	ATP-sensitive potassium channel
MM-IBMX	8-methoxymethyl-3-isobutyl-1-methylxanthine
PDE	Phosphodiesterase
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PI3-kinase	Phosphoinositide-3-OH-kinase
PKA	Protein kinase A
PFK	Phosphofructokinase
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} ATPase
TEA	Tetraethylammonium
VDCC	Voltage-dependent calcium channel
YFP	Enhanced yellow fluorescent protein

Introduction

The blood glucose-lowering hormone insulin is released from β-cells in the islets of Langerhans, which are endocrine micro-organs dispersed throughout the pancreas. Insulin acts by stimulating the uptake of glucose into fat and muscle and by stimulating glycogen formation and suppressing glycogenolysis and gluconeogenesis in the liver [1]. Insufficient release of insulin leads to impaired glucose tolerance or overt diabetes mellitus. There are two major forms of diabetes mellitus: type-1, which is caused by an autoimmune destruction of the pancreatic β-cells, and type-2, characterized by impaired insulin secretion and insulin resistance. Type-2 diabetes accounts for more than 90% of all diabetes and the incidence is increasing rapidly due to obesity and a Western life style [2]. The World Health Organization estimates that more than 180 million people globally suffer from diabetes and around year 2030 the number is estimated to double [3].

Glucose is the main physiological stimulus of insulin secretion. The normal insulin secretory response to glucose is biphasic with a marked initial peak lasting for 10-15 minutes, followed by a sustained or gradually increasing second phase [4]. However, studies of the detailed kinetics of glucose-stimulated insulin secretion have revealed that the hormone is released in pronounced pulses from the perfused pancreas [5], individual pancreatic islets [6] and single β-cells [7-9]. The resulting oscillations of insulin are very prominent in the portal vein but dampened in the peripheral circulation [10, 11]. The oscillations are likely important for maintaining insulin sensitivity in the liver. It has been demonstrated that insulin receptors on hepatocytes are rapidly internalized and recirculated within a few minutes [12]. Interestingly, loss of regular insulin oscillations is one of the earliest signs of developing diabetes [13, 14]. It is therefore important to understand the mechanisms underlying the generation of pulsatile insulin secretion.

In addition to glucose there are other physiological regulators of insulin secretion in β-cells. Hormones and neurotransmitters such as glucagon, glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide/glucose-dependent insulinotropic peptide (GIP), acetylcholine and ATP potentiate glucose-induced insulin secretion, whereas e.g. somatostatin, noradrenaline, ghrelin and galanin suppress secretion. The stimulatory effects of glucose and neuro-hormonal regulators on insulin secretion are largely mediated via the second messengers Ca^{2+} and 3',5'-cyclic adenosine monophosphate (cAMP). In contrast to Ca^{2+} signals, which have been extensively characterized, little is known

about the kinetics of cAMP signals. This thesis describes a novel technique for on-line measurements of cAMP concentration changes in individual cells, how cAMP is regulated in β-cells, and how different patterns of cAMP signals influence downstream responses, in particular insulin secretion.

Stimulus-secretion coupling in pancreatic β-cells

Glucose-induced insulin secretion

Following a rise of the blood glucose concentration the sugar rapidly enters the β-cell through GLUT transporters [15]. Glucose is subsequently phosphorylated by glucokinase [16] and further metabolized in the glycolysis to pyruvate, which enters the mitochondria where it is metabolized in the citric acid cycle [17]. The metabolism generates ATP at the expense of ADP and the resulting increase of the ATP/ADP ratio causes closure of ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane, depolarization and opening of voltage-dependent Ca^{2+} channels (VDCC) [18]. The influx of Ca^{2+} elevates the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and triggers exocytosis of insulin granules [18]. The L-type VDCC is the dominating type in human and rodent β-cells [19, 20], although the R-type VDCC recently was proposed to contribute to the $[Ca^{2+}]_i$ elevation during the late phase of insulin secretion [21].

The elevation of $[Ca^{2+}]_i$ is often manifested as oscillations. Different types of $[Ca^{2+}]_i$ oscillations have been described in glucose-stimulated β-cells [22, 23]. The dominating kind depends on slow periodic depolarization of the plasma membrane and influx of $[Ca^{2+}]_i$ through VDCC [24-26]. The slow oscillations have a frequency of 0.1-0.5 min^{-1} in individual β-cells. β-cells also exhibit short-lasting (<40 s) irregular $[Ca^{2+}]_i$ spikes superimposed on the slow oscillations or on a stable $[Ca^{2+}]_i$ elevation. These spikes are particularly prominent after elevation of the cAMP concentration and reflect gated release of Ca^{2+} from the endoplasmic reticulum (ER) via inositol 1,4,5-trisphosphate (IP_3) receptors [27-29]. Oscillations of $[Ca^{2+}]_i$ are also present in β-cells within intact pancreatic islets [30, 31]. Besides the slow oscillations, similar to those in isolated β-cells, the islets often show fast regular membrane oscillations of $[Ca^{2+}]_i$ (1-5 min^{-1}) or slow oscillations with superimposed fast ones [30, 31]. Also the fast regular islet oscillations depend on depolarization with influx of Ca^{2+} through L-type channels [32]. However, in some way they also co-dependent on the Ca^{2+} handling by the ER, since fast islet oscillations are immediately transformed into slow ones when preventing Ca^{2+} sequestration in the ER by inhibiting the sarco(endo)-plasmic reticulum Ca^{2+} ATPase (SERCA) [31].

Apart from the K_{ATP} channel-dependent triggering of insulin secretion, glucose also promotes insulin secretion by an amplifying mechanism [33]. This amplification becomes obvious when studying the effect of glucose on

insulin secretion triggered by elevated Ca^{2+} under conditions when the K_{ATP} channels are either kept closed or open. Glucose consequently promotes additional secretion from β-cells stimulated with sulfonylureas like tolbutamide [34], which cause depolarization and stable elevation of $[Ca^{2+}]_i$ by closing the K_{ATP} channels. Moreover, the sugar has a similar effect after raising $[Ca^{2+}]_i$ by K^+ depolarization in the presence of the K_{ATP} channel opener diazoxide [35]. Under these conditions glucose amplifies insulin secretion without changing the membrane potential. The mechanisms underlying the amplifying pathway is not entirely clear and many different factors, including ATP/ADP, GTP, cAMP, Ca^{2+}/calmodulin-dependent protein kinase II and acyl-CoAs have been suggested as putative mediators [33].

Neuro-hormonal control of insulin secretion

β-cells respond to acetylcholine, released from intrapancreatic nerve endings, and to ATP from nerves and β-cells. These transmitters bind to $G\alpha_q$-coupled receptors that activate phospholipase C, which catalyzes the degradation of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol [36]. IP_3 mobilizes Ca^{2+} from the ER and diacylglycerol activates protein kinase C, both effects contributing to stimulated secretion [37]. Glucagon from α-cells in the islets of Langerhans is secreted in response to hypoglycemia and is the major blood glucose counterregulatory hormone. Glucagon stimulates glucose output from the liver [38] but is paradoxically also a very potent insulinotropic hormone. GLP-1 and GIP are released from intestinal L- and K-cells, respectively, in response to food intake [39, 40]. The release of the gut hormones explains why insulin secretion is better stimulated by oral than intravenous glucose (the incretin effect) [41]. Glucagon, GLP-1 and GIP bind to $G\alpha_s$-coupled receptors [42-44] to stimulate adenylyl cyclase (AC) and increase cAMP. GLP-1 can improve β-cell function, restore first phase of insulin secretion and normalize blood glucose [45]. A drawback with GLP-1 as therapeutic agent is its short half-life (1.5-2 min) due to the degradation by dipeptidyl peptidase 4. The long-acting GLP-1 analogue Exenatide, which is resistant to dipeptidyl peptidase-4 degradation and has a higher affinity for the GLP-1 receptor, is a promising drug in the management of type 2 diabetes [46].

Neurohormonal factors not only mediate stimulatory influences on insulin secretion. For example, adrenaline, noradrenaline, somatostatin, ghrelin and galanin are potent inhibitors and at least part of their inhibitory effects are mediated by $G\alpha_i$-mediated inactivation of ACs and lowering of cAMP [47-49].

Oscillatory control of insulin secretion

Oscillations of $[Ca^{2+}]_i$ is probably the most important factor underlying pulsatile insulin release, although pulsatile secretion has also been detected under conditions when $[Ca^{2+}]_i$ is stable [50, 51]. The mechanism underlying these oscillations is not entirely clear, but it has been proposed to involve Ca^{2+} feedback on ion channels and mitochondrial metabolism as well as primary oscillations in β-cell metabolism [23, 52-54]. Glucose-induced $[Ca^{2+}]_i$ oscillations are paralleled by oscillations of the mitochondrial membrane potential [55, 56]. Elevation of $[Ca^{2+}]_i$ depolarizes mitochondria, thus reducing the driving force for ATP synthesis [55, 56]. Accordingly, Ca^{2+} entry may lead to transient inhibition of mitochondrial ATP production, a fall of the ATP/ADP ratio, opening of K_{ATP} channels with resulting repolarization of the plasma membrane and closure of the VDCCs. However, the situation is complicated because Ca^{2+} also stimulates metabolism by activating mitochondrial dehydrogenases [57, 58]. Further support for the idea of a link between oscillations of metabolism and $[Ca^{2+}]_i$ comes from the observation that the oxygen tension in islets oscillates in a manner coinciding with the slow oscillations in $[Ca^{2+}]_i$ and insulin secretion [59-61]. Moreover, NAD(P)H-levels have been found to oscillate in glucose-stimulated rat β-cells [62] and intact mouse islets [63] where they are coupled to oscillations of $[Ca^{2+}]_i$. It has been shown that free cytosolic ATP and the ATP/ADP ratio oscillate in isolated islets and individual primary mouse and human β-cells [59, 64]. The observation that the activity of the K_{ATP} channels, which probably are the most important ATP targets in insulin secretion, oscillates even in the presence of a sub-stimulatory concentration of glucose [65] indicates that there may be primary oscillations in ATP/ADP levels. Such inherent metabolic oscillations may arise from the glycolytic enzyme phosphofructokinase (PFK), whose interesting kinetics is known to generate oscillations in the levels of the PFK substrate fructose 6-phosphate and the product fructose 1, 6-bisphosphate [52]. Humans with an inherited deficiency of PFK show an impaired oscillatory insulin secretion pattern [66], suggesting that PFK indeed is important for generating pulsatile insulin secretion. The observation that citrate oscillates in isolated islet mitochondria [67] indicates that, in addition to metabolic oscillations arising from Ca^{2+} feedback or from glycolytic oscillations, there is an independent mitochondrial oscillator. Interestingly, citrate is a potent regulator of PFK and citrate exported from the mitochondria may thus feedback-coordinate mitochondrial and glycolytic oscillations [67]. Metabolic oscillations, which ultimately result in periodic variations in the ATP concentration, probably affect insulin secretion not only via K_{ATP} channels and $[Ca^{2+}]_i$ oscillations, but also via direct effects on the exocytosis machinery or via second messengers, such as cAMP.

cAMP signaling in β-cells

cAMP was discovered in the 1950s [68] and is recognized as an ubiquitous intracellular second messenger and key regulator of many cellular processes, including metabolism, secretion, gene expression, cell growth and survival [69]. In pancreatic β-cells, cAMP potentiates Ca^{2+}-dependent exocytosis [70-72] and mediates the amplification of insulin release exerted by glucagon and incretin hormones [73, 74]. Early studies indicated that also glucose stimulation leads to a modest elevation of islet cAMP [75-78], an effect that has been attributed to the elevation of $[Ca^{2+}]_i$ [79, 80]. From the observation that purified β-cells, lacking influence from the glucagon-producing α-cells, show reduced cAMP content and impaired secretion, it was suggested that cAMP has a permissive role in glucose-induced insulin secretion and that the effect of the sugar on cAMP content represents amplification of glucagon-induced cAMP production [81].

Adenylyl cyclases

cAMP is generated by ACs after activation of $G\alpha_s$-coupled hormone receptors. There are nine membrane bound (AC1-9) and one soluble AC (sAC) isoform in mammalian cells. The membrane bound ACs share a common 120-140 kDa structure with twelve transmembrane and two cytosolic domains which dimerize and form the catalytic core [82]. All the membrane-bound ACs are stimulated by $G\alpha_s$ and can be activated experimentally by the plant diterpene forskolin. The inhibitory G-protein $G\alpha_i$ negatively regulates AC1, AC3, AC5, AC6, AC8 and AC9. The $G\beta\gamma$ complex also exerts type-specific regulation of ACs. Accordingly, AC1 and AC8 are inhibited, whereas AC2, AC4 and AC7 are stimulated by the $G\beta\gamma$ complexes [82]. cAMP production is also subject to regulation by Ca^{2+} [83]. AC1 and AC8 are stimulated by Ca^{2+}/calmodulin, whereas AC3, AC5, AC6, and AC9 are inhibited. Inhibition of AC3 is mediated by Ca^{2+} through Ca^{2+}/calmodulin-dependent protein kinases II and IV and AC 9 is inhibited by the Ca^{2+}-dependent phosphatase calcineurin. Several AC isoforms are regulated by protein kinases. Protein kinase C has been found to stimulate AC2, AC4 and AC7 [84], whereas protein kinase A (PKA)-mediated phosphorylation feed-back-inhibits AC5/AC6 [85]. Analyses of mRNA expression in β-cells have demonstrated predominant expression of Ca^{2+}-regulated ACs. In one study AC5 and AC6 were most abundant in human islets and AC3, AC4 and AC6 in rat islets [86]. Another study identified AC6 and AC8 as the most highly expressed isoforms in rat β-cells [87], and an immunofluorescence study indicated that the levels of AC8 are particularly high in β-cells from spontaneously diabetic GK-rats [88]. Although both Ca^{2+}-inhibited and Ca^{2+}-stimulated isoforms are expressed in β-cells, most functional studies indicate that the ion has a stimulatory effect on cAMP production [80, 87, 89]. The

soluble isoform of AC is unaffected by G-proteins and forskolin but is activated by bicarbonate and Ca^{2+} [90]. Soluble AC is predominantly expressed in male germ cells, although recent studies have suggested expression in various somatic cell types, including those secreting insulin [91].

Phosphodiesterases

The discovery of phosphodiesterase (PDE) by Sutherland and colleagues [92] followed shortly after that of cAMP. The function of PDEs is to catalyze the hydrolysis of the 3'-phosphoester bond of cyclic AMP or cyclic GMP to 5'-AMP and 5'-GMP, thereby terminating the messenger signal. The PDEs constitute more than 60 isoforms divided into 11 sub-families (PDE1-11) that are structurally and genetically related but which differ in substrate specificity, kinetics and regulatory properties [90, 93]. The PDE1 family of enzymes are activated by Ca^{2+}/calmodulin and hydrolyze both cyclic nucleotides [94]. PDE2 is a cGMP-stimulated cAMP-degrading enzyme [93], and PDE3 is a dual specificity enzyme with kinetic properties resulting in cGMP-inhibited cAMP degradation [95]. PDEs 4, 7 and 8 are all cAMP-specific enzymes. The more than 20 different PDE4 family members are characterized by multiple molecular targeting interactions for subcellular localization as well as by regulation by PKA phosphorylation [96]. PDEs 5, 6 and 9 are cGMP-selective and the more recently described and yet poorly characterized PDE 10 and 11 family enzymes show dual substrate specificity for cAMP and cGMP [93]. PDE activity has been extensively characterized in many cell types using biochemical methods based on hydrolysis of radioactively labelled cyclic nucleotides. All isoforms, except the PDE 8 and 9 families, can be inhibited by 3-isobutyl-methylxanthine (IBMX) [90]. In addition, there are a number of family selective pharmacological PDE inhibitors [93, 97].

Early studies demonstrated that pancreatic islets contain Ca^{2+}/calmodulin-sensitive PDE activity [98, 99], and expression of PDE1 isoforms have subsequently been demonstrated with PCR in islets and insulin-secreting cell lines [97, 100]. Pharmacological inhibition of PDE1 has also been found to augment insulin secretion from βTC3 cells [100]. The PDE3 family consists of the two isoforms PDE3A and B. PDE3B is highly expressed in tissues involved in energy metabolism, including liver, fat and pancreatic islets [97]. Several studies have reported the involvement of PDE3B in insulin secretion [101-103]. Transgenic or adenovirus-mediated over-expression of PDE3B in β-cells and islets resulted in impaired insulin secretion in response to GLP-1 and glucose [101, 102, 104]. Being localized both to the insulin secretory granule and plasma membranes [104] the enzyme probably affects insulin secretion at distal steps of the granule-plasma membrane fusion during exocytosis [101, 104]. PDE3B has also been proposed to promote survival of insulin-secreting cells through a PI3-kinase-dependent pathway, where the

activation of PDE3B may reduce intracellular cAMP levels to protect cells from cAMP-induced apoptosis [105]. The PDE4 family is present and functionally active in islets and insulin-secreting cells, as indicated by mRNA expression and the observation that PDE4-selective inhibitors augment glucose dependent insulin secretion [97, 106]. Recent real-time PCR analysis have identified both mRNA and protein expression of PDE8B as well as mRNA of PDEs 10A and 11A in INS-1 cells and rat islets [97, 103]. Down-regulation of PDE8B expression with RNA interference was found to augment insulin secretion [103].

cAMP effector proteins

cAMP exerts its effects by PKA and cAMP-regulated guanine nucleotide exchange factors, also known as "Exchange proteins directly activated by cAMP" (Epacs). PKA is a ubiquitous serine/threonine protein kinase present as a tetrameric holoenzyme under basal conditions [107]. The holoenzyme consists of a regulatory subunit dimer and two catalytic subunits. Binding of cAMP to the regulatory subunits releases the catalytic subunits. Four regulatory subunit isoforms (RIα, RIβ, RIIα and RIIβ) and three catalytic subunit isoforms (Cα, Cβ, Cγ) have been identified [108, 109]. PKA holoenzyme tetramers with type I regulatory subunits are mostly cytoplasmic, whereas PKA with type II regulatory subunits typically binds to membranes and intracellular structures. The regulatory subunits are often targeted to specific intracellular locations via binding to A-kinase anchoring proteins [109, 110]. Apart from PKA, these scaffold proteins bind other signaling proteins including other kinases, phosphatases and PDEs [85, 110]. Such anchoring of closely linked signaling molecules enables highly localized PKA-signaling. Disruption of A-kinase anchoring complexes have been found to reduce GLP-1-induced stimulation of insulin secretion [111]. An immunocytochemical study of PKA localization in β-cells indicated that glucose stimulates translocation of the Cα-subunit to the nucleus, whereas Cβ was enriched in the plasma membrane [112]. Free PKA catalytic subunits can enter the nucleus via passive diffusion through nuclear pores [113] and phosphorylate the cAMP response element-binding protein (CREB) and related family members (ATF-1, CREM), which regulate transcription of genes containing cAMP response elements [114, 115].

Several proteins involved in insulin secretion are known PKA substrates. For example, PKA phosphorylates the K_{ATP} channels, which promotes depolarization [70]. PKA also phosphorylates the VDCCs and IP_3-receptors [19, 70] stimulating Ca^{2+} influx [116] and facilitating Ca^{2+} release from the ER [28, 29], respectively. Moreover, PKA phosphorylates proteins associated with the exocytosis machinery, including SNAP-25, synapsin, snapin and cystein string proteins [70]. Several of these mechanisms likely take part in the amplification of insulin secretion by cAMP. However, since PKA inhibi-

tors fail to inhibit a significant proportion of cAMP-stimulated exocytosis in β-cells, it was early concluded that cAMP stimulates secretion also via additional, non-PKA effectors [117].

The other major cAMP effector, Epac, was first described in 1998 as a cAMP regulated guanine nucleotide exchange factor for the small GTPase Rap1 [118, 119]. There are two Epac isoforms, and Epac2 was soon demonstrated to mediate PKA-independent amplification of exocytosis by cAMP [120]. This conclusion gained support by the recent demonstration that Epac2 knock-out mice show diminished cAMP potentiation of first phase insulin secretion [121]. The mechanisms by which Epac stimulates secretion remain unclear. It has been proposed that Epac promotes Ca^{2+} release from the ER [122], but this finding is controversial [29]. It seems more likely that Epac2 stimulates secretion in β-cells by direct interactions with exocytosis proteins. Whereas one study identified Rim2 and Piccolo as important Epac interaction partners [123], another study indicated that the effects of Epac2 on exocytosis rely on its interaction with the SUR1 subunit of the K_{ATP} channel [124]. Consistent with Epac acting as a guanine nucleotide exchange factor, PKA-independent potentiation of insulin secretion by cAMP was recently found to be associated with activation of Rap1 [121].

Measurements of cAMP

Soon after the discovery of cAMP, methods were developed to quantify intracellular concentrations of the messenger [69]. Most of the current knowledge about cAMP is based on immunoassays, which do not provide information about kinetics in single cells. It has long been known that cAMP undergoes rapid turnover and that many regulatory influences on ACs and PDEs make cAMP signals highly dynamic. The first single-cell technique providing spatio-temporal information about cAMP was developed by Tsien and co-workers and was based on recordings of Förster resonance energy transfer (FRET) between two fluorescence-labeled subunits of PKA [125]. Using this probe, Gorbunova and Spitzer were able to demonstrate that spontaneous electrical activity and $[Ca^{2+}]_i$ oscillations are associated with pronounced cAMP oscillations in *Xenopus* frog embryonal neurons [126]. In the original version of this biosensor membrane-impermeant rhodamine and fluorescein were conjugated to the regulatory and catalytic subunits, respectively, and the holoenzyme therefore had to be microinjected into cells [125]. Moreover, the recorded responses gradually become distorted when the labeled PKA subunits bind to non-fluorescent endogenous PKA. The creation of a genetically encoded biosensor based on the fusion of the PKA subunits with blue and green fluorescent proteins [127], and later, cyan and yellow fluorescent proteins (CFP and YFP) [128] represented major improvements. More recently, biosensors based on FRET between fluorescent proteins fused to

Epac or isolated cAMP binding domains from PKA and Epac have been developed [129-131]. An advantage with FRET-based single polypeptide chain biosensors is that the two fluorescent moieties are present at identical concentrations. Using such a probe, it was demonstrated that glucose induces elevation of the cytoplasmic cAMP concentration in MIN6 cells and that the K^+ channel inhibitor tetraethylammonium triggers anti-synchronous oscillations of cAMP and $[Ca^{2+}]_i$ [132]. A disadvantage with all FRET probes is that the signal changes are usually small, making it difficult to record modest variations in cAMP under physiological conditions. The sub-plasma membrane compartment is a particularly interesting cAMP site in secretory cells like β-cells, because this is where cAMP is formed by membrane-bound ACs, and where cAMP exerts its effect on the exocytosis of secretory granules. Therefore, genetically modified cyclic nucleotide-gated plasma membrane channels (CNGs) provide an interesting alternative to FRET sensors for detecting cAMP specifically in this compartment [133]. CNGs are directly opened by binding of the cyclic nucleotide and this allows Ca^{2+} to enter the cytoplasm. Ionic current or changes in $[Ca^{2+}]_i$ are then used as readout for CNG channel activity. However, it may be difficult to determine whether an increase in $[Ca^{2+}]_i$ reflects activation of CNG channels or other mechanisms of importance for secretion. Moreover, as described above, Ca^{2+} may affect the cAMP signal being measured. Thus, there is a need for novel sub-membrane biosensors for cAMP with improved signal responses to cAMP binding.

Aims

The aims of the present study were to:

1. Develop a fluorescent translocation biosensor reporting changes of the cAMP concentration beneath the plasma membrane in single cells.

2. Explore the kinetics of cAMP signals induced by glucose and hormones in insulin-secreting cells.

3. Determine how different phosphodiesterases are involved in the shaping of sub-membrane cAMP signals in insulin-secreting cells.

4. Clarify the relationship between cAMP and Ca^{2+} signals in insulin-secreting cells.

5. Study if of the kinetics of cAMP signals affects the distribution of the catalytic PKA subunit in insulin-secreting cells.

6. Investigate the relationship between cAMP signals and the kinetics of insulin secretion.

Material and methods

Islet isolation, cell culture and transfection

Insulin-secreting rat INS-1 cells (passage 90-120 [134] and the more glucose-responsive subclone INS-1E [135] were cultured in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 50 µM 2-mercaptoethanol, 100 U/mL penicillin and 100 µg/mL streptomycin. Clonal mouse MIN-6 cells (passage 17-30 [136]) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 15 % fetal calf serum, 2 mM glutamine, 70 µM 2-mercaptoethanol, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were seeded onto poly-L-lysine coated 25-mm coverslips and cultured to reach 50-60% confluence on the day of transfection. Transient transfections of fluorescent protein fusion constructs were performed using 2 µg of each plasmid DNA construct and 5 µg Lipofectamine™ 2000 in 1 mL serum-free DMEM or Optimem® transfection medium during 4 h followed by repeated washing of the cells and further culture in DMEM or RPMI 1640 for 12-48 h.

Islets of Langerhans were collagenase-isolated from pancreata of C57Bl/6 mice. All procedures were approved by a local ethical committee on animal experiments. Single cells were prepared by shaking the islets in a Ca^{2+}-deficient medium. After resuspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin, the cells were allowed to attach to the center of cirular 25 mm diameter coverslips during 2-5 days culture. The islet cells were transfected using adenoviruses at a multiplicity of infection of 60 fluorescence forming units/cell. After 1h incubation at 37 °C the inoculum was removed and the cells were washed twice, followed by further culture in complete medium supplemented with 4 µM doxycycline for 24 h. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Measurements of the cAMP concentration beneath the plasma membrane

A translocation biosensor for the submembrane cAMP concentration ($[cAMP]_{pm}$) based on fluorescent protein labeled PKA subunits was devel-

oped as described in the Results and Discussion section. In brief, PKA subunits were engineered so that a CFP-labelled or unlabelled regulatory subunit is fixed to the plasma membrane and a YFP or CFP-labelled catalytic subunit dissociates from the membrane-bound regulatory subunit and translocates into the cytoplasm upon elevation of $[cAMP]_{pm}$. The use of different spectral variants allowed simultaneous measurements of cAMP and phosphatidylinositol-3,4,5-triphosphate (PIP_3) or Ca^{2+}. Biosensor translocation was recorded with evanescent wave microscopy as detailed below. In experiments on primary islet cells, β-cells were identified based on their large size and low nuclear/cytoplasmic ratio in transmitted light, as well as on their negative cAMP response to adrenaline [47] .

Recombinant adenoviruses

Recombinant adenovirus vectors expressing the cAMP biosensor subunits under the transcriptional control of a tetracycline-regulated promoter were created by first amplifying the biosensor with new restriction sites generated by PCR. The products were ligated into the pShuttleTetTripLac-BamHI transfer vector [137]. The desired recombinant adenovirus constructs were subsequently generated by overlap recombination in BJ5183 *E. coli* cells expressing the pAd-Tet-ON plasmid [138]. The resulting constructions were then transfected into 293-Lacl cells to make recombinant viruses. After 7-10 days of cell culture virus particles were harvested and used for reinfection. After three rounds of re-infection high-titer virus stocks were produced in twelve 15-cm plates with 293-Lac1 cells. 2-3 days post-infection when a clear cytopathic effect was visible, the cells were harvested by low-speed centrifugation, resuspended in Tris-HCl and lysed. The lysate was sonicated and viruses purified by CsCl gradient centrifugation. Virus bands were collected and dialyzed against 100 volumes of PBS containing 1 mM of $CaCl_2$ and $MgCl_2$ as well as 10% glycerol using a Slide-A-Lyzer Cassette (Pierce, Rockford, IL, USA). Virus titers were determined by counting fluorescent forming units using a monoclonal antibody directed against the adenovirus-hexon protein and a FITC-conjugated secondary antibody.

Measurements of Ca^{2+}

In experiments with $[Ca^{2+}]_i$ measurements, cells were loaded with the Ca^{2+} indicator Fura-2 by pre-incubation for 30 min at 37 °C with 1 µM of its acetoxymethyl ester. $[Ca^{2+}]_i$ imaging of Fura-2-loaded cells was performed using a wide-field imaging system based on a Nikon Eclipse TE2000U inverted microscope with a 40x, NA 1.3, oil immersion objective. The microscope was equipped with an epifluorescence illuminator (Cairn Research

Ltd, Faversham, UK) connected through a 5-mm diameter liquid light guide to a monochromator (Optoscan, Cairn Research Ltd) with a 150-W xenon arc lamp. The monochromator provided excitation light at 340 nm (1.7 nm half-bandwidth) and 380 nm (1.4 nm half-bandwidth). Emission was measured through a 510 nm interference filter (40 nm half-bandwidth). Images were acquired with a Hamamatsu Orca-ER-1394 firewire digital charge-coupled device (CCD) camera. The monochromator and camera were controlled by Molecular Devices' Metafluor software. To measure Ca^{2+} concentrations in the submembrane space ($[Ca^{2+}]_{pm}$) the cells were loaded with the Ca^{2+} indicator Fura Red by pre-incubation for 30 min at 37 °C with 5 µM of its acetoxymethyl ester. Imaging of $[Ca^{2+}]_{pm}$ of the Fura Red-loaded cells was performed with an evanescent wave microscope as described below.

Recordings of insulin release kinetics from single cells

For recordings of insulin secretion kinetics from individual cells, advantage was taken by the fact that insulin release causes autocrine activation of insulin receptors on the β-cell surface [139, 140]. Binding of insulin to its receptor initiates a signaling cascade resulting in activation of PI3-kinase and formation of the membrane PIP_3. PIP_3 formation can be visualized in single cells as translocation of fluorescent protein-tagged protein domains, which selectively binds this lipid [7]. In the present studies, the PH domain from protein kinase B/Akt fused to CFP (CFP-PH$_{Akt}$) or fullength GRP1 (general receptor for phosphoinositides-1) ligated with four tandem copies of GFP (GRP1-GFP$_4$) were used in combination with evanescent wave microscopy to detect PIP_3 (see below). In some experiments, the vesicle membrane protein VAMP2 with a luminal YFP tag was used to visualize the fusion of insulin granules with the plasma membrane.

FlAsH labelling of PKA catalytic α subunit

Since tagging with a bulky fluorescent protein may restrict the penetration of PKA catalytic subunits through nuclear pores, a different labeling strategy was used to study nuclear translocation of PKA. The PKA catalytic Cα subunit was tagged with a tetracysteine motif by ligating Cα cDNA into the pENTR4 Gateway® entry vector followed by site-specific recombination into the pcDNA6.2-cLumio vector (Invitrogen). The resulting tetracysteine-tagged PKA Cα was expressed in INS-1 cells and subsequently labelled with 0.5 µM of the membrane-permeable fluorescent biarsenical dye FlAsH (fluorescein arsenical helix binder) [141] and incubated for 20 min at 37 °C. To reduce non-specific labeling the cells were subsequently rinsed with dye-free buffer, incubated for 10-15 min in 250 µM 1,2-ethanedithiol and again

rinsed with buffer. Fluorescence was excited with wide-filed epifluorescence illumination using the microscope setup described below.

Fluorescence microscopy

Translocation of the fluorescent reporter constructs was measured using custom-built evanescent wave microscope setups. In evanescent wave microscopy laser light is used to excite fluorescence from cells attaching to a glass coverslip that is the bottom of a superfusion chamber with physiological medium. A unique feature of this technique is that the excitation light hits the glass/water interface at an angle causing total internal reflection. A rapidly fading (evanescent) wave of light is generated above the coverslip, which can excite fluorescence about 150 nm into the cytoplasm of the attaching cells [142].

One evanescent wave setup was built around a Nikon Eclipse TE2000 microscope equipped with a 60x, 1.45-NA oil immersion objective. A multiline argon laser provided excitation light for CFP at 458 nm and for YFP and Fura Red at 514 nm using 458 nm/10 nm half-bandwidth and 514.5 nm/10 nm interference filters, respectively. The beam was homogenized and expanded by a rotating light-shaping diffuser and moved towards the periphery of the objective aperture until the critical angle was exceeded and total internal reflection occurred at the interface between the coverslip and the attached cells. CFP fluorescenc was measured through a 485 nm/25 nm filter, YFP through a 550 nm/30 nm filter and Fura Red through a 630 nm long-pass filter. FlAsH-labelled PKA Cα was imaged using the same setup in epifluorescence illumination mode with excitation at 488 nm/10 nm and emission at 530 nm/50 nm. Fluorescence was monitored with a Hamamatsu Orca-ER camera controlled by MetaFluor software (Molecular Devices).

Another evanescent wave system was built around a Nikon E600FN upright microscope. A helium-cadmium laser provided 442 nm light for excitation of CFP and the 488 and 514 nm lines of an argon laser were used to excite GFP and YFP, respectively. The beams were merged with a dichroic mirror, homogenized and expanded by a rotating light shaping diffuser and subsequently refocused through a modified quartz dove prism with a 70° angle to achieve total internal reflection. The chamber was mounted on the custom-built stage of the microscope such that the cover slip was maintained in contact with the dove prism by a layer of immersion oil. Regular "widefield" excitation of Fura-2 at 340 and 380 nm was achieved via an epifluorescence illuminator connected through a liquid light guide to an Optoscan monochromator (Cairn Research Ltd) equipped with a 150-W xenon arc lamp. Irrespective of the mode of illumination, fluorescence light was collected through 40x 0.8-NA or 60x 1.0-NA water immersion objectives and detected with an Andor iXon DU-887 EMCCD camera under MetaFluor

software control. In both setups excitation and emission wavelengths were selected with filters mounted in Sutter Lambda 10-3 filter wheels. Images were acquired every 2-5 s using exposure times in the 30-200 ms range. To minimize exposure to the potentially harmful excitation light, the beam was blocked with a shutter between image captures.

Before experiments the cells were transferred to a buffer containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 25 mM HEPES with pH adjusted to 7.40 with NaOH, and incubated for 30 min at 37°C. After incubation the coverslips with the attached cells were mounted as exchangeable bottoms of an open 50-µL chamber and superfused with buffer at a rate of 0.3 mL/min.

Permeabilization of cells

Cell permeabilization in some experiments was preceded by superfusion in an intracellular-like medium containing 140 mM KCl, 6 mM NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 0.465 mM $CaCl_2$, and 10 mM HEPES with pH adjusted to 7.00 with KOH. Cells were then permeabilized with 5 µL of 0.46 mg/mL α-toxin from *Staphylococcus aureus* (PhPlate, Stockholm, Sweden) added directly into the 50-µl superfusion chamber. After permeabilization 1-4 mM MgATP was added to the medium and the concentrations of free Mg^{2+} and Ca^{2+} were maintained a 1 mM and 350 nM, respectively.

Data analysis

Image analysis was made using MetaFluor or ImageJ (W.S.Rasband, National Institute of Health, (http://rsb.info.nih.gov/ij) software. The cAMP concentration was expressed as the ratio of CFP over YFP fluorescence after background subtraction. To compensate for differences in expression levels, the basal ratio was normalized to unity. The magnitudes of the responses were calculated by time-averaging the signal over the stimulation period. Fluorescence intensities were otherwise expressed as changes relative to initial fluorescence after subtraction of background (F/F_0). Data are presented as means ± s.e.m. Statistical comparisons were assessed with Student's t-test.

Results and Discussion

Construction of a translocation biosensor reporting cAMP beneath the plasma membrane (*I, II*)

To explore cAMP signals in β-cells, a fluorescent biosensor was developed that reports changes in $[cAMP]_{pm}$. The RIIβ regulatory subunit of PKA was labelled with cyan fluorescent protein (CFP) and targeted to the plasma membrane with a polybasic sequence and a farnesylation motif from Ki-Ras. To minimize anchoring to other intracellular locations, the first 80 amino acid residues, including the dimerization and anchoring protein binding domains, were deleted from the N-terminus. The fullength PKA catalytic Cα subunit was labelled with yellow fluorescent protein (YFP). Because of the high affinity interaction between the catalytic and regulatory subunits under basal conditions, the engineered PKA holoenzyme was located to the plasma membrane when co-expressed in INS-1-, INS-1E-, MIN6- and primary mouse pancreatic β-cells. Elevation of $[cAMP]_{pm}$ by forskolin stimulation of ACs or IBMX inhibition of PDEs caused holoenzyme dissociation with redistribution of Cα-YFP to the cytoplasm. When recorded with evanescent wave microscopy, which selectively visualizes a thin volume within ~150 nm from the plasma membrane [142], this translocation resulted in a rise of the CFP/YFP ratio due to the selective loss of YFP from the membrane. The time to half maximal change of the CFP/YFP ratio in INS-1 cells after addition or removal of IBMX averaged approximately 20 s, demonstrating that there is high turnover of cAMP beneath the plasma membrane. Application of 10 µM cAMP to cells permeabilized with staphylococcal α-toxin resulted in even faster translocation with a half-time of <12 s. The probe response was specific, since the membrane-permeable cyclic nucleotide analogue 8Br-cGMP lacked effect under conditions when 8Br-cAMP induced a pronounced increase of the CFP/YFP ratio.

This new biosensor has advantages over previously used cAMP reporters. The plasma membrane targeting makes the biosensor sensitive to cAMP changes in the cell compartment where the messenger is produced and where it affects ion channels and exocytosis. In contrast to CNG channel reporters, which also record the sub-membrane cAMP concentration [133], the translocation biosensor does not induce confounding changes of $[Ca^{2+}]_i$. When detected with evanescent wave microscopy, the translocation of the construct results in a large fluorescence change that can be measured with minimal

photobleaching and phototoxic effects and with a signal-to-noise ratio superior to that of other fluorescence microscopy approaches.

GLP-1 and glucagon trigger oscillations of [cAMP]$_{pm}$ (*I*)

When INS-1 cells were stimulated with GLP-1 in the presence of 3 mM glucose there was a prompt increase in [cAMP]$_{pm}$. Cells of the INS-1E subclone showed a markedly enhanced response when re-stimulated in the presence of 20 mM glucose. The GLP-1-induced [cAMP]$_{pm}$ elevation was rapidly reversed when GLP-1 was removed or when ACs were inhibited with noradrenaline, which acts mainly via Gα_i-coupled α_2 adrenergic receptors in β-cells [47]. Intermediate concentrations of GLP-1 (0.3-1.0 nM) induced [cAMP]$_{pm}$ oscillations from the baseline or a slightly elevated level in >80% of the cells, whereas higher concentrations typically induced a sustained [cAMP]$_{pm}$ elevation. The oscillatory pattern varied with frequencies ranging from 0.16 to 1.5 min^{-1} and amplitudes from 0.08 to 1.7 normalized ratio units. Glucagon also induced [cAMP]$_{pm}$ elevation with oscillations in 22% of the cells exposed to 0.3-30 nM of the hormone. It has long been known that glucagon and GLP-1 stimulate cAMP production in β-cells, but the time-course of the cAMP signal has never been studied. Oscillatory cAMP signaling involving the excretion of cAMP and the activation of an extracellular cAMP receptor is previously known from the slime mould *Dictyostelium* [143]. cAMP levels have also been found to vary during the cardiac contraction cycle [144] and in *Xenopus* embryonal neurons [126]. The present data is the first demonstration that hormone receptor activation can evoke oscillations of [cAMP]$_{pm}$.

Interplay between cAMP and Ca^{2+} in hormone-stimulated cells (*I*)

cAMP-generating stimuli are known to promote [Ca^{2+}]$_i$ signaling in β-cells by PKA-mediated modulation of K$_{ATP}$ channels, VDCCs and IP$_3$ receptors [28, 29, 72-74, 116]. Accordingly, GLP-1 induced pronounced spiking of [Ca^{2+}]$_i$ in INS-1 cells. It is also well-known that Ca^{2+} can affect the formation and breakdown of cAMP via Ca^{2+}-sensitive ACs and PDEs [83, 94]. Indeed, the hormone-induced [cAMP]$_{pm}$ oscillations immediately disappeared when extracellular Ca^{2+} was removed. Simultaneous measurements of [cAMP]$_{pm}$ and [Ca^{2+}]$_{pm}$ revealed that the oscillations of [cAMP]$_{pm}$ were synchronized with those of [Ca^{2+}]$_{pm}$. This synchronization of the triggering Ca^{2+} and amplifying

[cAMP]$_{pm}$ signals may explain how GLP-1 selectively enhances the pulsatile component of insulin secretion in healthy and diabetic subjects [145, 146].

Glucose induces [cAMP]$_{pm}$ oscillations in β-cells (*II*)

Elevation of the glucose concentration from 3 to 11 mM induced a slight decrease of [cAMP]$_{pm}$ followed after a delay by a rapid rise and pronounced oscillations in both MIN6 cells and primary mouse β-cells. The oscillatory pattern varied between cells with frequencies ranging from 0.07 to 1.8 min^{-1} and amplitudes from 0.13 to 2.4 ratio units. The nadirs between the oscillations often did not reach the baseline, but after lowering of the glucose concentration the ratio returned to the pre-stimulatory level. The glucose-induced [cAMP]$_{pm}$ oscillations were abolished by adrenaline, noradrenaline and by direct AC inhibition with 2',5'-dideoxyadenosine (DDA). The [cAMP]$_{pm}$-lowering effect of adrenaline was not secondary to its hyperpolarizing action [147], since the hormone was equally efficient in cells depolarized with a high concentration of K$^+$ in the presence of the K$_{ATP}$ channel opener diazoxide.

Although cAMP is a key regulator of exocytosis in many cells and has long been known as a potent amplifier of insulin secretion it has not been clear to what extent changes in cAMP take part in glucose-induced insulin secretion. The present findings challenge the traditional view that glucose only triggers modest elevation of cAMP in β-cells [75, 77, 81]. Glucose-induced changes of cAMP might have been underestimated in previous studies based on traditional biochemical detection of cAMP because the time-average cAMP concentration is lower than the peaks reached during oscillations. Another possibility is that the cAMP concentration is lower in the bulk cytoplasm than in the vicinity of the plasma membrane. The absence of cAMP oscillations in a recent imaging study of MIN6 cells stimulated with glucose alone [132] may be due to the use of a lower-affinity biosensor reporting cAMP concentration in the entire cytoplasm.

Ca^{2+} amplifies, but is not essential for the glucose-induced [cAMP]$_{pm}$ oscillations (*II*)

Investigation of the Ca^{2+} dependence of the glucose-induced [cAMP]$_{pm}$ signaling showed that the [cAMP]$_{pm}$ oscillations often persisted, albeit significantly suppressed, in the presence of the voltage-dependent Ca^{2+}-channel inhibitor methoxyverapamil or in Ca^{2+}-deficient medium containing 2 mM of the Ca^{2+} chelator EGTA. Similar results were obtained in primary mouse β-

cells, supporting the conclusion that glucose regulation of $[cAMP]_{pm}$ in β-cells is at least partially independent of Ca^{2+}.

Simultaneous measurements of $[Ca^{2+}]_i$ and $[cAMP]_{pm}$ in transfected MIN6-cells loaded with the fluorescent Ca^{2+} indicator Fura-2 demonstrated that oscillations of the two messengers were synchronized. The temporal relationship varied somewhat, with $[cAMP]_{pm}$ sometimes increasing before, but more often after, the initial glucose-induced rise of $[Ca^{2+}]_i$. $[cAMP]_{pm}$ always increased after $[Ca^{2+}]_i$ during subsequent oscillations with an average time difference of 40 s between elevations. A relatively large fraction of cells (63%) showed $[cAMP]_{pm}$ oscillations without corresponding changes in $[Ca^{2+}]_i$. The dissociation between the cAMP and Ca^{2+} signals was not due to the epifluorescence $[Ca^{2+}]_i$ measurements with Fura-2 being performed in a larger cell volume than the evanescent-wave microscopy recordings of $[cAMP]_{pm}$. Accordingly, a similar discrepancy between Ca^{2+} and cAMP were observed in parallel evanescent wave microscopy recordings of $[Ca^{2+}]_{pm}$ and $[cAMP]_{pm}$ using cells transfected with a modified cAMP bio-sensor with CFP-labelled catalytic subunit and unlabelled regulatory subunit to enable $[cAMP]_{pm}$ measurements without spectral overlap with the Ca^{2+} indicator Fura Red.

The glucose-induced changes in $[cAMP]_{pm}$ resemble those of $[Ca^{2+}]_i$ with a small initial lowering followed by a pronounced rise and slow oscillations with a period of 2-10 min. β-cells express Ca^{2+}-calmodulin-sensitive ACs [80, 86, 87] and phosphodiesterases [100, 132, 148] and changes in $[Ca^{2+}]_i$ can therefore be expected to influence cAMP formation and degradation. Indeed, the GLP-1-induced $[cAMP]_{pm}$ oscillations in insulinoma cells depended on Ca^{2+}. Also insulinoma cells stimulated with a combination of high glucose and the K^+ channel inhibitor TEA have previously been found to respond with Ca^{2+}-dependent cAMP oscillations [132]. However, while the presently observed hormone- and glucose-evoked $[cAMP]_{pm}$ oscillations were synchronized with Ca^{2+} oscillations, the TEA-induced oscillations were anti-synchronous, with the peaks of $[Ca^{2+}]_i$ coinciding with the nadirs of cAMP, interpreted as periodic activation of the Ca^{2+}-sensitive PDE1 family enzymes. The effect of TEA may be due to prolongation of the action potentials with excessive elevation of $[Ca^{2+}]_i$.

cAMP production is directly stimulated by cell metabolism (*II*)

There is evidence that the β-cell metabolism oscillates and that these oscillations may underlie those of $[Ca^{2+}]_i$ [59, 60]. To investigate how cAMP production in β-cells is influenced by cell metabolism, MIN6-cells were hyperpolarized with the K_{ATP} channel opener diazoxide. Elevation of glucose from

3 to 11 mM failed to affect $[cAMP]_{pm}$ under these conditions, indicating that stimulation of metabolism alone is not sufficient to trigger cAMP production in the presence of basal $[Ca^{2+}]_i$. In contrast, depolarization of the membrane with 30 mM K^+ in the presence of 3 mM glucose and diazoxide induced a rapid but modest elevation of $[cAMP]_{pm}$. This response was significantly enhanced when the cells were re-stimulated with 30 mM K^+ after elevating glucose to 11 mM in the continued presence of diazoxide. Similarly, when the glucose concentration was elevated from 3 to 11 mM during K^+ depolarization in the presence of diazoxide, there was a pronounced rise of $[cAMP]_{pm}$, often with superimposed oscillations, in both MIN6 and primary mouse β-cells. Also α-ketoisocaproic acid, which stimulates mitochondrial ATP production [149, 150], caused a marked elevation of $[cAMP]_{pm}$. The effects of glucose and α-ketoisocaproic acid on $[cAMP]_{pm}$ were not paralleled by increases of $[Ca^{2+}]_i$. Instead, elevation of glucose or addition of KIC induced a transient lowering of $[Ca^{2+}]_i$ from the levels obtained by K^+ depolarization, probably reflecting fuelling of the SERCAs that pump Ca^{2+} into the ER. Mitochondrial metabolism seems essential for nutrient-induced elevation of $[cAMP]_{pm}$, since the uncoupler carbonylcyanid-p-trifluoromethoxyphenylhydrazone immediately reversed the effect of glucose.

Among putative mediators of the glucose effect on cAMP production, ATP appears as an attractive candidate, since it is the substrate for ACs. To investigate if ATP directly stimulates cAMP production, cellular ATP consumption was acutely suppressed by ouabain inhibition of the Na^+/K^+-ATPase. Ouabain has previously been found to suppress K_{ATP} channel activity in β-cells by increasing the ATP concentration beneath the plasma membrane [151]. Consistent with the hypothesis that ATP stimulates cAMP formation, ouabain induced a rise in $[cAMP]_{pm}$ without apparent effects on $[Ca^{2+}]_i$. Direct evidence for the involvement of ATP as a regulator of cAMP production came from experiments in biosensor-expressing MIN6 cells permeabilized with staphylococcal α-toxin. Despite the fact that cAMP can be washed out from permeabilized cells, there was a significant and reversible elevation of $[cAMP]_{pm}$ when the ATP concentration was increased from 1 to 4 mM, and this response was further enhanced by application of forskolin and IBMX.

Taken together, the data show that cell metabolism is a strong stimulus for cAMP production when $[Ca^{2+}]_i$ is elevated. Even though other metabolites cannot be excluded, the present data underscore the importance of ATP as a regulator of ACs. It may be argued that cytoplasmic ATP varies relatively little and that physiological concentrations of the nucleotide are higher than the K_m of mouse islet ACs, which has been estimated to be 0.32 mM in vitro [152]. In this context, it is interesting to note that a recent study proposed that glucose-induced cAMP formation in INS-1 cells is mediated by sAC, which has a K_m for ATP in the millimolar range [91]. However, this explanation is difficult to reconcile with the present observation that essen-

tially all cAMP formation is inhibited by the membrane cyclase inhibitor DDA. Also the K_{ATP} channels [153, 154] and the SERCA pump [155] are regulated by cytoplasmic ATP, although the in vitro sensitivities to ATP are in the micromolar range. In the case of the K_{ATP} channels the ATP sensitivity is reduced by ADP, which can be formed by local ATP hydrolysis [153] or phosphotransfer reactions [154]. Metabolism and the ATP/ADP ratio are known to undergo periodic variations in β-cells [59, 60, 156]. The present results are consistent with the idea that such variations underlie the glucose-induced $[cAMP]_{pm}$ oscillations.

Constitutive PDE activity masks periodic cAMP production (*III*)

The high turnover of cAMP in many types of cells is the result of a high basal production by ACs balanced by constitutive degradation by PDEs [90]. To investigate the involvement of PDEs in maintaining low basal $[cAMP]_{pm}$ in insulin-secreting cells, various inhibitors of PDEs were applied to MIN6-cells exposed to 3 mM glucose. Non-specific inhibition of PDEs with IBMX invariably resulted in $[cAMP]_{pm}$ elevation. At a concentration of 50 µM IBMX, 55% of the cells showed a stable $[cAMP]_{pm}$ elevation. In the remaining cells, $[cAMP]_{pm}$ either increased transiently and returned to baseline after 5-10 min, or oscillated from an elevated level. At 300 µM, IBMX induced stable $[cAMP]_{pm}$ elevation in 81% of the cells and oscillations in the remaining fraction. While there was no difference in the steady-state $[cAMP]_{pm}$ level reached after IBMX stimulation, the maximal rate of cAMP elevation was more than doubled in the presence of 300µM compared to 50 µM. Analysis of the IBMX-induced $[cAMP]_{pm}$ elevation revealed that it sometimes was delayed or occurred in two steps with a rapid rise leveling off and followed by a new increase to a steady-state level. DDA inhibition of AC activity resulted in a mono-exponential lowering of $[cAMP]_{pm}$ without oscillations to a level corresponding to 36% or 56% of that obtained with 50 µM or 300 µM IBMX, respectively, indicating that up to ~40% of cAMP degradation in the sub-membrane space may be accounted for by IBMX-insensitive mechanisms.

The transient responses and the $[cAMP]_{pm}$ oscillations observed with IBMX probably reflect incomplete inhibition of cAMP degradation in combination with natural variations in the rate of cAMP production. Such variations may also explain the biphasic rise of $[cAMP]_{pm}$ upon PDE inhibition, where the plateau at an intermediate $[cAMP]_{pm}$ level may reflect a period of minimal cAMP production. As discussed above, periodic cAMP production could result from variations in metabolism [59, 60]. Since ATP seems to oscillate in both glucose-stimulated cells [64] and under basal conditions

[65], it is conceivable that the $[cAMP]_{pm}$ oscillations reflect those of ATP. The possibility that the IBMX-induced $[cAMP]_{pm}$ oscillations instead are due to variations in PDE activity appears unlikely, since general inhibition of PDE activity would then be expected to reduce $[cAMP]_{pm}$ oscillations and since $[cAMP]_{pm}$ oscillations were never observed after inhibition of cAMP production.

Under basal non-stimulatory conditions with 3 mM glucose, selective inhibition of PDE4 family isoforms with 8 µM rolipram induced a very modest $[cAMP]_{pm}$ elevation in only 11% of the cells. In contrast, the PDE1 family inhibitor MM-IBMX (100 µM) and the PDE3 family inhibitor cilostamide (0.7-8 µM) triggered a distinct $[cAMP]_{pm}$ elevation in one third and about half of the cells, respectively, and in the presence of the highest concentration of cilostamide some cells showed oscillations of $[cAMP]_{pm}$. The lack of response to inhibitors of a single PDE family in many cells may be due to other isoforms showing sufficiently high activity to maintain low $[cAMP]_{pm}$ levels. Accordingly, when MM-IBMX and cilostamide were combined there was a $[cAMP]_{pm}$ response in nearly all tested cells (95%). The magnitude of the response to combined inhibition of PDE1 and PDE3 suggested that these isoforms account for at least 75% of the basal IBMX-sensitive PDE activity in MIN6-cells. Together, these data indicate that constitutive PDE activity masks periodic basal cAMP production beneath the plasma membrane. Most of the IBMX-sensitive cAMP degradation is accounted for by PDE1 and PDE3 isoforms and IBMX-insensitive mechanisms also contribute.

PDE1, 3 and 4 are active in glucose-stimulated cells, but are not required for glucose generation of $[cAMP]_{pm}$ oscillations (*III*)

Family-selective PDE inhibitors were also used to clarify which PDEs are involved to shape the $[cAMP]_{pm}$ oscillations in glucose-stimulated MIN6 cells. In contrast to its minimal effect under basal conditions, the PDE4 inhibitor rolipram induced a distinct increase of $[cAMP]_{pm}$ in cells exposed to 20 mM glucose. However, the effect was transient and the glucose-induced $[cAMP]_{pm}$ oscillations typically continued unaltered after the initial increase. Similarly, when the glucose concentration was elevated from 3 to 20 mM in the presence of rolipram, a majority of the cells responded with $[cAMP]_{pm}$ oscillations identical to those observed in the absence of the PDE inhibitor. The mechanism underlying activation of PDE4 remains unclear, but contrary to findings in other types of cells [90], it does not involve PKA, since the rolipram-induced $[cAMP]_{pm}$ elevation was unaffected by 1-5 µM of the PKA inhibitor H-89. From these results, it can be concluded that PDE4 is active in

insulin-secreting cells after glucose stimulation, but it is not required for glucose generation of [cAMP]$_{pm}$ oscillations.

Inhibition of PDE3 with cilostamide caused [cAMP]$_{pm}$ elevation in more than 80% of the glucose-stimulated cells and in about 50% of these cells the [cAMP]$_{pm}$ oscillations remained. The peaks of these oscillations reached similar levels as those observed with glucose alone, but the nadirs were higher, which caused a significant increase of the integrated [cAMP]$_{pm}$ response. Similar results were obtained with the PDE1 inhibitor MM-IBMX in glucose-stimulated cells. Most cells thus responded with [cAMP]$_{pm}$ elevation. In some cases the glucose-induced [cAMP]$_{pm}$ oscillations were perturbed by the drug, but in other cases the oscillations continued. There were also cells in which a stable [cAMP]$_{pm}$ response was transformed into oscillations by MM-IBMX. The glucose dependence of the MM-IBMX response is not surprising, since PDE1 is activated by Ca^{2+}/calmodulin [94]. As the glucose-induced [cAMP]$_{pm}$ oscillations persisted in the presence of MM-IBMX it seems unlikely that Ca^{2+} regulation of PDE1 underlies the oscillations as has been suggested for MIN6 cells stimulated with a combination of glucose and the K^+ channel inhibitor TEA [132]. Therefore, it is not surprising that the glucose-induced oscillations often persist when Ca^{2+} entry was prevented.

The present results indicate that no single PDE isoform is essential for the generation of cAMP oscillations in the sub-membrane space. Instead, [cAMP]$_{pm}$ depends on periodic cAMP production in combination with constitutive cAMP degradation via both IBMX-sensitive and-insensitive mechanisms. Interestingly, it was recently demonstrated that the IBMX-insensitive PDE8B is expressed in islets and insulin-secreting cell lines and that down-regulation of the enzyme with RNA interference results in amplification of insulin secretion [97, 103]. Investigation of the involvement of PDE8B in the generation of [cAMP]$_{pm}$ oscillations will require tools for rapid modulation of its activity, which unfortunately are still unavailable.

[cAMP]$_{pm}$ oscillations are important for pulsatile insulin secretion (*II*)

To test if the glucose-induced oscillations of [cAMP]$_{pm}$ in β-cells are important for the kinetics of pulsatile insulin release, secretion was monitored in single cells using an optical assay. Insulin release is associated with autocrine activation of insulin receptors on the β-cell surface [139, 157], which in turn leads to activation of PI3-kinase and formation of PIP$_3$ in the plasma membrane [7, 158, 159]. It has previously been demonstrated that the changes of membrane PIP$_3$ reflects the kinetics of insulin secretion [7]. The link between exocytosis of insulin granules and autocrine activation of insu-

lin receptors was further substantiated by simultaneous evanescent wave microscopy of PIP_3 using the CFP-tagged PH domain from protein kinase B/Akt (CFP-PH_{Akt}) and plasma membrane insertion of the YFP-tagged insulin secretory vesicle protein VAMP2, a well-established marker for exocytosis [160]. As expected, the MIN6 cells responded to K^+ depolarization with prompt VAMP2-YFP membrane insertion followed by elevation of PIP_3. PIP_3 was also monitored with another biosensor, based on the "general receptor for phosphoinositides-1" (GRP-1) tagged with four tandem copies of GFP (GFP$_4$-GRP1). Compared to previously used constructs, this biosensor provides improved responses by its brighter fluorescence and nuclear exclusion. Elevation of the glucose concentration from 3 to 11 mM induced a rise of evanescent wave-excited GFP$_4$-GRP1 fluorescence with pronounced oscillations. The glucose-induced GFP$_4$-GRP1 fluorescence response was completely abolished by inhibition of the insulin receptor tyrosine kinase with hydroxyl-2-naphthalenylmethylphosphonic acid tris-acetoxymethyl ester (HNMPA-AM$_3$), by inhibition of PI3-kinase with LY294002 or insulin receptor antibodies. Similarly, the PIP_3 signal immediately returned to baseline when insulin secretion was inhibited by preventing voltage-dependent Ca^{2+} influx. Moreover, amplification of insulin secretion with the cAMP-elevating agents forskolin or IBMX enhanced the PIP_3 response. Interestingly, inhibition of ACs with DDA resulted in strong suppression or even abolishment of the glucose-induced PIP_3 oscillations without affecting those of $[Ca^{2+}]_i$, indicating that $[cAMP]_{pm}$ oscillations play an important role for pulsatile insulin release. Simultaneous measurements of PIP_3 and $[cAMP]_{pm}$ with CFP-PH_{Akt} along with non-fluorescent regulatory PKA subunit and YFP-labelled catalytic subunit showed that the oscillations of $[cAMP]_{pm}$ and PIP_3 were synchronized. The data also revealed that each $[cAMP]_{pm}$ oscillation slightly preceded the rise of PIP_3 and that there was a linear correlation between the amplitudes of $[cAMP]_{pm}$ and PIP_3 oscillations.

cAMP in the submembrane space has important effects on ion channels and exocytosis of insulin granules [29, 70, 72, 117]. The present results indicate that $[cAMP]_{pm}$ oscillations are critical for the magnitude of pulsatile insulin secretion. The glucose-induced cAMP formation, directly stimulated by sub-membrane ATP, seems to potentiate insulin secretion by sensitizing the exocytosis machinery, since inhibition of $[cAMP]_{pm}$ oscillations suppressed insulin secretion without affecting the $[Ca^{2+}]_i$ oscillations. The interplay between Ca^{2+}, ATP and cAMP may thus contribute both to the triggering and amplifying pathways of insulin secretion and help to explain how insulin secretion from isolated islets can be pulsatile at stable $[Ca^{2+}]_i$ elevation [51].

Effect of oscillatory and stable $[cAMP]_{pm}$ elevations on $[Ca^{2+}]_i$ and nuclear entry of PKA (*I*)

Signaling with oscillations have been suggested to improve low-level signal detection and to contribute to specificity in down-stream cellular responses [161-163]. To test if the temporal pattern of $[cAMP]_{pm}$ influences down-stream responses, $[Ca^{2+}]_i$ and PKA catalytic subunit localization were analyzed in INS-1 cells exposed to stable or oscillatory $[cAMP]_{pm}$ elevations. Since the large YFP tag (238 amino acids) may restrict the diffusion of catalytic subunits into the nucleus, the Cα subunit was instead labeled with 37 amino acid residues, including a tetracysteine motif that can be specifically stained with the membrane-permeable fluorescent biarsenical dye FlAsH [141]. Both sustained and oscillatory $[cAMP]_{pm}$ elevations, induced by stable or intermittent IBMX application, respectively, triggered $[Ca^{2+}]_i$ spiking in the INS-1 cells. The Ca^{2+} spikes were grouped and coincided precisely with the $[cAMP]_{pm}$ oscillations induced by intermittent IBMX stimulation. FlasH-labelled PKA-Cα was clearly excluded from the nucleus under basal conditions, but significant nuclear translocation was detected after 25 minutes of continuous IBMX application. In contrast, $[cAMP]_{pm}$ oscillations imposed by repetitive 1 min applications of IBMX followed by 3 min wash periods failed to cause nuclear PKA translocation even after exposure to similar total doses of IBMX. The results indicate that transient elevations of $[cAMP]_{pm}$ can selectively regulate local cytoplasmic events, such as ion channel activity and exocytosis, without inducing PKA signaling in the nucleus. Only prolonged $[cAMP]_{pm}$ elevations, sufficient for PKA to enter the nucleus, will activate PKA-dependent transcription factors that regulate long-term cellular effects, like changes in cell survival or differentiation (Fig.1).
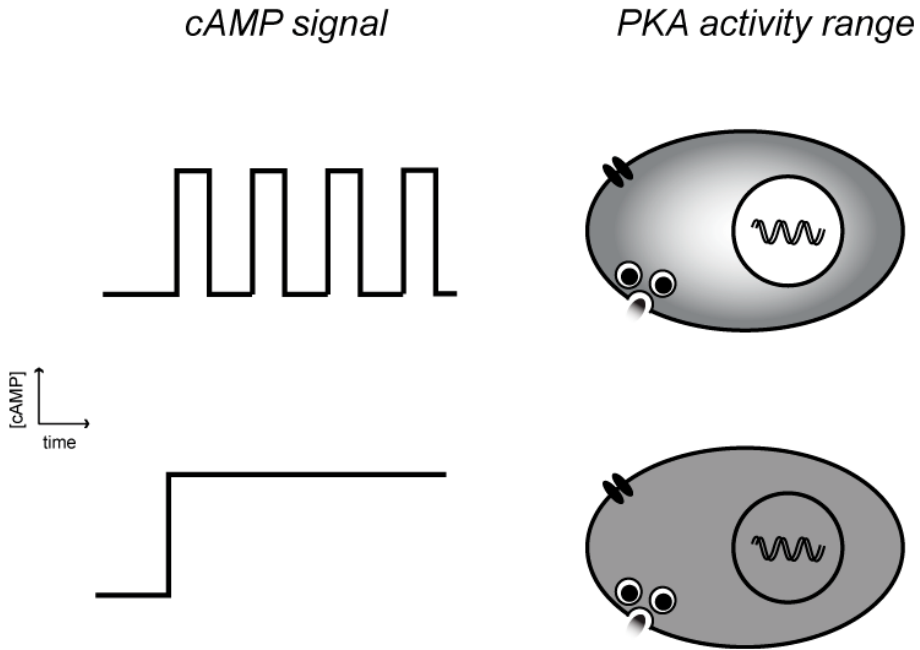
Figure 1. Schematic illustration of the effect of $[cAMP]_{pm}$ oscillations and stable $[cAMP]_{pm}$ elevation on the spatial distribution of PKA. During $[cAMP]_{pm}$ oscillations, PKA activity will be restricted to the cytoplasm due to diffusional restriction of the entry of catalytic subunits into the nucleus. Cytoplasmic PKA will e.g. affect insulin secretion in β-cells by acting on ion channels or proteins involved in insulin granule exocytosis. Extended $[cAMP]_{pm}$ elevations allow PKA to enter the nucleus, where the enzyme may regulate gene expression via CREB and other transcription factors. Adopted from [164].

Conclusions

1. Changes of $[cAMP]_{pm}$ can be recorded in individual cells using a PKA-based fluorescent translocation biosensor and ratiometric evanescent wave microscopy.

2. Stimulation of insulin-secreting cells with the G_s-coupled receptor agonists GLP-1 and glucagon trigger pronounced oscillations of $[cAMP]_{pm}$, whereas the G_i-activating hormones adrenaline and noradrenaline suppress $[cAMP]_{pm}$ levels. Both MIN6-cells and primary mouse β-cells respond to glucose stimulation with a marked elevation and prominent $[cAMP]_{pm}$ oscillations.

3. Constitutive PDE activity masks periodic basal cAMP production beneath the plasma membrane. cAMP degradation in non-stimulated cells is mainly accounted for by PDE1 and PDE3, but also by IBMX-insensitive mechanisms. The PDE1, -3 and -4 families are active in glucose-stimulated cells, but no single PDE isoform is required for glucose generation of $[cAMP]_{pm}$ oscillations.

4. GLP-1-induced $[cAMP]_{pm}$ oscillations are Ca^{2+}-dependent and synchronized with $[Ca^{2+}]_{pm}$ oscillations and there is a mutual dependence between Ca^{2+} and cAMP in hormone-stimulated insulinoma cells. $[Ca^{2+}]_i$ and $[cAMP]_{pm}$ oscillations are synchronized also in glucose-stimulated cells. However, although Ca^{2+} amplifies the glucose-induced cAMP response, $[Ca^{2+}]_i$ oscillations are not required for the glucose-induced $[cAMP]_{pm}$ oscillations.

5. Cell metabolism is a strong stimulus for cAMP production when $[Ca^{2+}]_i$ is elevated, an effect that seems mediated by intracellular ATP. Glucose-induced $[cAMP]_{pm}$ oscillations depend, at least in part, on periodic stimulation of cAMP formation by metabolically generated ATP.

6. Brief elevation of $[cAMP]_{pm}$ is sufficient to induce cytoplasmic effects, whereas sustained elevation is required to induce translocation of PKA catalytic subunit into the nucleus. Signaling with $[cAMP]_{pm}$ oscillations may consequently contribute to specificity in downstream responses by restricting the spatial distribution of PKA.

7. Glucose-induced cAMP formation stimulates insulin secretion by sensitizing the exocytosis machinery to the Ca^{2+} signal. Oscillations of $[cAMP]_{pm}$ are critical for the magnitude of pulsatile insulin secretion.

Svensk sammanfattning

Hormonet insulin frisätts från β-celler i de så kallade Langerhanska öarna i bukspottkörteln. Insulin sänker blodets glukoskoncentration genom att stimulera sockerupptaget i fett- och muskelceller och genom att minska leverns glukosproduktion. Otillräcklig frisättning av insulin leder till försämrad glukostolerans eller sockersjuka (diabetes mellitus). Det finns två former av diabetes mellitus: typ-1, som orsakas av autoimmun destruktion av β-cellerna, och typ-2, som karaktäriseras av otillräcklig insulinsekretion och insulinresistens. Den senare typen svarar för omkring 90% av all diabetes och antalet drabbade ökar i snabb takt.

Insulinfrisättningen stimuleras av glukos, andra näringsämnen, hormoner och nervsignalämnen. När glukoskoncentrationen ökar transporteras sockret snabbt in i β-cellen och bryts ned i ämnesomsättningen. I denna process bildas den energirika föreningen ATP som genom inverkan på kaliumkanaler i plasmamembranet depolariserar membranet, vilket i sin tur resulterar i inflöde av kalciumjoner genom spänningsberoende kanaler. Det är ökningen av kalciumkoncentrationen i cytoplasman som utlöser frisättningen av insulininnehållande sekretkorn från β-cellen. Kalciumkoncentrationen uppvisar ofta regelbundna variationer, så kallade oscillationer. Kalciumoscillationer i β-cellerna tros vara en viktig förklaring till att insulinfrisättningen pulserar hos friska individer.

Många hormoner och nervsignalämnen förstärker insulinsekretionen genom att binda till cellytereceptorer som aktiverar en signaleringskaskad som resulterar i frisättning av kalciumjoner från intracellulära förråd eller i bildning av budbärarmolekylen cykliskt adenosinmonofosfat (cAMP). cAMP reglerar många cellfunktioner, däribland insulinfrisättningen, bland annat genom att göra sekretionsmaskineriet mera känsligt för kalciumsignalen. Hormonerna glukagon och GLP-1 stimulerar insulinfrisättningen framför allt genom att öka koncentrationen av cAMP i β-cellerna. Däremot är oklart i vilken utsträckning cAMP har betydelse för glukosstimulerad insulinsekretion. En av förklaringarna till denna oklarhet är att det tills helt nyligen varit omöjligt att mäta cAMP i enskilda levande celler.

I avhandlingens första delarbete beskrivs hur cAMP-koncentrationen kan mätas direkt under plasmamembranet på enskilda celler med totalreflexionsmikroskopi och en nykonstruerad biosensor som baseras på de regulatoriska och katalytiska subenheterna av proteinkinas A (PKA) som märkts med fluorescerande protein. Studierna visar att stimulering av klonala INS-1 β-

celler med glukagon och GLP-1 inte bara leder till en ökning av cAMP-koncentrationen utan att den oscillerar. Samtidiga mätningar av kalcium visar att cAMP-oscillationerna är synkroniserade med kalciumoscillationer och de båda budbärarna är ömsesidigt beroende av varandra. Vidare studeras effekterna av oscillerande respektive stabila cAMP-ökningar på kalciumsignalering och på translokering av PKA till cellkärnan. Medan kortvariga cAMP-ökningar omdelebart initierade effekter i cellmembranet krävdes långvariga ökningar för att PKA skulle kunna komma in i cellkärnan. Resultaten tyder sålunda på att cAMP-oscillationer kan vara viktiga för att begränsa PKA-aktiviteten till cytoplasman, vilket kan bidra till specificitet i signaleringen.

Den andra uppsatsen beskriver effekten av glukos på de insulinproducerande cellernas cAMP-signalering. I motsats till den gängse uppfattningen att glukos endast stimulerar en blygsam cAMP-ökning i β-cellerna visas här att sockret orsakar kraftiga cAMP-oscillationer i såväl klonala celler som i primära β-celler från mus. cAMP-oscillationerna är ofta, men inte alltid, samordnade med kalciumoscillationer. I arbetet prövas hypotesen att intracellulära ATP-ökningar kan stimulera cAMP-bildningen. Sammantaget visas att kalciumökningar är tillräckliga för att orsaka cAMP-bildning, men då intracellulära kalciumkoncentrationen är förhöjd kommer ATP som genererats av ämnesomsättningen att kraftigt stimulera cAMP-bildningen. Det är väl dokumenterat att ämnesomsättningen i insulin-frisättande celler är cyklisk med en period som motsvarar den som observeras för cAMP-oscillationerna. I uppsatsen visas också att glukos-inducerade cAMP-oscillationer sammanfaller med oscillerande frisättning av insulin från isolerade β-celler. När insulin frisätts aktiveras insulinreceptorer på β-cellytan, vilket i sin tur stimulerar bildning av fosfolipiden PIP_3 i cellmembranet. PIP_3 kan mätas i totalreflexionsmikroskopet med en fluorescerande markör och det kan visas att fluorescensförändringarna mycket väl avspeglar insulinfrisättningens kinetik. De glukosinducerade insulinoscillationerna försvinner då cAMP-bildningen hämmas och förstärks då cAMP-nivåerna ökas på farmakologisk väg. Resultaten påvisar vikten av ett fungerande samspel mellan cAMP, Ca^{2+} och ATP invid cellmembranet för normal insulinfrisättningskinetik.

Mekanismerna bakom β-cellens cAMP-oscillationer undersöktes vidare i avhandlingens tredje delarbete. Den stora grupp av enzymer som kallas fosfodiesteraser, och som negativt reglerar cAMP-signalering genom att bryta ner cAMP till inaktivt AMP, undersöktes med hjälp av isoform-specifika farmakologiska inhibitorer. Tidigare biokemiska studier har identifierat PDE1, PDE3 och PDE4 som de viktigaste fosfodiesteraserna i insulinproducerande celler. Det visar sig nu att en partiell hämning av fosfodiesteraserna med isobutylmetylxantin (IBMX) kan framkalla cAMP-oscillationer, vilket tolkas som att oscillationerna beror på periodisk cAMP-bildning som normalt balanseras av hög fosfodiesterasaktivitet. Hämning av fosfodieste-

rasgrupperna PDE1 och PDE3, men inte PDE4, leder till ökning av cAMP-koncentrationen under plasmamembranet under basala betingelser. Efter glukosstimulering av cellerna har också PDE4-hämmaren en cAMP-höjande effekt, men den påverkade inte cAMP-oscillationerna. I påfallande många celler kvarstod cAMP-oscillationerna också efter hämning av PDE1 och PDE3. Eftersom hämning av cAMP-bildningen leder till kraftig sänkning av cAMP-koncentrationen även i närvaro av en hög koncentration av IBMX, verkar också en IBMX-okänslig mekanism spela en viktig roll för cAMP-nedbrytning i insulinfrisättande celler. Denna mekanism, tillsammans med PDE1 och PDE3, bidrar sålunda till regleringen av cAMP-koncentrationen invid plasmamembranet i β-cellen, men tycks inte förklara uppkomsten av cAMP-oscillationer. Slutsatsen att cAMP-oscillationerna istället genereras genom variationer i bildningshastigheten stämmer väl med resultaten i det föregående arbetet som visar att cellmetabolismen kan reglera cAMP-produktionen.

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