

Role of phosphodiesterases in the shaping of sub-plasma-membrane cAMP oscillations and pulsatile insulin secretion

Geng Tian¹, Jenny Sâgetorp¹, Yunjian Xu¹, Hongyan Shuai¹, Eva Degerman² and Anders Tengholm^{1,*}

¹Department of Medical Cell Biology, Uppsala University, Biomedical Centre, Box 571, SE-751 23 Uppsala, Sweden

²Department of Experimental Medical Science, Division for Diabetes, Metabolism and Endocrinology, Lund University, Lund, Sweden

*Author for correspondence (anders.tengholm@mcb.uu.se)

Accepted 20 July 2012

Journal of Cell Science 125, 5084–5095

© 2012. Published by The Company of Biologists Ltd

doi: 10.1242/jcs.107201

Summary

Specificity and versatility in cyclic AMP (cAMP) signalling are governed by the spatial localisation and temporal dynamics of the signal. Phosphodiesterases (PDEs) are important for shaping cAMP signals by hydrolyzing the nucleotide. In pancreatic β -cells, glucose triggers sub-plasma-membrane cAMP oscillations, which are important for insulin secretion, but the mechanisms underlying the oscillations are poorly understood. Here, we investigated the role of different PDEs in the generation of cAMP oscillations by monitoring the concentration of cAMP in the sub-plasma-membrane space ($[cAMP]_{pm}$) with ratiometric evanescent wave microscopy in MIN6 cells or mouse pancreatic β -cells expressing a fluorescent translocation biosensor. The general PDE inhibitor IBMX increased $[cAMP]_{pm}$, and whereas oscillations were frequently observed at 50 μ M IBMX, 300 μ M–1 mM of the inhibitor caused a stable increase in $[cAMP]_{pm}$. The $[cAMP]_{pm}$ was nevertheless markedly suppressed by the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine, indicating IBMX-insensitive cAMP degradation. Among IBMX-sensitive PDEs, PDE3 was most important for maintaining a low basal level of $[cAMP]_{pm}$ in unstimulated cells. After glucose induction of $[cAMP]_{pm}$ oscillations, inhibitors of PDE1, PDE3 and PDE4 inhibited the average cAMP level, often without disturbing the $[cAMP]_{pm}$ rhythmicity. Knockdown of the IBMX-insensitive PDE8B by shRNA in MIN6 cells increased the basal level of $[cAMP]_{pm}$ and prevented the $[cAMP]_{pm}$ -lowering effect of 2',5'-dideoxyadenosine after exposure to IBMX. Moreover, PDE8B-knockdown cells showed reduced glucose-induced $[cAMP]_{pm}$ oscillations and loss of the normal pulsatile pattern of insulin secretion. It is concluded that $[cAMP]_{pm}$ oscillations in β -cells are caused by periodic variations in cAMP generation, and that several PDEs, including PDE1, PDE3 and the IBMX-insensitive PDE8B, are required for shaping the sub-membrane cAMP signals and pulsatile insulin release.

Key words: Islets, Pancreatic β -cells, Adenylyl cyclase, PDE3, PDE8, PDE4, PDE1

Introduction

Cyclic AMP (cAMP) is a ubiquitous intracellular messenger, which regulates numerous cell functions, including metabolism, gene expression and secretion. Changes of cAMP concentration are typically spatially confined and precisely regulated in time. The tight spatio-temporal control is an important prerequisite for the versatility and specificity of cAMP signalling pathways, and is, at least in part, obtained by subcellular compartmentalization of adenylyl cyclases and phosphodiesterases (PDEs), the enzymes generating and degrading the nucleotide, respectively (Conti and Beavo, 2007; Willoughby and Cooper, 2007; Houslay, 2010). The subcellular localisation of adenylyl cyclases and PDEs is determined by protein–protein and protein–lipid interactions, often through anchoring proteins that also bind regulating factors and downstream effectors, such as protein kinases, phosphatases and their substrates, thereby generating spatially compartmentalized signalling complexes (Taskén and Aandahl, 2004; Wong and Scott, 2004).

The PDEs constitute a large group of enzymes that are divided into 11 subfamilies, which differ with regard to substrate specificity (some of them hydrolyzing cyclic GMP), kinetics and regulatory properties (Bender and Beavo, 2006; Lugnier,

2006; Conti and Beavo, 2007). The PDE1 family of enzymes is activated by Ca^{2+} -calmodulin (Goraya and Cooper, 2005), and cAMP hydrolysis by PDE2 is stimulated by cGMP. PDE3 is a dual-specificity enzyme with kinetic properties resulting in cGMP-inhibited cAMP degradation (Thompson et al., 2007; Murata et al., 2009). PDE4, PDE7 and PDE8 are all cAMP-specific enzymes. The more than 20 different PDE4 family members are characterised by multiple different molecular targeting interactions for subcellular localisation as well as by regulation by phosphorylation mediated through protein kinase A (PKA), extracellular signal-regulated kinase (ERK) and MAP-kinase-activated protein kinase 2 (MK2) (Houslay and Adams, 2003; Houslay, 2010). PDE5, PDE6 and PDE9 are cGMP-selective, and the relatively recently described and yet poorly characterised PDE10 and 11 family enzymes show dual substrate specificity (Bender and Beavo, 2006).

PDE activity has been extensively characterised in many cell types by using biochemical methods based on hydrolysis of radioactive nucleotides. All isoforms, except for PDE8 and PDE9, can be inhibited by 3-isobutyl-methylxanthine (IBMX) (Lugnier, 2006). The recent development of tools for online monitoring of cAMP concentration (Zaccolo et al., 2000; Rich

et al., 2001; Zaccolo and Pozzan, 2002; DiPilato et al., 2004; Nikolaev et al., 2004; Dyachok et al., 2006a; Herget et al., 2008) or PDE activity (Takakusa et al., 2002) in living cells has enabled more detailed studies of the function of PDEs in compartmentalization of cAMP signalling. For example, cAMP measurements with cyclic nucleotide gated channels or fluorescence sensors have shown that the cAMP concentration displays varying kinetics in different subcellular compartments owing to compartmentalization of PDEs (Rich et al., 2001; Terrin et al., 2006). Real-time cAMP measurements have also revealed complex temporal patterns, including oscillations. cAMP oscillations were first described as an important property of developing *Dictyostelium* slime moulds (Gerisch and Wick, 1975). These oscillations involve the excretion of cAMP with binding to extracellular cell surface receptors (Maeda et al., 2004). cAMP oscillations have subsequently been demonstrated to occur spontaneously in developing neural circuits (Gorbunova and Spitzer, 2002; Dunn et al., 2006) and after treatment of insulin-secreting MIN6 cells with a combination of high concentrations of glucose and a K^+ channel inhibitor (Landa et al., 2005). We recently developed a ratiometric evanescent wave microscopy approach to determine the concentration of cAMP in the sub-plasma-membrane space ($[cAMP]_{pm}$) and found that $[cAMP]_{pm}$ undergoes pronounced oscillations in isolated hormone- (Dyachok et al., 2006a) and glucose-stimulated (Dyachok et al., 2008) insulin-secreting cells as well as in α - and β -cells within intact pancreatic islets (Tian et al., 2011). However, the mechanisms underlying these oscillations are poorly understood.

In pancreatic β -cells, cAMP strongly amplifies the secretion of insulin. The nucleotide acts through both PKA and the cAMP-dependent guanine nucleotide exchange factor EPAC at multiple levels to potentiate Ca^{2+} -dependent exocytosis (Ämmälä et al., 1993; Renström et al., 1997; Dyachok and Gylfe, 2004; Seino and Shibasaki, 2005). The coordination of Ca^{2+} and cAMP oscillations are important to generate pulsatile insulin secretion (Dyachok et al., 2008). Pancreatic islets and insulin-secreting β -cells express several PDE isozymes. Early studies demonstrated that pancreatic islets show Ca^{2+} -calmodulin-sensitive PDE activity (Sugden and Ashcroft, 1981; Lipson and Oldham, 1983; Capito et al., 1986), and expression of PDE1 isoforms has subsequently been demonstrated by RT-PCR analysis in islets and β -cell lines (Han et al., 1999; Dov et al., 2008; Waddleton et al., 2008). PDE3 exists in two isoforms, PDE3A and PDE3B. PDE3B is expressed in β -cells and has been demonstrated to be important for insulin secretion (Parker et al., 1995; Zhao et al., 1997; Härndahl et al., 2002; Pyne and Furman, 2003; Choi et al., 2006; Waddleton et al., 2008), probably by affecting the most distal steps of granule fusion (Walz et al., 2007). Furthermore, PDE4 has been found to have a function in islets and insulin-secreting cells (Parker et al., 1995; Shafiee-Nick et al., 1995; Waddleton et al., 2008). Recent studies have also identified members of the PDE5, PDE7, PDE8, PDE9, PDE10 and PDE11 families in rodent and human islets and in insulin-secreting cell lines (Waddleton et al., 2008; Heimann et al., 2010). Silencing of PDE8B expression with RNA interference was found to potentiate insulin secretion in rat islets and insulinoma cells (Dov et al., 2008; Waddleton et al., 2008). An increasing number of family-selective pharmacological PDE inhibitors have been reported, and PDEs are attractive targets for enhanced insulin secretion in type 2 diabetes. The aim of the present study was to

determine how different PDE families contribute to the regulation of cAMP concentration in the sub-plasma-membrane space and, in particular, their functions in the generation of the pronounced $[cAMP]_{pm}$ oscillations in β -cells that underlie the glucose-stimulated pulsatile release of insulin.

Results

Constitutive PDE activity masks a periodic production of cAMP

In β -cells exposed to a basal medium containing 3 mM glucose, the $[cAMP]_{pm}$ was low and stable. Inhibition of PDEs with IBMX invariably resulted in an increase in $[cAMP]_{pm}$. At a concentration of 50 μ M, IBMX caused a stable increase in $[cAMP]_{pm}$ in 11 out of 20 MIN6 cells (55%, Fig. 1A). In the remaining cells, the $[cAMP]_{pm}$ increase was either transient with return to baseline levels after 5–10 minutes, or showed oscillations typically occurring from an elevated level (Fig. 1B). At 300 μ M, IBMX induced a stable increase in $[cAMP]_{pm}$ in 29 out of 36 cells (81%) and oscillations in the remaining seven cells (not shown). Although there was no difference in the apparent $[cAMP]_{pm}$ level reached after stimulation with IBMX (50 μ M, 1.46 ± 0.07 ratio units; 300 μ M, 1.46 ± 0.05 ratio units), the maximal rate of increase in $[cAMP]_{pm}$ was more than doubled in the presence of 300 μ M IBMX (Fig. 1C). Similar results were obtained in primary mouse β -cells. Thus, 50 μ M IBMX induced a stable increase in $[cAMP]_{pm}$ in 13 out of 18 cells (72%, Fig. 1D) and oscillations in the remaining cells (Fig. 1E). The IBMX-induced $[cAMP]_{pm}$ increase was reversed by 5 μ M adrenaline, which suppresses the formation of cAMP in β -cells by activating G_i -coupled α_2 -adrenoceptors (Schuit and Pipeleers, 1986). This response can be used to distinguish β -cells from α -cells, in which adrenaline increases $[cAMP]_{pm}$ by activating G_s -coupled β -adrenoceptors (Schuit and Pipeleers, 1986; Tian et al., 2011) (Fig. 1D,E). The oscillatory $[cAMP]_{pm}$ responses probably reflect incomplete inhibition of PDEs in combination with natural variations in the rate of cAMP production. Analysis of the IBMX-induced increase in $[cAMP]_{pm}$ revealed that it sometimes occurred in two steps, with a rapid rise that levelled off before increasing again to a steady-state level (Fig. 1D,F). In other cases, the opposite pattern was observed, that is, a slow initial response followed by a sudden acceleration (data not shown). These biphasic responses were seen in 55% ($n=20$) of MIN6 cells and 50% ($n=18$) of primary β -cells at 50 μ M IBMX, and in 30.5% ($n=36$) of MIN6 cells at 300 μ M of the PDE inhibitor. Periodic changes of $[cAMP]_{pm}$ were never observed when cAMP production was suppressed. Thus, inhibition of membrane adenylyl cyclase activity with 100 μ M 2',5'-dideoxyadenosine (DDA) resulted in a mono-exponential reduction in $[cAMP]_{pm}$ without oscillations to a level corresponding to $36 \pm 4\%$ ($n=6$) or $56 \pm 4\%$ ($n=17$) of that obtained with 50 μ M (data not shown) or 300 μ M IBMX (Fig. 1G). A similar reduction in $[cAMP]_{pm}$ (to $61 \pm 5\%$; $n=9$) was obtained even in the presence of 1 mM of the PDE inhibitor (Fig. 1H). Together, these data indicate that constitutive PDE activity masks periodic basal levels of cAMP production beneath the plasma membrane and that the cAMP degradation is accounted for both by IBMX-sensitive and -insensitive mechanisms.

PDE3 accounts for most of the basal levels of IBMX-sensitive PDE activity in β -cells

To determine the PDE isoforms that maintain low $[cAMP]_{pm}$ under basal (i.e. non-stimulated) conditions, PDE family-specific pharmacological inhibitors were applied to MIN6 cells or islets in

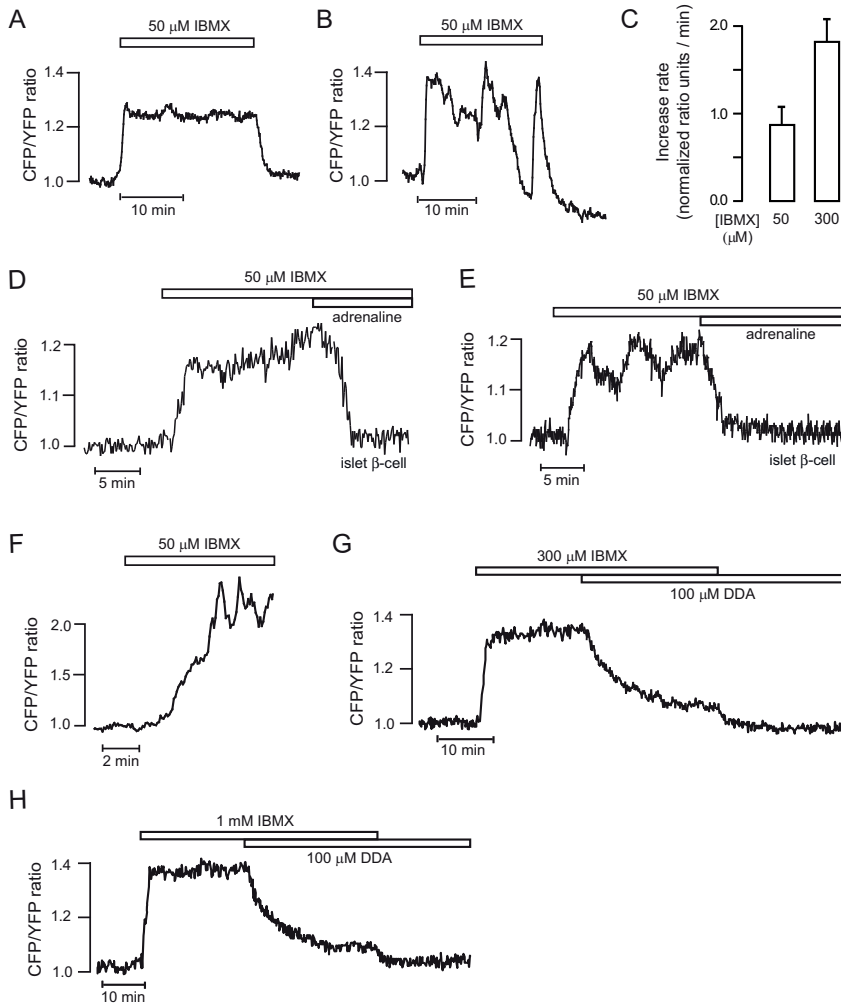


Fig. 1. Constitutive PDE activity masks periodic basal levels of cAMP production beneath the plasma membrane. Ratiometric evanescent wave microscopy recordings of $[cAMP]_{pm}$ in individual insulin-secreting MIN6 cells or mouse islet beta-cells, expressing a fluorescent translocation and maintained in buffer containing 3 μM glucose. (A) PDE inhibition with 50 μM IBMX induces stable $[cAMP]_{pm}$ elevation in a single MIN6 cell. (B) Example of a MIN6 cell showing fluctuations of $[cAMP]_{pm}$ in response to 50 μM IBMX. (C) Mean $\pm$ s.e.m. for the maximal rates of initial $[cAMP]_{pm}$ elevation in MIN6 cells after PDE inhibition with 50 ($n=18$) or 300 μM ($n=14$) IBMX. (D) Stable $[cAMP]_{pm}$ elevation induced by 50 μM IBMX in a mouse islet β -cell identified by the $[cAMP]_{pm}$ -lowering effect of adrenaline. (E) Oscillations of $[cAMP]_{pm}$ induced by 50 μM IBMX in an adrenaline-identified mouse islet β -cell. (F) Illustration of the step-wise elevation of $[cAMP]_{pm}$ in a MIN6 cell after application of 50 μM IBMX. (G,H) Suppression of $[cAMP]_{pm}$ after inhibition of transmembrane adenylyl cyclases with 100 μM 2',5'-dideoxyadenosine (DDA) in a MIN6 cell exposed to 300 μM (G) or 1 mM (H) IBMX.

the presence of 3 mM glucose. In MIN6 cells, inhibition of isoforms from the PDE4 family with 8 μM rolipram caused only a very modest $[cAMP]_{pm}$ elevation in 11% of the cells ($n=44$; Fig. 2A,K,L). A larger fraction of the cells (33%, $n=15$) responded to 100 μM of the PDE1 inhibitor 8-methoxymethyl-IBMX (MM-IBMX), showing a CFP:YFP ratio increase of 0.13 ± 0.03 ($n=5$; Fig. 2B,K,L). The PDE3 inhibitor cilostamide (0.7–1 μM) caused an increase in $[cAMP]_{pm}$ in 52% of the cells ($n=29$; ratio increase 0.16 ± 0.04 ; Fig. 2C,K,L). Increasing the concentration to 8 μM did not increase the number of responding cells, but at this concentration, cilostamide induced oscillations of $[cAMP]_{pm}$ in five out of 12 cells (Fig. 2D). Similar results were obtained with primary β -cells within mouse islets, but compared with MIN6 cells, the primary cells were more responsive to PDE4 inhibition (Fig. 2E–G; Fig. 2K,L). The inhibitors of PDE4, PDE1 and PDE3 thus induced an increase in $[cAMP]_{pm}$ in 39% (five out of 13 cells; 0.16 ± 0.014 ratio units; Fig. 2E,K,L), 27% (three out of 11 cells; 0.19 ± 0.009 ratio units; Fig. 2F,K,L) and 50% (five out of 10 cells; 0.23 ± 0.03 ratio units; Fig. 2G,K,L), respectively, of β -cells identified with 5 μM adrenaline.

The lack of response to inhibitors of a single PDE family in many cells might be owing to other isoforms showing sufficiently high activity to maintain low $[cAMP]_{pm}$. Accordingly, when MM-IBMX and cilostamide were

combined, there was a response in 95% of the MIN6 cells with an average CFP:YFP increase of 0.34 ± 0.04 ratio units ($n=19$; Fig. 2H,K,L). Addition of IBMX increased the $[cAMP]_{pm}$ by an additional 0.10 ± 0.03 units. In a similar manner, the combination of cilostamide and rolipram evoked a response in 79% of the cells, averaging 0.31 ± 0.03 ratio units ($n=21$; Fig. 2I–L). These findings indicate that PDE3 accounts for most of the basal levels of IBMX-sensitive PDE activity in the sub-membrane space of β -cells. Although PDE1 is the second most important isoform in MIN6 cells, PDE4 and PDE1 both contribute substantially in primary mouse β -cells.

PDE4 is active in glucose-stimulated cells, but is not required for glucose generation of $[cAMP]_{pm}$ oscillations

Next, we investigated which PDE isoforms are involved to shape the $[cAMP]_{pm}$ signals in glucose-stimulated cells. As previously demonstrated in our laboratory, for individual β -cells (Dyachok et al., 2008) and β -cells within intact islets (Tian et al., 2011), elevation of the glucose concentration often results in pronounced oscillations of $[cAMP]_{pm}$. The amplitudes of the oscillations in MIN6 cells averaged 0.27 ± 0.02 ratio units and the period was 4.7 ± 0.4 minutes (Fig. 3A; $n=21$). In contrast to its minimal effect in non-stimulated MIN6 cells, the PDE4 inhibitor rolipram induced a transient increase in $[cAMP]_{pm}$ in cells exposed to 20 mM glucose (Fig. 3B; $n=7$). After the first peak,

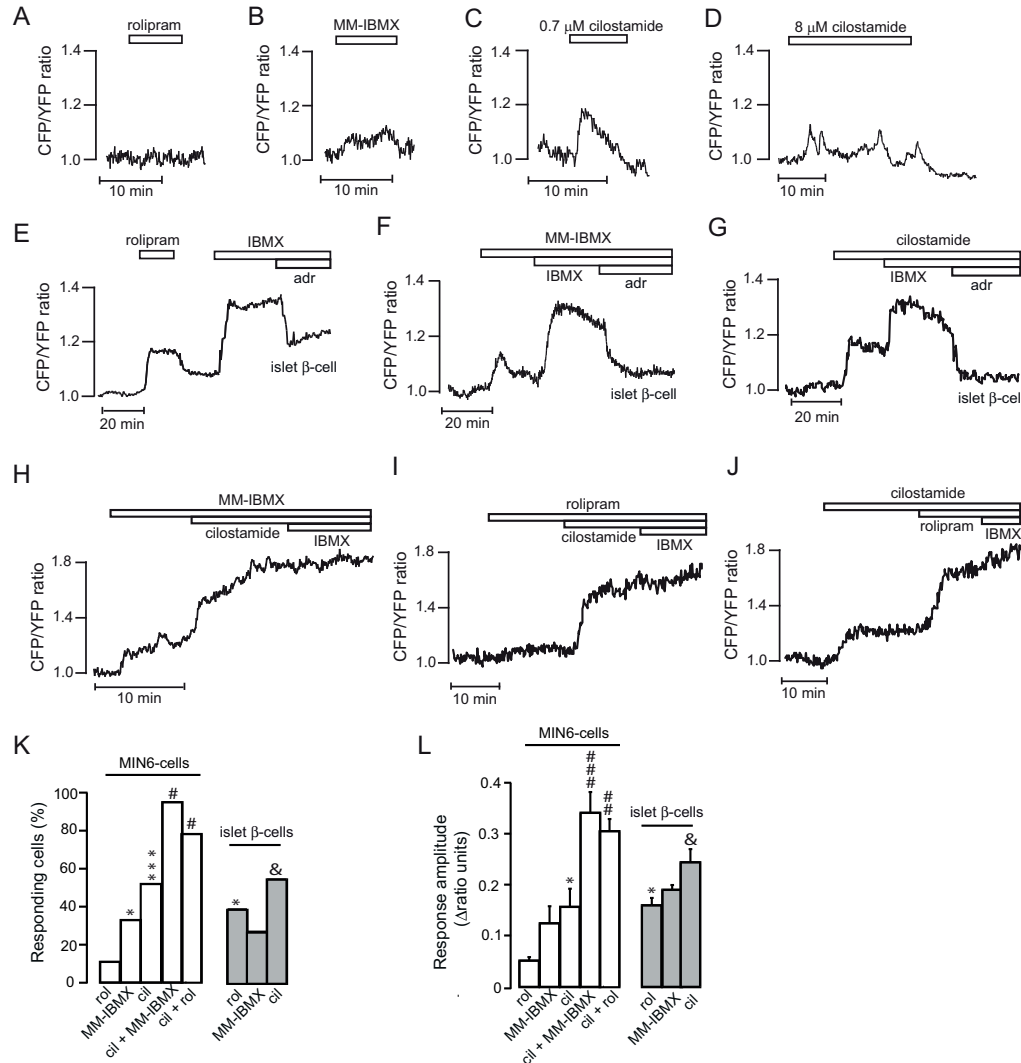


Fig. 2. PDE1 and PDE3 isoforms account for most of the basal levels of PDE activity. Ratiometric evanescent wave microscopy recordings of $[cAMP]_{pm}$ in individual insulin-secreting MIN6 cells or mouse islet β -cells expressing a fluorescent translocation biosensor, which are maintained in buffer containing 3 mM glucose. (A) The small effect of PDE4 inhibition with 8 μ M rolipram on $[cAMP]_{pm}$ in a MIN6 cell. (B) Inhibition of PDE1 with 100 μ M MM-IBMX induces a modest increase in $[cAMP]_{pm}$ in a MIN6 cell. (C) An increase in $[cAMP]_{pm}$ induced by PDE3 inhibition with 0.7 μ M cilostamide in a MIN6 cell. (D) $[cAMP]_{pm}$ oscillations induced by 8 μ M cilostamide in a MIN6 cell. (E) PDE4 inhibition with 10 μ M rolipram induces an increase in $[cAMP]_{pm}$ in a mouse islet β -cell identified by the $[cAMP]_{pm}$ -lowering effect of adrenaline. (F) $[cAMP]_{pm}$ responses to sequential additions of 100 μ M MM-IBMX, 50 μ M IBMX and 5 μ M adrenaline in a mouse islet β -cell. (G) An increase in $[cAMP]_{pm}$ induced by PDE3 inhibition with 1 μ M cilostamide in an adrenaline-identified mouse islet β -cell. (H) $[cAMP]_{pm}$ responses in a MIN6 cell after sequential additions of 100 μ M MM-IBMX, 0.7 μ M cilostamide and 50 μ M IBMX. (I) $[cAMP]_{pm}$ responses in a MIN6 cell after sequential additions of 10 μ M rolipram, 1 μ M cilostamide and 50 μ M IBMX. (J) $[cAMP]_{pm}$ responses in a MIN6 cell after sequential additions of 1 μ M cilostamide, 10 μ M rolipram, and 50 μ M IBMX. (K) Fractions of MIN6 cells and mouse islet β -cells that respond by $[cAMP]_{pm}$ elevation to different isoform-selective PDE inhibitors. *, $P < 0.05$, ***, $P < 0.005$ for the difference between the effect of each PDE inhibitor and that of rolipram in MIN6 cells; #, $P < 0.05$ for the difference between the effect of the PDE inhibitor combination and that of cilostamide in MIN6 cells; &, $P < 0.05$ compared with the effect of MM-IBMX in mouse islet β -cells. (L) Mean amplitude $\pm$ s.e.m. of the increase in $[cAMP]_{pm}$ in the responding MIN6 cells and mouse islet β -cells. *, $P < 0.05$ for the difference from the effect of rolipram in MIN6 cells; ##, $P < 0.01$, ###, $P < 0.005$ for the difference from the effect of cilostamide in MIN6 cells; &, $P < 0.05$ for the difference from the effect of rolipram in mouse islet β -cells.

$[cAMP]_{pm}$ oscillations typically continued unaltered in the presence of the PDE4 inhibitor (Fig. 3B,G). In cells showing an initial glucose-induced $[cAMP]_{pm}$ transient followed by a stable increase at suprabasal levels of $[cAMP]_{pm}$, rolipram sometimes evoked a sustained oscillatory pattern (Fig. 3C, $n=6$). Overall, rolipram significantly increased the time-average level of $[cAMP]_{pm}$ (Fig. 3H). Primary mouse islet β -cells responded in a similar way to the MIN6 cells, and in the presence of 20 mM

glucose, rolipram caused a modest amplification of the sugar-induced $[cAMP]_{pm}$ oscillations (Fig. 3D; $n=4$) or evoked pronounced oscillations from a slightly elevated baseline level (Fig. 3E; $n=2$). Although PDE4 activity is known to be regulated by PKA (Houslay and Adams, 2003), the rolipram-induced elevation of $[cAMP]_{pm}$ in glucose-stimulated MIN6 cells was not affected by 1–5 μ M of the PKA inhibitor H89 (0.14 ± 0.03 , $n=15$ with 5 μ M H89 vs 0.14 ± 0.02 , $n=16$ in control; Fig. 3F). Thus,

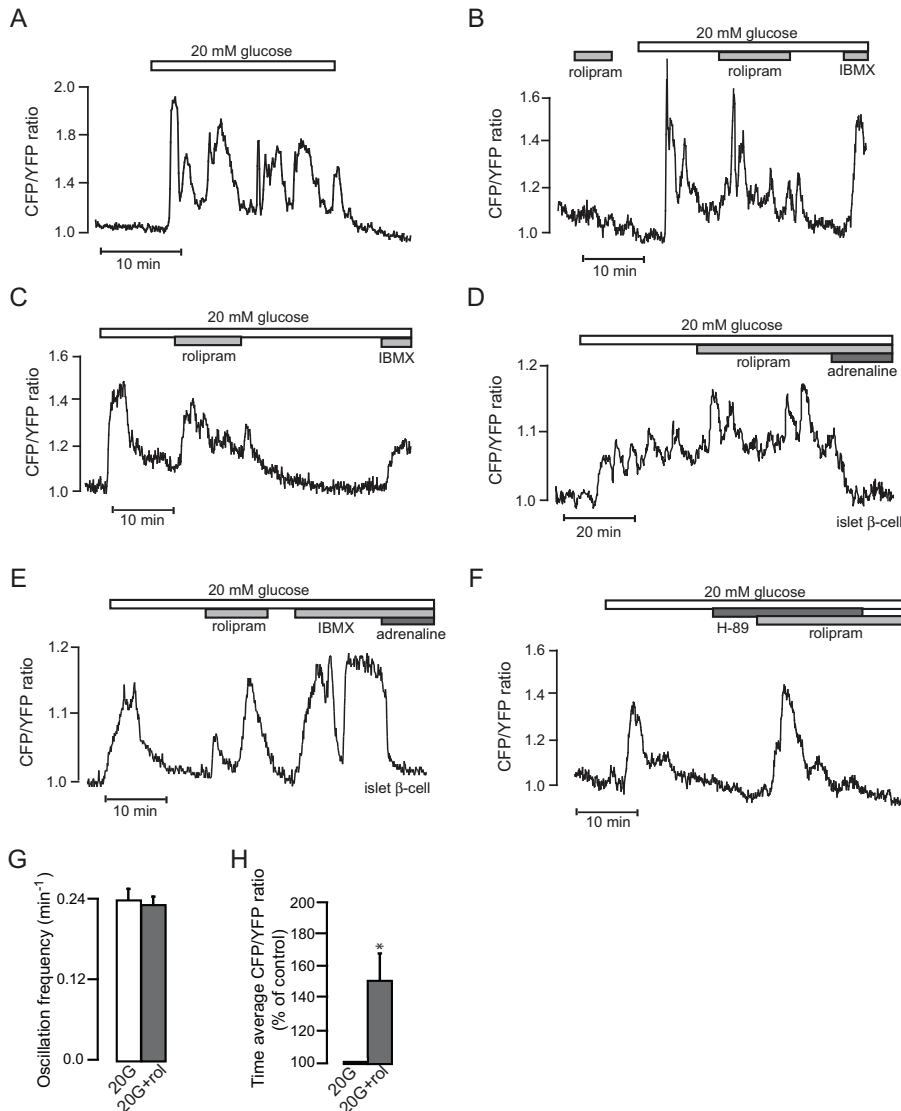


Fig. 3. PDE4 is active in glucose-stimulated cells, but is not required for glucose generation of $[cAMP]_{pm}$ oscillations. Ratiometric evanescent wave microscopy recordings of $[cAMP]_{pm}$ in individual insulin-secreting MIN6 cells or mouse islet β -cells expressing a fluorescent translocation biosensor. (A) Elevation of the glucose concentration from 3 to 20 mM triggers oscillations of $[cAMP]_{pm}$ in a MIN6 cell. (B) The effect of 8 μ M of the PDE4 inhibitor rolipram in a MIN6 cell before and after elevation of glucose from 3 to 20 mM. (C) Induction of $[cAMP]_{pm}$ oscillations by 8 μ M rolipram in a MIN6 cell exposed to 20 mM glucose. (D) Modest amplification of glucose-induced $[cAMP]_{pm}$ oscillations by 10 μ M rolipram in an adrenaline-identified mouse β -cell. (E) Induction of $[cAMP]_{pm}$ oscillations by 10 μ M rolipram in an adrenaline-identified mouse islet β -cell exposed to 20 mM glucose. (F) The PKA inhibitor H89 fails to prevent the rolipram-induced $[cAMP]_{pm}$ elevation in glucose-stimulated cells. (G) Mean $\pm$ s.e.m. for the frequency of $[cAMP]_{pm}$ oscillations induced by 20 mM glucose in MIN6 cells in the absence and presence of 10 μ M rolipram. (H) Mean $\pm$ s.e.m. for the effect of rolipram on the time-average $[cAMP]_{pm}$ level in MIN6 cells expressed as a percentage of the control level at 20 mM glucose. *, $P < 0.05$.

PDE4 seems to be activated by glucose stimulation in a PKA-independent manner in insulin-secreting cells, but is not required for the generation of $[cAMP]_{pm}$ oscillations.

PDE1 and PDE3 shape glucose-induced $[cAMP]_{pm}$ signals, but are not required for the generation of oscillations

The majority of MIN6 β -cells stimulated with 20 mM glucose responded to 0.7 μ M of the PDE3 inhibitor cilostamide with an increase in $[cAMP]_{pm}$ (83%, $n=24$), and in 50% of the responding cells there were oscillations in $[cAMP]_{pm}$ (Fig. 4A). The frequency of these oscillations did not differ from the control, but the time-integrated $[cAMP]_{pm}$ response was significantly amplified (Fig. 4G,H). Similar responses were seen with 8 μ M cilostamide, with some cells showing continued oscillations from an elevated level ($n=12$; Fig. 4B), and others showing a stable increase in $[cAMP]_{pm}$ ($n=11$; not shown). Primary mouse islet β -cells responded essentially as MIN6 cells. Thus, five out of eight cells responded to 1 μ M cilostamide with an increase in $[cAMP]_{pm}$ and continued oscillations (Fig. 4C).

In the presence of 20 mM glucose, the responses to the PDE1 inhibitor MM-IBMX were similar to those of cilostamide in both

MIN6 and primary mouse islet β -cells. Accordingly, most of the cells responded with an increase in $[cAMP]_{pm}$ (18 of 24 MIN6 cells, and six out of eight primary β -cells). In some MIN6 cells, the glucose-induced $[cAMP]_{pm}$ oscillations were perturbed by the drug ($n=5$; Fig. 4D), but in other cases the oscillations continued ($n=4$) or were even restored by MM-IBMX with maintained frequency and an increased average $[cAMP]_{pm}$ level ($n=8$; Fig. 4E,G,H). An example of a primary mouse islet β -cell where MM-IBMX increases the average $[cAMP]_{pm}$ level without inhibiting the oscillatory pattern is shown in Fig. 4F. Exposure to IBMX induced further $[cAMP]_{pm}$ elevation, which was counteracted by adrenaline. These results demonstrate that PDE1 and PDE3 contribute to shaping the glucose-induced $[cAMP]_{pm}$ signals, but neither of them are essential for the generation of cAMP oscillations.

IBMX-insensitive PDE8B has an important role in shaping $[cAMP]_{pm}$ in β -cells

We next assessed the involvement of IBMX-insensitive PDEs in shaping $[cAMP]_{pm}$ signals in β -cells. Measurements of PDE activity in MIN6 β -cell lysates showed that $7.2 \pm 0.3\%$ ($n=5$) of the

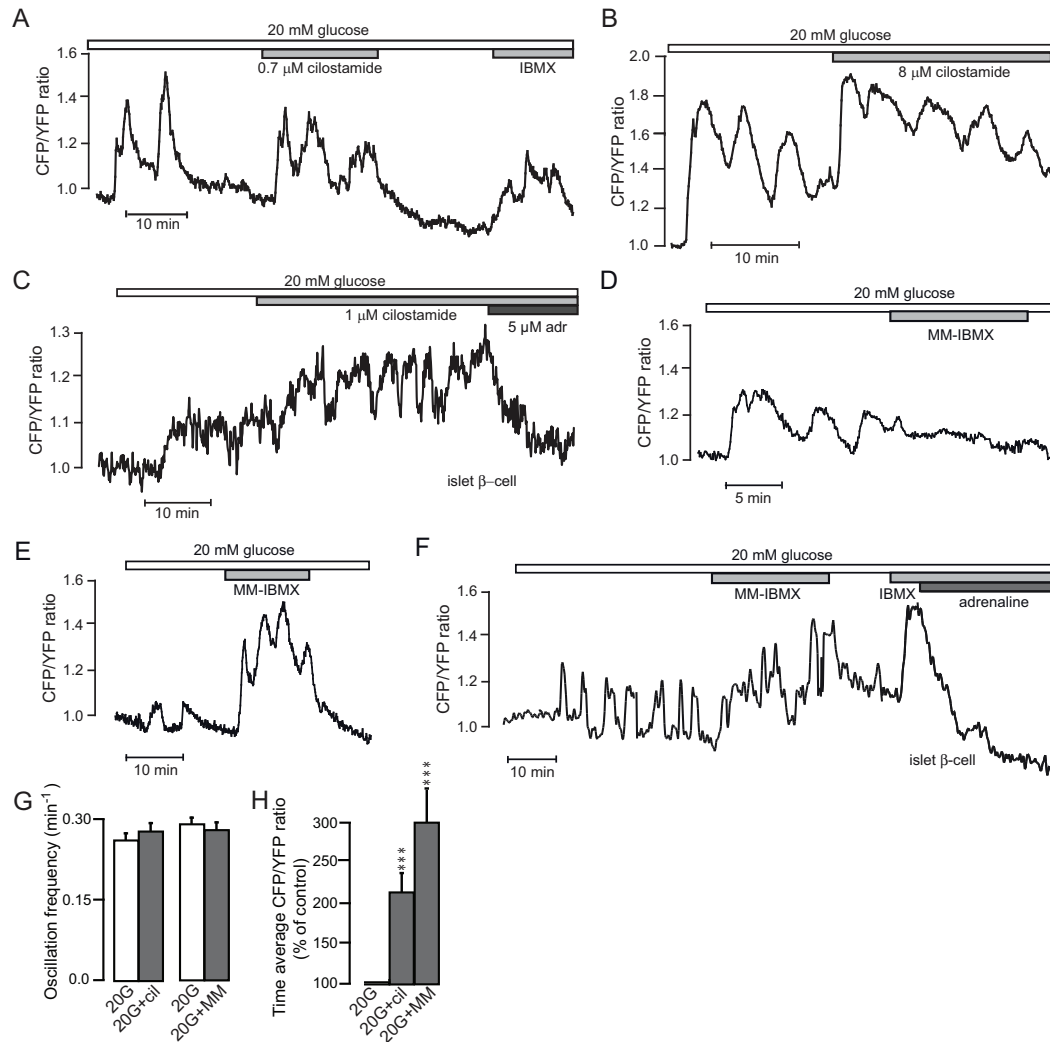


Fig. 4. Glucose-induced [cAMP]_{pm} oscillations require neither PDE1 nor PDE3 activity. Ratiometric evanescent wave microscopy recordings of [cAMP]_{pm} in individual insulin-secreting MIN6 cells or mouse islet β -cells expressing a fluorescent translocation biosensor. (A) Induction of [cAMP]_{pm} oscillations by 0.7 μ M cilostamide in a MIN6 cell exposed to 20 mM glucose. (B) Elevation of the average [cAMP]_{pm} level with maintenance of glucose-induced [cAMP]_{pm} in a MIN6 cell after the addition of 8 μ M cilostamide. (C) Induction of [cAMP]_{pm} oscillations by 1 μ M cilostamide in the presence of 20 mM glucose in a primary islet β -cell identified by the [cAMP]_{pm}-lowering effect of adrenaline. (D) Perturbation of glucose-induced [cAMP]_{pm} oscillations in a MIN6 cell by inhibition of PDE1 with 100 μ M MM-IBMX. (E) Elevation of the average [cAMP]_{pm} level with maintenance of glucose-induced [cAMP]_{pm} oscillations in a MIN6 cell after addition of 100 μ M MM-IBMX. (F) Elevation of the average [cAMP]_{pm} level with maintenance of glucose-induced [cAMP]_{pm} oscillations in an adrenaline-identified mouse islet β -cell after addition of 100 μ M MM-IBMX. (G) Mean $\pm$ s.e.m. for the frequency of [cAMP]_{pm} oscillations induced by 20 mM glucose in MIN6 cells in the absence and presence of 1 μ M cilostamide and 100 μ M MM-IBMX. (H) Mean $\pm$ s.e.m. for the effect of cilostamide and MM-IBMX on the time-average [cAMP]_{pm} level in MIN6 cells expressed as a percentage of the control level at 20 mM glucose. ***, $P < 0.005$.

total PDE activity remained after inhibition of PDE1, PDE3 and PDE4 with MM-IBMX, cilostamide and rolipram. IBMX inhibited an additional $54 \pm 6\%$ showing that the IBMX-insensitive activity only constitutes a few percent of the total PDE activity. Nevertheless, IBMX-insensitive PDEs might be functionally important in specific subcellular compartments. Among IBMX-insensitive isoforms, PDE8B is expressed in islets and insulin-secreting cell lines (Dov et al., 2008; Waddleton et al., 2008). The limited availability of useful pharmacological inhibitors of PDE8 makes it difficult to study this family of enzymes. Dipyrindamole can be used to inhibit PDE8, but the drug is fluorescent and interfered spectrally with the present [cAMP]_{pm} biosensor measurements (data not shown). In the PDE assay, dipyrindamole

inhibited $63 \pm 3\%$ ($n=5$) of the small fraction remaining after inhibition of PDE1, PDE3 and PDE4. The non-specific PDE inhibitor papaverine (100 μ M) induced increases in [cAMP]_{pm} in the presence of IBMX and reduced the [cAMP]_{pm}-lowering effect of adrenaline, consistent with the presence of IBMX-insensitive PDE activity in primary mouse β -cells (Fig. 5A). To pinpoint the involvement of enzymes of the PDE8 family, MIN6 cells were treated with lentiviral vectors expressing shRNA directed against PDE8B. After 48 hours, the PDE8B mRNA level was approximately half of that in control cells expressing non-target shRNA, whereas the levels of PDE1C, PDE3B and PDE4A were unaffected (Fig. 5B). Knockdown of PDE8B did not result in any significant change of the small dipyrindamole-sensitive or

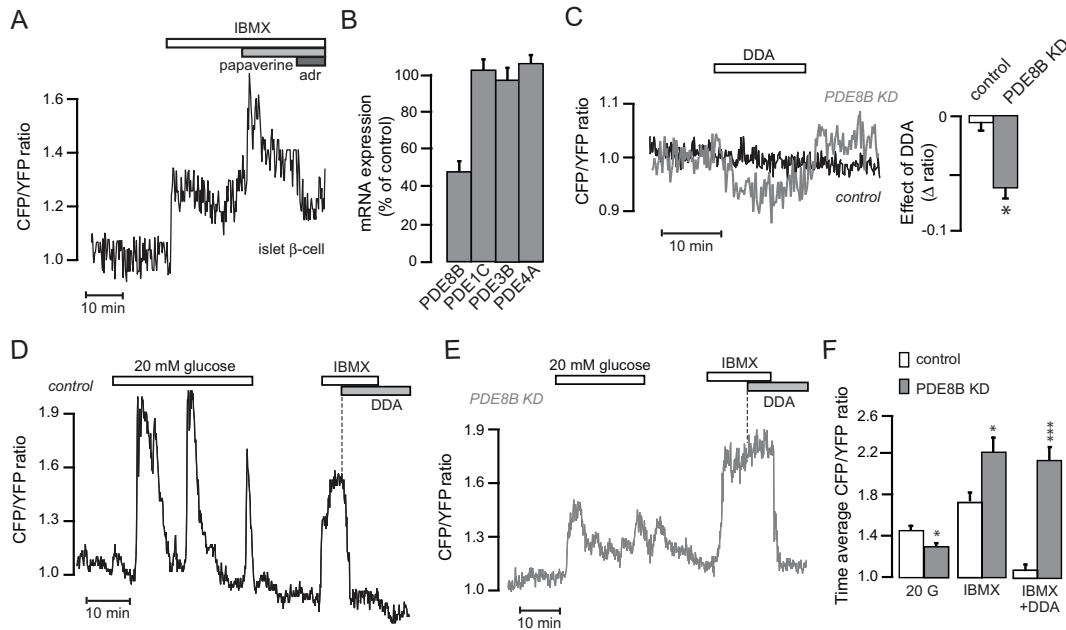


Fig. 5. IBMX-insensitive PDE8B has an important role in shaping $[cAMP]_{pm}$ in β -cells. Ratiometric evanescent wave microscopy recordings of $[cAMP]_{pm}$ in individual insulin-secreting MIN6 cells or primary mouse islet β -cells expressing a fluorescent translocation biosensor. (A) Amplification of the $[cAMP]_{pm}$ elevation induced by 100 μM IBMX by the non-selective PDE inhibitor papaverine (100 μM) in a mouse islet β -cell identified by the $[cAMP]_{pm}$ -lowering effect of adrenaline. (B) Expression of PDE1C, PDE3B, PDE4A and PDE8B mRNA in MIN6 cells detected with real-time PCR 48 hours after treatment with a lentivirus expressing shRNA directed against PDE8B, or non-targeted shRNA as a control. (C) Inhibition of adenylyl cyclases with 100 μM DDA lowers the basal level of $[cAMP]_{pm}$ in unstimulated MIN6 cells, which are maintained at 3 mM glucose after knockdown of PDE8B, but not in the control. The bar diagram shows the mean $\pm$ s.e.m. for the effect of DDA. (D) Glucose-induced oscillations of $[cAMP]_{pm}$ in a non-target shRNA-expressing MIN6 cell. IBMX induces a stable increase in $[cAMP]_{pm}$, which is reversed by 100 μM DDA. (E) Glucose-induced elevation of $[cAMP]_{pm}$ in a MIN6 cell treated with a lentivirus expressing shRNA against PDE8B. IBMX induces a stable increase in $[cAMP]_{pm}$, which is not affected by DDA. (F) Mean $\pm$ s.e.m. for the time-average $[cAMP]_{pm}$ after exposure to 20 mM glucose, IBMX or the combination of IBMX and DDA in PDE8B-shRNA expressing and control MIN6 cells. *, $P < 0.05$, ***, $P < 0.001$ for the difference between PDE8B KD and the control.

IBMX-insensitive PDE activity in the cell lysates (data not shown). However, in the single-cell measurements, the basal level of $[cAMP]_{pm}$ was elevated in knockdown cells as indicated by a distinct and reversible $[cAMP]_{pm}$ -lowering effect of adenylyl cyclase inhibition with DDA (100 μM), which was not observed in control cells (Fig. 5C).

The glucose response in MIN6 cells treated with non-target shRNA lentivirus was unaffected, and 25 out of 29 cells (86%) reacted to a step increase of glucose from 3 to 20 mM with pronounced $[cAMP]_{pm}$ oscillations (Fig. 5D). Elevation of $[cAMP]_{pm}$ induced by 50 μM IBMX was almost completely suppressed by DDA (Fig. 5D,F). After knockdown of PDE8B, the increase in $[cAMP]_{pm}$ in response to glucose was less pronounced (Fig. 5E,F) and only 45% of the cells ($n=31$) showed oscillations, which in many cases occurred from an elevated level. In 32% of the cells, the glucose-induced increase in $[cAMP]_{pm}$ was stable. IBMX induced a higher response in knockdown cells compared with that of the control (Fig. 5F) and 100 μM DDA failed to reduce the level of $[cAMP]_{pm}$ in the presence of IBMX (Fig. 5E,F). These data indicate that PDE8B accounts for most of the IBMX-insensitive PDE activity in the β -cell sub-plasma-membrane space, and that this isoform has an important function both for determining the basal level of $[cAMP]_{pm}$ and for shaping glucose-induced $[cAMP]_{pm}$ oscillations.

PDE effects on insulin secretion kinetics

Because $[cAMP]_{pm}$ oscillations are important for the magnitude and kinetics of insulin secretion (Dyachok et al., 2008), we

investigated the effect of the various PDEs on insulin-release kinetics. The time-course of insulin secretion from single MIN6 cells was monitored using the fluorescent translocation sensor GFP₄-GRP1, which reports formation of PtdIns(3,4,5) P_3 in the plasma membrane following insulin receptor activation (Dyachok et al., 2008). Most cells that express the sensor responded to a rise of glucose from 3 to 20 mM with pronounced PtdIns(3,4,5) P_3 oscillations reflecting pulsatile secretion of insulin (46 of 54 cells; Fig. 6A,F,G). Inhibition of PDE3 with cilostamide did not affect the frequency of oscillations but caused a modest, though significant, increase in the peak amplitude of the oscillations and a marked increase in the time-average levels of PtdIns(3,4,5) P_3 (Fig. 6A; Fig. 6F–H). Removal of cilostamide was sometimes associated with reduction of PtdIns(3,4,5) P_3 and loss of the oscillations, with restoration of the response only occurring after reintroduction of the drug (Fig. 6A). In eight of the 54 cells, glucose triggered an initial increase in the level of PtdIns(3,4,5) P_3 followed by a modest stable elevation. Cilostamide induced oscillations of PtdIns(3,4,5) P_3 from an elevated level (data not shown). Rolipram affected neither the amplitude nor the frequency of the PtdIns(3,4,5) P_3 oscillations, but in a similar manner to cilostamide, removal of the drug sometimes resulted in loss of the oscillatory response (Fig. 6B; Fig. 6F–H; $n=47$).

Inhibition of PDE1 with 100 μM MM-IBMX resulted in a marked increase in the level of PtdIns(3,4,5) P_3 and loss of the oscillations ($n=39$; Fig. 6C,H). A similar perturbation of the oscillatory response was obtained with 30 μM of the drug, but

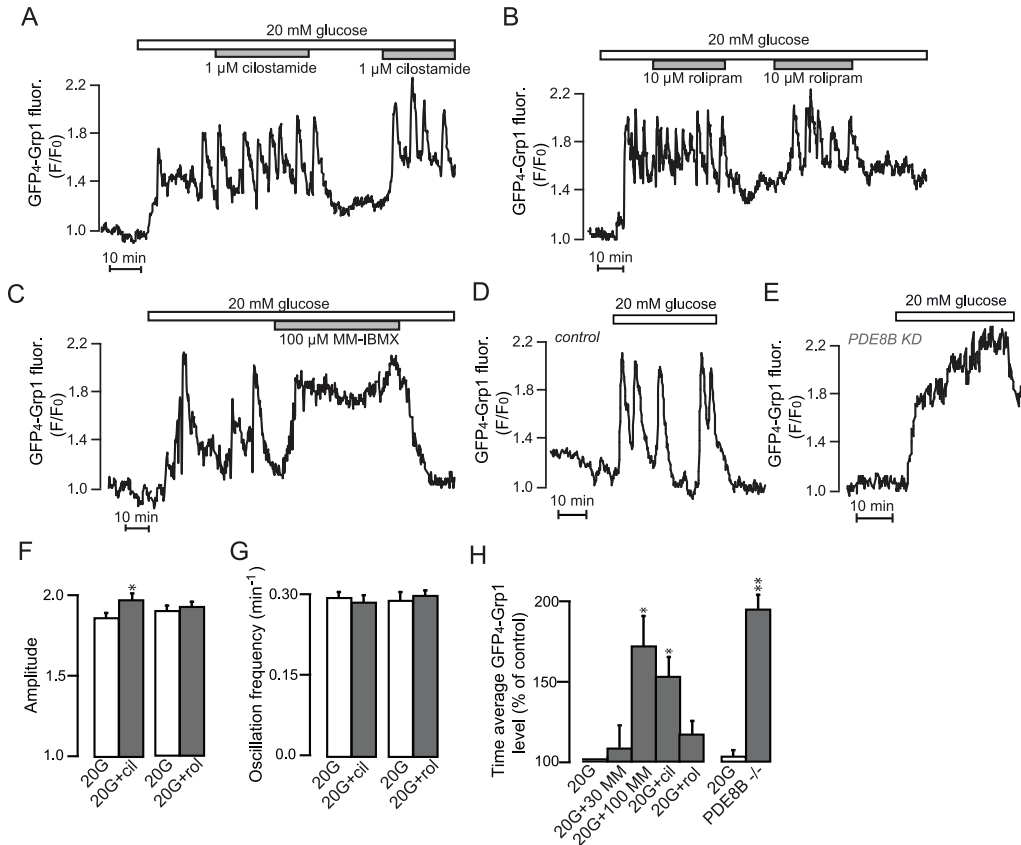


Fig. 6. PDE effects on insulin secretion kinetics. Evanescent wave microscopy recordings of PtdIns(3,4,5)P₃, which reflects insulin secretion kinetics, using the GFP₄-GRP1 translocation reporter expressed in single MIN6 cells. (A–C) The effect of 1 μM cilostamide (A), 10 μM rolipram (B) and 100 μM MM-IBMX (C) on the PtdIns(3,4,5)P₃ response induced by an increase in the glucose concentration from 3 to 20 mM. (D) Glucose-induced PtdIns(3,4,5)P₃ oscillations in a MIN6 cell expressing non-targeted control shRNA. (E) Glucose triggers a pronounced, stable increase in PtdIns(3,4,5)P₃ in a MIN6 cell expressing shRNA against PDE8B. (F,G) Mean ± s.e.m. for the amplitude (F) and frequency (G) of glucose-induced PtdIns(3,4,5)P₃ oscillations in the absence and presence of cilostamide and rolipram. *, $P < 0.05$. (H) Mean ± s.e.m. for the effect of family-selective PDE inhibitors or PDE8B-shRNA expression on the time-average PtdIns(3,4,5)P₃ levels in MIN6 cells. Paired data from inhibitor experiments are expressed as the time-average level in the presence of the PDE inhibitor as a percentage of the control level at 20 mM glucose in the same cell. The unpaired shRNA data are expressed as the time-average level as a percentage of the mean value from all control cells. *, $P < 0.05$; **, $P < 0.01$ for the effect of PDE inhibition or knockdown.

this was not associated with elevation of the time-average level of PtdIns(3,4,5)P₃ (Fig. 6H; $n = 61$). Similarly, after PDE8B knockdown, the glucose-induced PtdIns(3,4,5)P₃ response was typically stable (Fig. 6D,E; 25 of 27 cells) and the time-average GFP₄-GRP1 fluorescence was nearly doubled compared with the control (Fig. 6H), consistent with a marked amplification of insulin secretion.

Discussion

The specificity and versatility in cAMP signalling pathways depend on spatial compartmentalization and precise temporal control of the cAMP concentration. Pancreatic β -cells constitute an interesting model to study the spatio-temporal dynamics of cAMP because these cells display pronounced cAMP oscillations in the sub-membrane space after stimulation with physiological regulators of insulin secretion (Dyachok et al., 2006a; Dyachok et al., 2008). In the present study, we investigated the involvement of PDEs in shaping sub-membrane cAMP signals and found that no single PDE isoform is essential for the generation of cAMP oscillations, but that [cAMP]_{pm} depends on periodic cAMP production in combination with constitutive

cAMP degradation through both IBMX-sensitive and -insensitive mechanisms. PDE8B was found to account for most of the IBMX-insensitive PDE activity in the sub-membrane space, and together with PDE1 and PDE3, has a key function in the regulation of insulin-secretion kinetics.

Inhibition of most PDEs by IBMX resulted in dose-dependent elevation of [cAMP]_{pm} at 3 mM glucose, consistent with cAMP production being balanced by PDE-mediated degradation. Although IBMX is also an adenosine receptor inhibitor, and β -cells express adenosine A₁ receptors, the activation of which results in a reduction in cAMP (Bertrand et al., 1989), it is unlikely that A₁-receptor antagonism mediated by IBMX would contribute to the [cAMP]_{pm} elevation in unstimulated cells. The observation that [cAMP]_{pm} often fluctuates in the presence of low IBMX concentrations indicates that there are variations in the basal rate of cAMP production that become unmasked upon partial suppression of cAMP degradation. As expected, further suppression of cAMP degradation resulted in the disappearance of the oscillations. Variations in the rate of cAMP production should also be reflected in the rate of [cAMP]_{pm} elevation. Indeed, the IBMX-induced increase of [cAMP]_{pm} was sometimes

delayed or occurred in two steps. The temporary 'plateau' at an intermediate $[cAMP]_{pm}$ level might thus reflect a period of relatively low cAMP production.

Periodic cAMP production could result from variations in metabolism. It is well established that metabolism in β -cells and other types of cells oscillates (Hess and Boiteux, 1971; Longo et al., 1991; Jung et al., 2000). We have recently shown that glucose-induced cAMP production is mediated by an increase in the levels of metabolically derived ATP (Dyachok et al., 2008). Because ATP seems to oscillate in both glucose-stimulated β -cells (Ainscow and Rutter, 2002) and under basal conditions (Dryselius et al., 1994), it is conceivable that the oscillations in $[cAMP]_{pm}$ reflect those of ATP. Apart from being a precursor for cAMP, it remains to be established if ATP also has other regulating influences on adenylyl cyclases. The possibility that oscillations in $[cAMP]_{pm}$ instead are due to inherent variations in PDE activity appears unlikely. The presence of $[cAMP]_{pm}$ oscillations when most PDEs were inhibited by IBMX does not exclude the fact that IBMX-insensitive mechanisms show periodic activity. However, $[cAMP]_{pm}$ oscillations were not observed after inhibition of cAMP production, which would have been expected if PDE activity showed intrinsic oscillations.

Pharmacological inhibitors were used to identify PDE families involved in cAMP degradation under basal and glucose-stimulated conditions. MIN6 cells and β -cells within intact mouse islets overall responded in a similar manner. The effects of the PDE1 inhibitor MM-IBMX were modest in the presence of 3 mM glucose, which does not exclude that this enzyme is active under basal conditions. An increase in cAMP might escape detection if it occurs outside the sub-membrane compartment where the biosensor is located. The PDE activity that remains when a single isoform is inhibited might still be sufficient to maintain low levels of $[cAMP]_{pm}$. Consistent with the latter idea, it was found that 95% of the cells responded to a combination of MM-IBMX and the PDE3 inhibitor cilostamide, whereas only 33 and 52%, respectively, responded to either of the drugs alone. A similar synergistic effect was observed with combined inhibition of PDE3 and PDE4. The MM-IBMX response was more pronounced in glucose-stimulated cells, which is not surprising, because glucose stimulation of β -cells is associated with elevation of the cytoplasmic Ca^{2+} concentration (Grapengiesser et al., 1988) and PDE1 is activated by Ca^{2+} -calmodulin (Goraya and Cooper, 2005). As the glucose-induced $[cAMP]_{pm}$ oscillations continued in many cells exposed to 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 tetraethylammonium (Landa et al., 2005). Their independence of the Ca^{2+} -calmodulin-sensitive PDE1 is in line with our previous observation that the oscillations often persist when Ca^{2+} entry is prevented (Dyachok et al., 2008). Nevertheless, consistent with previous studies (Han et al., 1999; Waddleton et al., 2008) inhibition of PDE1 markedly amplified insulin release, and this effect was associated with a disturbed pulsatile secretion pattern.

The rise of $[cAMP]_{pm}$ after inhibition of PDE4 in primary β -cells is consistent with the previously reported role of this isoform in islets (Parker et al., 1995; Shafiee-Nick et al., 1995; Waddleton et al., 2008). In the MIN6 cells, PDE4 seemed active only after stimulation with glucose. The mechanism underlying the activation of PDE4 by glucose is unclear. The enzyme shows

a relatively low affinity for cAMP (Bender and Beavo, 2006), and activity is therefore stimulated by the elevated levels of the nucleotide. Although some PDE4 isoforms are regulated by PKA (Houslay and Adams, 2003), the glucose-induced PDE4 activity did not depend on this kinase, as rolipram also readily increased $[cAMP]_{pm}$ in the presence of H89. Inhibition of PDE3 gave the most pronounced increases of $[cAMP]_{pm}$ in both resting and glucose-stimulated cells, which is consistent with previous studies identifying PDE3B as a main regulator of cAMP in cellular compartments that are relevant for insulin secretion. For example, β -cells or transgenic mice overexpressing PDE3B show reduced insulin secretion (Härndahl et al., 2002; Härndahl et al., 2004), and genetic or pharmacological reduction of PDE3B activity amplifies secretion (Choi et al., 2006; Waddleton et al., 2008). The strong effect of a PDE3 inhibitor on the concentration of cAMP in the sub-membrane space is also in line with the observation that PDE3 is often associated with membranes (Shakur et al., 2001), including the plasma membrane and secretory-granule membranes (Walz et al., 2007). Although $[cAMP]_{pm}$ levels and insulin release were greatly increased by cilostamide, the drug neither prevented $[cAMP]_{pm}$ oscillations nor interfered with pulsatile insulin secretion.

Previous biochemical studies have indicated that 90% of total PDE activity in insulin-secreting cells can be inhibited by IBMX (Pyne and Furman, 2003), and we have now found that an even higher percentage can be inhibited in MIN6 β -cells. However, this does not mean that the remaining fraction is unimportant. The observation that inhibition of adenylyl cyclases suppressed $[cAMP]_{pm}$ by ~40% even in the presence of 1 mM IBMX indicates that the cAMP degradation in the sub-membrane space accounted for by IBMX-insensitive mechanisms is functionally important. Interestingly, it was recently demonstrated that the IBMX-insensitive PDE8B is expressed in islets and insulin-secreting cell lines, and that downregulation of the enzyme by RNA interference results in amplification of insulin secretion (Dov et al., 2008; Waddleton et al., 2008). We now found that PDE8B accounts for most, if not all, of the IBMX-insensitive PDE activity in the sub-membrane space. Accordingly, inhibition of adenylyl cyclases failed to counteract the IBMX-induced $[cAMP]_{pm}$ elevation after knockdown of PDE8B. PDE8B is a cAMP-specific enzyme with a K_m of 0.15 μ M (Soderling et al., 1998). Consistent with its high affinity for cAMP, this isoform contributed substantially to maintaining low basal levels of cAMP. Elevated basal $[cAMP]_{pm}$ might explain why the magnitude of the glucose-induced $[cAMP]_{pm}$ response appeared reduced in the knockdown cells. The amplitude of the IBMX responses were nevertheless elevated, which should not be surprising under conditions when most of the cAMP degrading capacity of the cell is inhibited. Furthermore, the oscillatory $[cAMP]_{pm}$ signalling pattern was disturbed when expression of PDE8B was suppressed. The functional importance of this isoform was underlined by the finding that insulin pulsatility was abolished in glucose-stimulated knockdown cells. The loss of insulin pulsatility was associated with increased average secretion, which is in line with the previous observation that knockdown of PDE8B amplifies insulin secretion in rat islets (Dov et al., 2008).

cAMP oscillations have been observed in several systems and might be a widespread phenomenon fulfilling diverse functions in various cell types. In developing neurons, the motility response to axon guidance cues has been found to depend on cAMP oscillations (Nicol et al., 2007), and in insulin-secreting cells,

[cAMP]_{pm} oscillations are translated to oscillations in insulin exocytosis (Dyachok et al., 2008). Moreover, the duration of a cAMP signal has been found to be crucial for the translocation of PKA catalytic subunits from the cytoplasm into the nucleus (Dyachok et al., 2006a; Dyachok et al., 2006b; Ni et al., 2011). cAMP oscillations could thereby provide a mechanism for spatially confining the activation of PKA-dependent effectors. The present study in insulin-secreting β -cells highlights an example where oscillations of cAMP in the sub-membrane space are generated by the periodic formation of the messenger by Adenylyl cyclases. Several PDE isoforms, including IBMX-sensitive PDE1 and PDE3 as well as the IBMX-insensitive PDE8B, contribute to shaping the [cAMP]_{pm} signal, but are not essential for generating the oscillations.

Materials and Methods

Materials

Analytical grade reagents and deionized water were used. Adrenaline, cilostamide, DDA, dipyrindamole, dithiothreitol, EDTA, EGTA, HEPES, IBMX, MM-IBMX, 2-mercaptoethanol, papaverine, phenylmethanesulphonyl fluoride, poly-L-lysine, rolipram, sodium orthovanadate and Tris-HCl were purchased from Sigma (St Louis, MO, USA). Lipofectamine 2000, DMEM, trypsin, penicillin, streptomycin, glutamine and fetal calf serum were from Invitrogen (Carlsbad, CA, USA). Plasmid or adenoviral vectors encoding the two moieties of a cAMP translocation biosensor were used as previously described (Dyachok et al., 2006a; Dyachok et al., 2008). The sensor consists of a truncated and membrane-anchored PKA regulatory RII β subunit tagged with CFP and a PKA catalytic α subunit tagged with YFP. A plasmid encoding GRP1 (General receptor for phosphoinositides-1) fused to 4 tandem copies of (GFP₄-GRP1) was used to monitor plasma membrane phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] levels, which reflect insulin secretion with concomitant autocrine activation of insulin receptors and PI3-kinase (Dyachok et al., 2008). Lentiviruses encoding shRNA directed against PDE8B and a non-target control vector were purchased from Sigma. The PDE family-specific inhibitors were typically used at concentrations that were approximately ten times above their reported IC₅₀, but sometimes more than one concentration was tested.

Islet isolation, cell culture and transfection

Insulin-secreting MIN6 cells of passages 17–30 (Miyazaki et al., 1990) were cultured in DMEM containing 25 mM glucose and supplemented with 15% fetal calf serum, 2 mM glutamine, 70 μ M 2-mercaptoethanol, 100 U/mL penicillin and 100 μ g/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 transfection of the cAMP or PtdIns(3,4,5)P₃ biosensor plasmids was performed using Lipofectamine 2000 during 4 hours followed by culture in DMEM for 12–24 hours. Where indicated, MIN6 cells were treated with a mix of lentiviral vectors (at a multiplicity of infection of 10) expressing shRNA against PDE8B (5'-CCGGCCCAAACCTTCATTTCAGAAACTCGAGTTTCTGGAAA-TGAAGTTTGGGTTTTTG-3', 5'-CCGGCCCATCACAAAGGTTATAAATCT-CGAGATTATAACCTTTGTGATGGGTTTTTG-3', 5'-CCGGGCCATAGAA-ATAACAAGTGATCTCGAGATCACTTGTTATTTCTATGGCTTTTTG-3') or the shRNA control vector (5'-CCGGCAACAAGTGAAGAGCACCAAC-TCGAGTTGGTGCTCTTCATCTTGTGTTTTT-3'), 48 hours prior to the experiments. Islets of Langerhans were isolated from C57Bl6J female mice as described previously (Vieira et al., 2007). All procedures for animal handling and islet isolation were approved by the local animal ethics committee. After isolation, the islets were cultured for 1–4 days in RPMI-1640 medium containing 5.5 mM glucose, 10% fetal calf serum, 100 μ g/ml penicillin and 100 μ g/ml streptomycin at 37°C in an atmosphere of 5% CO₂ in humidified air. Data were obtained with cells from at least three independent islet isolations. The islets were infected with cAMP biosensor adenoviruses at a concentration of 10⁵ fluorescence forming units (FFU)/islet as described previously (Tian et al., 2011). Before the experiments, the cells or islets were transferred to a buffer containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 MgCl₂ and 25 mM HEPES with a pH adjusted to 7.40 with NaOH, and pre-incubated for 30 minutes at 37°C in a humidified atmosphere with 5% CO₂. After the pre-incubation, the islets were applied onto poly-lysine-coated 25-mm coverslips where they immediately attached. β -cells were identified on the basis of their large size and negative response to adrenaline (Tian et al., 2011).

RNA isolation and RT-PCR

Total RNA was extracted from MIN6 cells using the RNeasy micro kit (Qiagen, Hilden, Germany). Real-time PCR was performed using Quanti Tect SYBR^(R) Green RT-PCR kit (Qiagen, Hilden, Germany), and the following primers were designed from the coding sequence of PDE8B: forward, 5'-GACTGATG-

AAGAGAAGAG-3'; reverse, 5'-ATGTCTGTTATGAAGTAGT-3'; PDE1C: forward, 5'-AAGCAGCAGAACGGTGACTT-3'; reverse, 5'-GGCAAGGTAA-TGCGACTTGT-3'; PDE3B: forward, 5'-CCAATTCCTGGCTTACCTCA-3'; reverse, 5'-GTGATCGTAATCGTGCATGG-3'; PDE4A: forward, 5'-CA-TCAATGTCCCACGATTG-3'; reverse, 5'-TAAGTCCCGCTCCTGGAATA-3'; and β -actin: forward, 5'-GTTACAGGAAGTCCTCACC-3'; reverse, 5'-GGAGACCAAAGCCTTCATAC-3'. PCR products were normalised to the housekeeping gene β -actin, and expression levels are given relative to the control according to the following formula: fold change = 2 ^{$\Delta\Delta Ct$} , where $\Delta\Delta Ct = [Ct(PDE8B \text{ shRNA}) - Ct(\beta\text{-actin shRNA})] - [Ct(PDE8B \text{ control}) - Ct(\beta\text{-actin control})].$

Single-cell recordings of [cAMP]_{pm} and plasma membrane PtdIns(3,4,5)P₃

Measurements of [cAMP]_{pm} or PtdIns(3,4,5)P₃ were performed as described previously with evanescent wave [total internal reflection fluorescence, (TIRF)] microscopy, using either a custom-built prism-based system (Idevall-Hagren et al., 2010) or an objective-based setup (Tian et al., 2011). The prism setup was built around an E600FN upright microscope (Nikon Corp. Tokyo, Japan). A helium-cadmium laser (Kimmon, Tokyo, Japan) provided 442 nm light for excitation of CFP, and the 514 nm line of an argon laser (ALC 60X, Creative Laser Production, Munich, Germany) was used to excite YFP. Interference filters (Semrock, Rochester, NY, USA) mounted in a filter wheel (Sutter Instruments, Novato, CA, USA) were used to select the appropriate wavelength. The merged laser beam was homogenised and expanded by a rotating light-shaping diffuser (Physical Optics Corp. Torrance, CA, USA) and refocused through a modified quartz dove prism (Axicon, Minsk, Belarus) with a 70° angle to achieve total internal reflection. The chamber was mounted on the custom-built stage of the microscope, such that the coverslip maintained contact with the dove prism by a layer of immersion oil. Fluorescence light was collected through a 40 $\times$, 0.8-NA water immersion objective (Nikon). The objective-based system consisted of an Eclipse Ti microscope (Nikon) with a TIRF illuminator (Nikon) and a 60 $\times$, 1.45-NA objective. The 458, 488 and 514 nm lines of an argon laser (ALC60X, Creative Laser Production) were used to excite CFP, GFP and YFP, respectively. The beam was coupled to the TIRF illuminator through an optical fibre (Oz Optics, Ottawa, Canada). In both evanescent wave microscope setups, fluorescence was detected with back-illuminated EMCCD cameras (DU-897, Andor Technology, Belfast, Northern Ireland) under MetaFluor (Molecular Devices Corp. Downingtown, PA) software control. Emission wavelengths were selected using filters [485 nm/25 nm half-bandwidth for CFP, 527/27 nm for GFP and 560/40 nm for YFP (Semrock Rochester, NY)] mounted in a filter wheel (Sutter Instruments). For time-lapse recordings, images or image pairs were acquired every 5 seconds. To minimize exposure of the cells to the potentially harmful laser light, the beam was blocked by a mechanical shutter (Sutter Instruments) between image captures.

PDE activity in MIN6 β -cell homogenates

Cells infected with control or PDE8B shRNA lentivirus were sonicated in a buffer containing 50 mM Tris pH 7.4, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 0.05 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl fluoride, protease inhibitor cocktail (Sigma) and PhoSTOP (Roche). PDE activity was measured in duplicates as described previously (Murad et al., 1971). In order to determine non-PDE1/3/4 activity in the homogenates, assays were performed in the presence or absence of 50 μ M MM-IBMX, 3 μ M cilostamide and 10 μ M rolipram. The effects of the non-selective PDE inhibitors, dipyrindole (100 μ M) and IBMX (50 μ M), were tested in the presence of the family-selective inhibitors.

Data analysis

Image analysis was performed using MetaFluor. The cAMP concentration was expressed as the ratio of CFP over YFP fluorescence after subtraction of the background. To compensate for variability in expression levels between different cells, the basal level ratio was normalised to 1. The GFP₄-GRP1 concentration in the plasma membrane was evaluated as the fluorescence intensity F in relation to the initial fluorescence intensity F₀ after subtraction of background (F/F₀). Time-average levels of [cAMP]_{pm} or PtdIns(3,4,5)P₃ were calculated by measuring the area under the curve followed by normalization for the elapsed time. All traces show original data, which has not been filtered or processed except for the traces in Fig. 1G,H, which have been corrected for base-line drift. Data are presented as means $\pm$ s.e.m. Statistical comparisons were assessed using the chi-square test or Student's t-test as appropriate.

Acknowledgements

We thank Heléne Dansk, Ing-Marie Mörsare and Ann-Kristin Holmén-Pålbrink for skilfull technical assistance. Author contributions were as follows: G.T. – experimental design, [cAMP]_{pm} measurements in mouse islets and shRNA-treated MIN6 cells, PtdIns(3,4,5)P₃ measurements, data analysis, manuscript preparation; J.S. – experimental design, [cAMP]_{pm} measurements in MIN6 cells, data analysis; Y.X. – experimental design, shRNA

and PDE8B expression; H.S. – real-time PCR experiments; E.D. – experimental design, PDE assay experiments and data analysis; A.T. – conception of study, experimental design, data analysis and writing of manuscript. All authors read and approved the final version of the manuscript.

Funding

This study was supported by grants from Åke Wiberg's Foundation; the European Foundation for the Study of Diabetes/MSD; the Family Ernfrors Foundation; the Harald and Greta Jeansson Foundations; Novo Nordisk Foundation; the Swedish Diabetes Association; the Swedish national strategic grant initiative EXODIAB (Excellence of Diabetes Research in Sweden); and the Swedish Research Council [grant numbers 32X-14643, 32BI-15333, 32P-15439 and 12X-6240 and 2010-3362 to E.D.].

References

- Ainscow, E. K. and Rutter, G. A. (2002). Glucose-stimulated oscillations in free cytosolic ATP concentration imaged in single islet beta-cells: evidence for a Ca^{2+} -dependent mechanism. *Diabetes* **51**, S162-S170.
- Ämmälä, C., Ashcroft, F. M. and Rorsman, P. (1993). Calcium-independent potentiation of insulin release by cyclic AMP in single β -cells. *Nature* **363**, 356-358.
- Bender, A. T. and Beavo, J. A. (2006). Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol. Rev.* **58**, 488-520.
- Bertrand, G., Petit, P., Bozem, M. and Henquin, J. C. (1989). Membrane and intracellular effects of adenosine in mouse pancreatic β -cells. *Am. J. Physiol.* **257**, E473-E478.
- Capito, K., Hedekov, C. J. and Thams, P. (1986). Cyclic AMP phosphodiesterase activity in mouse pancreatic islets. Effects of calmodulin and phospholipids. *Acta Endocrinol. (Copenh.)* **111**, 533-538.
- Choi, Y. H., Park, S., Hockman, S., Zmuda-Trzebiatowska, E., Svennelid, F., Haluzik, M., Gavrilova, O., Ahmad, F., Pepin, L., Napolitano, M. et al. (2006). Alterations in regulation of energy homeostasis in cyclic nucleotide phosphodiesterase 3B-null mice. *J. Clin. Invest.* **116**, 3240-3251.
- Conti, M. and Beavo, J. (2007). Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu. Rev. Biochem.* **76**, 481-511.
- DiPilato, L. M., Cheng, X. and Zhang, J. (2004). Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc. Natl. Acad. Sci. USA* **101**, 16513-16518.
- Dov, A., Abramovitch, E., Warwar, N. and Nesher, R. (2008). Diminished phosphodiesterase-8B potentiates biphasic insulin response to glucose. *Endocrinology* **149**, 741-748.
- Dryselius, S., Lund, P. E., Gylfe, E. and Hellman, B. (1994). Variations in ATP-sensitive K^+ channel activity provide evidence for inherent metabolic oscillations in pancreatic β -cells. *Biochem. Biophys. Res. Commun.* **205**, 880-885.
- Dunn, T. A., Wang, C. T., Colicos, M. A., Zaccolo, M., DiPilato, L. M., Zhang, J., Tsien, R. Y. and Feller, M. B. (2006). Imaging of cAMP levels and protein kinase A activity reveals that retinal waves drive oscillations in second-messenger cascades. *J. Neurosci.* **26**, 12807-12815.
- Dyachok, O. and Gylfe, E. (2004). Ca^{2+} -induced Ca^{2+} release via inositol 1,4,5-trisphosphate receptors is amplified by protein kinase A and triggers exocytosis in pancreatic β -cells. *J. Biol. Chem.* **279**, 45455-45461.
- Dyachok, O., Isakov, Y., Sægetorp, J. and Tengholm, A. (2006a). Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β -cells. *Nature* **439**, 349-352.
- Dyachok, O., Sægetorp, J., Isakov, Y. and Tengholm, A. (2006b). cAMP oscillations restrict protein kinase A redistribution in insulin-secreting cells. *Biochem. Soc. Trans.* **34**, 498-501.
- Dyachok, O., Idevall-Hagren, O., Sægetorp, J., Tian, G., Wuttke, A., Arriëmerlouw, C., Akusjärvi, G., Gylfe, E. and Tengholm, A. (2008). Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metab.* **8**, 26-37.
- Gerisch, G. and Wick, U. (1975). Intracellular oscillations and release of cyclic AMP from Dictyostelium cells. *Biochem. Biophys. Res. Commun.* **65**, 364-370.
- Goraya, T. A. and Cooper, D. M. (2005). Ca^{2+} -calmodulin-dependent phosphodiesterase (PDE1): current perspectives. *Cell. Signal.* **17**, 789-797.
- Gorbunova, Y. V. and Spitzer, N. C. (2002). Dynamic interactions of cyclic AMP transients and spontaneous Ca^{2+} spikes. *Nature* **418**, 93-96.
- Grapenhiesser, E., Gylfe, E. and Hellman, B. (1988). Glucose-induced oscillations of cytoplasmic Ca^{2+} in the pancreatic β -cell. *Biochem. Biophys. Res. Commun.* **151**, 1299-1304.
- Han, P., Werber, J., Surana, M., Fleischer, N. and Michaeli, T. (1999). The calcium/calmodulin-dependent phosphodiesterase PDE1C down-regulates glucose-induced insulin secretion. *J. Biol. Chem.* **274**, 22337-22344.
- Härndahl, L., Jing, X. J., Ivarsson, R., Degerman, E., Åhrén, B., Manganiello, V. C., Renström, E. and Holst, L. S. (2002). Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell exocytosis and release of insulin. *J. Biol. Chem.* **277**, 37446-37455.
- Härndahl, L., Wierup, N., Enerbäck, S., Mulder, H., Manganiello, V. C., Sundler, F., Degerman, E., Åhrén, B. and Holst, L. S. (2004). Beta-cell-targeted overexpression of phosphodiesterase 3B in mice causes impaired insulin secretion, glucose intolerance, and deranged islet morphology. *J. Biol. Chem.* **279**, 15214-15222.
- Heimann, E., Jones, H. A., Resjö, S., Manganiello, V. C., Stenson, L. and Degerman, E. (2010). Expression and regulation of cyclic nucleotide phosphodiesterases in human and rat pancreatic islets. *PLoS ONE* **5**, e14191.
- Herget, S., Lohse, M. J. and Nikolaev, V. O. (2008). Real-time monitoring of phosphodiesterase inhibition in intact cells. *Cell. Signal.* **20**, 1423-1431.
- Hess, B. and Boiteux, A. (1971). Oscillatory phenomena in biochemistry. *Annu. Rev. Biochem.* **40**, 237-258.
- Houslay, M. D. (2010). Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem. Sci.* **35**, 91-100.
- Houslay, M. D. and Adams, D. R. (2003). PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem. J.* **370**, 1-18.
- Idevall-Hagren, O., Barg, S., Gylfe, E. and Tengholm, A. (2010). cAMP mediators of pulsatile insulin secretion from glucose-stimulated single β -cells. *J. Biol. Chem.* **285**, 23007-23018.
- Jung, S. K., Kauri, L. M., Qian, W. J. and Kennedy, R. T. (2000). Correlated oscillations in glucose consumption, oxygen consumption, and intracellular free Ca^{2+} in single islets of Langerhans. *J. Biol. Chem.* **275**, 6642-6650.
- Landa, L. R., Jr, Harbeck, M., Kaihara, K., Chepur, O., Kitiphongspattana, K., Graf, O., Nikolaev, V. O., Lohse, M. J., Holz, G. G. and Roe, M. W. (2005). Interplay of Ca^{2+} and cAMP signaling in the insulin-secreting MIN6 β -cell line. *J. Biol. Chem.* **280**, 31294-31302.
- Lipson, L. G. and Oldham, S. B. (1983). The role of calmodulin in insulin secretion: the presence of a calmodulin-stimulatable phosphodiesterase in pancreatic islets of normal and pregnant rats. *Life Sci.* **32**, 775-780.
- Longo, E. A., Tornheim, K., Deeney, J. T., Varnum, B. A., Tillotson, D., Prentki, M. and Corkey, B. E. (1991). Oscillations in cytosolic free Ca^{2+} , oxygen consumption, and insulin secretion in glucose-stimulated rat pancreatic islets. *J. Biol. Chem.* **266**, 9314-9319.
- Lugnier, C. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol. Ther.* **109**, 366-398.
- Maeda, M., Lu, S., Shaulsky, G., Miyazaki, Y., Kuwayama, H., Tanaka, Y., Kuspa, A. and Loomis, W. F. (2004). Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA. *Science* **304**, 875-878.
- Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y. and Yamamura, K. (1990). Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* **127**, 126-132.
- Murad, F., Manganiello, V. and Vaughan, M. (1971). A simple, sensitive protein-binding assay for guanosine 3':5'-monophosphate. *Proc. Natl. Acad. Sci. USA* **68**, 736-739.
- Murata, T., Shimizu, K., Hiramoto, K. and Tagawa, T. (2009). Phosphodiesterase 3 (PDE3): structure, localization and function. *Cardiovasc. Hematol. Agents Med. Chem.* **7**, 206-211.
- Ni, Q., Ganesan, A., Aye-Han, N. N., Gao, X., Allen, M. D., Levchenko, A. and Zhang, J. (2011). Signaling diversity of PKA achieved via a Ca^{2+} -cAMP-PKA oscillatory circuit. *Nat. Chem. Biol.* **7**, 34-40.
- Nicol, X., Voyatzis, S., Muzerelle, A., Narboux-Nême, N., Südhof, T. C., Miles, R. and Gaspar, P. (2007). cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map. *Nat. Neurosci.* **10**, 340-347.
- Nikolaev, V. O., Bünnemann, M., Hein, L., Hannawacker, A. and Lohse, M. J. (2004). Novel chain cAMP sensors for receptor-induced signal propagation. *J. Biol. Chem.* **279**, 37215-37218.
- Parker, J. C., VanVolkenburg, M. A., Ketchum, R. J., Brayman, K. L. and Andrews, K. M. (1995). Cyclic AMP phosphodiesterases of human and rat islets of Langerhans: contributions of types III and IV to the modulation of insulin secretion. *Biochem. Biophys. Res. Commun.* **217**, 916-923.
- Pyne, N. J. and Furman, B. L. (2003). Cyclic nucleotide phosphodiesterases in pancreatic islets. *Diabetologia* **46**, 1179-1189.
- Renström, E., Eliasson, L. and Rorsman, P. (1997). Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic B-cells. *J. Physiol.* **502**, 105-118.
- Rich, T. C., Fagan, K. A., Tse, T. E., Schaack, J., Cooper, D. M. and Karpen, J. W. (2001). A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a single cell. *Proc. Natl. Acad. Sci. USA* **98**, 13049-13054.
- Schuit, F. C. and Pipeleers, D. G. (1986). Differences in adrenergic recognition by pancreatic A and B cells. *Science* **232**, 875-877.
- Seino, S. and Shibasaki, T. (2005). PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol. Rev.* **85**, 1303-1342.
- Shafiee-Nick, R., Pyne, N. J. and Furman, B. L. (1995). Effects of type-selective phosphodiesterase inhibitors on glucose-induced insulin secretion and islet phosphodiesterase activity. *Br. J. Pharmacol.* **115**, 1486-1492.
- Shakur, Y., Holst, L. S., Landstrom, T. R., Movsesian, M., Degerman, E. and Manganiello, V. (2001). Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. *Prog. Nucleic Acid Res. Mol. Biol.* **66**, 241-277.

- Soderling, S. H., Bayuga, S. J. and Beavo, J. A. (1998). Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **95**, 8991-8996.
- Sugden, M. C. and Ashcroft, S. J. (1981). Cyclic nucleotide phosphodiesterase of rat pancreatic islets. Effects of Ca^{2+} , calmodulin and trifluoperazine. *Biochem. J.* **197**, 459-464.
- Takakusa, H., Kikuchi, K., Urano, Y., Sakamoto, S., Yamaguchi, K. and Nagano, T. (2002). Design and synthesis of an enzyme-cleavable sensor molecule for phosphodiesterase activity based on fluorescence resonance energy transfer. *J. Am. Chem. Soc.* **124**, 1653-1657.
- Taskén, K. and Aandahl, E. M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol. Rev.* **84**, 137-167.
- Terrin, A., Di Benedetto, G., Pertegato, V., Cheung, Y. F., Baillie, G., Lynch, M. J., Elvassore, N., Prinz, A., Herberg, F. W., Houslay, M. D. et al. (2006). PGE(1) stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases. *J. Cell Biol.* **175**, 441-451.
- Thompson, P. E., Manganiello, V. and Degerman, E. (2007). Re-discovering PDE3 inhibitors—new opportunities for a long neglected target. *Curr. Top. Med. Chem.* **7**, 421-436.
- Tian, G., Sandler, S., Gylfe, E. and Tengholm, A. (2011). Glucose- and hormone-induced cAMP oscillations in α - and β -cells within intact pancreatic islets. *Diabetes* **60**, 1535-1543.
- Vieira, E., Salehi, A. and Gylfe, E. (2007). Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia* **50**, 370-379.
- Waddleton, D., Wu, W., Feng, Y., Thompson, C., Wu, M., Zhou, Y. P., Howard, A., Thornberry, N., Li, J. and Mancini, J. A. (2008). Phosphodiesterase 3 and 4 comprise the major cAMP metabolizing enzymes responsible for insulin secretion in INS-1 (832/13) cells and rat islets. *Biochem. Pharmacol.* **76**, 884-893.
- Walz, H. A., Wierup, N., Vikman, J., Manganiello, V. C., Degerman, E., Eliasson, L. and Holst, L. S. (2007). β -cell PDE3B regulates Ca^{2+} -stimulated exocytosis of insulin. *Cell. Signal.* **19**, 1505-1513.
- Willoughby, D. and Cooper, D. M. (2007). Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. *Physiol. Rev.* **87**, 965-1010.
- Wong, W. and Scott, J. D. (2004). AKAP signalling complexes: focal points in space and time. *Nat. Rev. Mol. Cell Biol.* **5**, 959-970.
- Zaccolo, M. and Pozzan, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* **295**, 1711-1715.
- Zaccolo, M., De Giorgi, F., Cho, C. Y., Feng, L., Knapp, T., Negulescu, P. A., Taylor, S. S., Tsien, R. Y. and Pozzan, T. (2000). A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Nat. Cell Biol.* **2**, 25-29.
- Zhao, A. Z., Zhao, H., Teague, J., Fujimoto, W. and Beavo, J. A. (1997). Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. *Proc. Natl. Acad. Sci. USA* **94**, 3223-3228.