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Phosphodiesterase 3 and 4 comprise the major cAMP metabolizing enzymes responsible for insulin secretion in INS-1 (832/13) cells and rat islets

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ABSTRACT

cAMP is a key modulator for glucose-dependent insulin secretion (GDIS). Members of the phosphodiesterase (PDEs) gene family regulate intracellular levels of cAMP by hydrolyzing cAMP to the corresponding inactive 5′AMP derivative. These studies examined the expression and function of all 18 cAMP-specific PDEs in the rat insulinoma derived INS-1 (832/13) cell and isolated rat islets using quantitative PCR and siRNA-mediated gene-specific knockdown. PDE1C, PDE3B, PDE4C, PDE8B, PDE10A, and PDE11A were significantly expressed in rat islets and INS-1 (832/13) cells at the mRNA level. PDE1C, PDE10A and PDE11A were also expressed in brain, along with PDE3B, PDE4C and PDE8B which were also highly expressed in liver, and PDE3B was present in adipose tissue and PDE4C in skeletal muscle. siRNA mediated knockdown of PDE1C, PDE3B, PDE8B and PDE4C, but not PDE10A and PDE11A, significantly enhanced GDIS in rat INS-1 (832/13) cells. Also, selective inhibitors of PDE3 (trequinsin) and PDE4 (roflumilast and L-826,141) significantly augmented GDIS in both INS-1 (832/13) cells and rat islets. The combination of PDE3 and PDE4 selective inhibitors demonstrate that these enzymes comprise a significant proportion of the cAMP metabolizing activity in INS-1 cells and rat islets.

1. Introduction

Insulin release from the pancreatic β-cell is under tight control by arrays of positive and negative regulators at a precise level that is needed to maintain glucose homeostasis in vivo [1,2]. Intracellular cyclic AMP (cAMP) is an important amplifier by which many of these modulators affect glucose dependent insulin secretion (GDIS) in β cells [3]. For instance, it is well known that incretin hormones (GLP-1 and GIP) [4] and certain neurotransmittors such as PACAP [5] enhance GDIS by increasing cAMP levels in β cells via the activation of their corresponding G_s coupled receptors and subsequent activation of adenylate cyclase. While the mechanisms by which cAMP enhances GDIS are not fully understood, the activation of protein kinase A (PKA) and its downstream targets involved in glucose metabolism, including ion channel activation and

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insulin exocytosis most likely play key roles in the augmentation of GDIS [6–8]. Furthermore, activation of the cAMP-PKA pathway in β cells with GLP-1 and its analogues [9], or DPP-4 inhibitors [10] have been demonstrated to increase β-cell mass in animal models, probably via phosphorylation of cAMP-responsive element binding protein (CREB) and subsequent transactivation of genes involved in the regulation of β-cell proliferation and/or survival, such as the insulin receptor substrate 2 (IRS-2) [11,12]. Thus, increasing cAMP levels in pancreatic β cells may have far-reaching therapeutic importance for the treatment of type 2 diabetes.

Members of the cyclic nucleotide phosphodiesterases (PDEs) family play a critical role in regulating intracellular levels of cAMP by hydrolyzing cAMP to 5′AMP [13,14]. The role of PDE in the regulation of GDIS has been well established by numerous studies using various non-selective inhibitors of members of the PDE family [15–17]. Thus there have been significant efforts to determine the role of various PDE inhibitors as GDIS agents for the treatment of type 2 diabetes [18,19]. Nevertheless, to date no PDE inhibitors have actually advanced to late stage clinical development and emerged as novel therapeutics for diabetes [20]. The key challenge in developing novel GDIS enhancers (or secretagogues) targeting PDEs is to achieve β-cell selective inhibition as most of the PDEs are broadly expressed in many cell types in the body. For instance, the beneficial effects of PDE3B inhibitors on insulin secretion were completely overshadowed by their adverse effects on lipolysis and hepatic glucose output [21,22]. We thus set to determine which members of the PDE family are the key enzymes governing the cAMP levels in rat pancreatic β-cells, and their relative expression levels in tissues relevant to glucose homeostasis using quantitative PCR based mRNA profiling. The functional importance of various PDE enzymes in the context of GDIS were studied with siRNA-based gene-specific knockdown in the INS-1 (832/13) cells and subtype-selective inhibitors of PDE enzymes. Our results indicate that PDE3, and PDE4 enzymes appear to be the major phosphodiesterases metabolizing cAMP in the β-cell, with PDE8B also potentially playing a role.

2. Materials and methods

2.1. INS-1 (832/13) cells

The clonal rat insulinoma cell line was derived from the original INS-1 cell [23] by selecting individual clones based on the robustness of GDIS after a stable transfection with the human insulin gene and geneticin (Mediatech, Manassas, VA)) as selection marker, and was obtained from the Newgard Laboratory [24]. The 832/13 line was maintained in RPMI-1640 (Mediatech) containing 11 mM D-glucose and supplemented with 10% fetal bovine serum (HyClone, ThermoFisher, Pittsburg, PA) 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (Mediatech) at 37 °C.

2.2. Quantitative gene expression analysis

The expression levels of all 18 rat PDE isoforms in various rat tissues including islet and the INS-1 (832/13) cells were determined by Taqman analysis. Fluorogenic Taqman probe/primer sets specific for each rat PDE gene were purchased from Applied Biosystems (Foster City, CA). Absolute mRNA levels for the target genes were determined by real-time reverse transcription reaction using the ABI PRISM 7900 Sequence Detection System from Applied Biosystems (Foster City, CA) through 40 cycles. Gene specific probes were manually gated at the same fluorescent intensity to calculate the Cycle Threshold (CT). The CT values were then compared to the standard curve to generate the absolute concentration, with the β-actin probe (Applied Biosystems) used as a reference. The ratio of the concentrations between the gene specific probe and the β-actin was used as the relative mRNA level.

2.3. siRNA oligo and siRNA transfection

Gene specific siRNA duplexes were purchased from Dharmacon (Chicago, IL) as the Smartpool or self-designed using in-house software developed by Rosetta Inpharmatics (Sigma-Proligo, St. Louis, MO). Chemically modified siRNA were also purchased from Dharmacon as OnTargetPlus. All genes were screened using a pool of three (Sigma) or four (Dharmacon) duplexes at a final concentration of 60 nM. A pool of three siRNA duplexes against firefly luciferase was used as a control (Dharmacon). Sequences of our siRNA oligos targeting each gene are available upon request. The INS-1 (832/13) cells were detached and resuspended in 100 μl Nucleofector solution V (2.25×10^6 cells per reaction) (Amaxa, Gaithersburg, MD). The siRNA duplex pool (5 μl) was added to the cell suspension followed by immediate electroporation with Amaxa Nucleofector Device program T21. After electroporation, the cells were diluted 20-fold with regular RPMI 1640 medium and seeded into 9 wells of a 96-well plate.

2.4. Insulin secretion assay in INS-1 cells

A static insulin secretion assay was performed 48 h post-electroporation. The cells were washed once with PBS and incubated in glucose-free Krebs–Ringer Bicarbonate (KRB) medium (Sigma–Aldrich) for 2 h. The KRB medium contains 143.5 mM Na^+, 5.8 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 124.1 mM Cl^-, 1.2 mM PO_4^{3-}, 1.2 mM SO_4^{2+}, 25 mM CO_3^{2-}, 2 mg/ml bovine serum albumin (Sigma–Aldrich) (pH 7.4). The medium was replaced with fresh KRB supplemented with 2, 8, and 16 mM glucose and incubated for another 2 h at 37 °C. An aliquot of the KRB medium was retained at the end of the incubation for insulin measurement by ultra-sensitive rat insulin ELISA kit (ALPCO, Salem, NH). The extent of gene knockdown by siRNA transfection was quantified by Taqman real-time PCR also at 48-h post-electroporation.

2.5. Statistical analysis

The majority of the data is expressed as means ± standard error of the mean (S.E.M.), unless otherwise indicated. Statistical analysis was conducted using Student's *t*-test. Statistical significance was defined as $P < 0.05$.

2.6. Pharmacological inhibition of PDE isoforms

The effects of PDE inhibitors on GDIS were examined in non-transfected INS-1 (832/13) cells. Compounds were either synthesized in house or purchased from Sigma Chemicals. INS-1 cells were plated (60,000 cells) and grown to ~90% confluency in 96 well tissue culture plates. Following a 90 min incubation at 37 °C in glucose-free KRB buffer, the cells were stimulated with KRB buffer supplemented with 2 mM or 8 mM glucose. PDE inhibitors diluted in DMSO (Sigma–Aldrich) (1%, final) were added for 2 h at 37 °C. The supernatant was assayed for insulin using a rat insulin ELISA (Crystal Chem, Downers Grove, IL) or mouse/rat insulin ELISA from Meso Scale Discoveries (Gaithersburg, MD). Both methodologies have been verified to obtain similar results. EC_{50} values were calculated from the inflection point of half maximal activation expressed as a % control (1% DMSO).

2.7. Insulin secretion in isolated pancreatic Islets

Islets were isolated from normal Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) using standard collagenase-based isolation procedure described previously [25]. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Merck Research Laboratories, Rahway, NJ, USA. The islets were cultured for 1–2 h in RPMI1640 medium (11 mM glucose and 10% FBS) before being used for the insulin secretion assay. The effects of various PDE inhibitors on GDIS were determined by static incubation in KRB medium in 96-well format as described previously [26,27]. Briefly, islets were pre-incubated for 30 min in KRB with 2 mM glucose in Petri dishes (Fisher Scientific, Pittsburg, PA). Size-matched islets were then transferred to a round-bottomed 96-well plate (one islet/well in 200 μl of KRB) and incubated for 60 min at 37 °C in a CO_2 incubator. All compounds were first dissolved in DMSO as 1000× stock and were added to the KRB right before the 60 min incubation. Control wells received 0.1% DMSO as vehicle control. Aliquots of the incubation medium were carefully transferred to clean plates for insulin measurement.

2.8. cAMP Determination

Cells were grown and stimulated as outlined above except incubation time with PDE inhibitors was 30 min. The supernatant was removed, the cells were lysed and cAMP content was determined using cAMP BioTrak EIA (Amersham, UK), as previously reported [35].

3. Results

3.1. Relative mRNA abundance of PDEs in rat islet and the INS-1 (832/13) cell

The expression level of 18 PDE isoforms in rat islet and INS-1 (832/13) cells, a clonal rat insulinoma cell line, were quantified using real time TaqMan RT-PCR analysis. Of the specific isozymes from the 11 subfamilies of PDE enzymes examined, PDE1C, PDE3B, PDE4C, PDE8B, PDE10A and PDE11A were all expressed at reasonable levels in both rat islets and INS-1 (832/13) cells (Fig. 1). The mRNA levels of PDE1C, PDE3B, PDE10A and PDE11A in rat islets and in INS-1 (832/13) cells were at least fivefold greater than that in total RNA samples extracted from whole pancreas, indicating that they are selectively expressed in rat islets relative to the exocrine tissue in pancreas. The islet/pancreas ratios for PDE4C and PDE8B were 1.5 and 0.5, respectively, suggesting these two isoforms are present in both exocrine pancreas and islets.

3.2. Relative expression levels of islet PDEs in other tissues involved in glucose homeostasis

The tight control of normoglycemia is maintained by adequate insulin release in pancreatic β-cells and insulin actions in fat, liver, brain and muscle, all of which could be partially regulated by the PDE-cAMP signaling pathway. We also

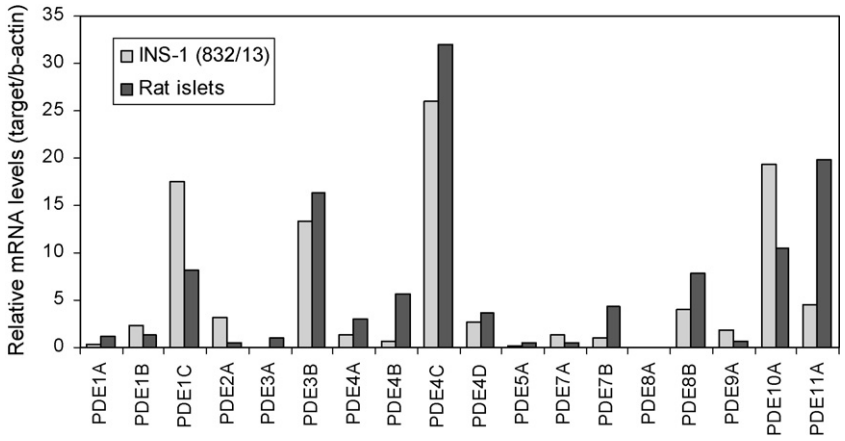


Fig. 1 – Relative PDE mRNA levels in INS-1 (832/13) and rat islets. The relative PDE mRNA level in rat islets and INS-1 (832/13) cells was quantified by real time quantitative PCR. The data were normalized against absolute fluorescence intensity and beta-actin expression level.

Table 1 – Distribution of key PDE isozymes in islets and other tissues involved in glucose homeostasis in rat

	PDE1C	PDE3B	PDE4C	PDE8B	PDE10A	PDE11A
Adipose	0.11	3.52	1.87	0.99	0.21	0.67
Brain	4.58	2.74	5.23	4.05	23.98	27.59
Colon	0.27	0.80	3.36	3.28	0.19	2.73
Small intestines	0.49	1.03	2.21	1.31	0.43	1.20
Liver	0.01	13.57	13.78	18.30	0.01	4.80
Heart	1.05	1.78	28.32	5.19	1.12	7.27
Skeletal muscle	0.19	0.90	17.55	1.74	1.99	0.09
Pancreas	1.01	3.21	20.80	15.66	0.22	0.12
Islet	8.23	16.37	32.06	7.80	10.53	19.76

Relative PDE mRNA levels in various tissues were measured by real time quantitative PCR and normalized against absolute fluorescence intensity and beta-actin expression level.

examined the expression level in the key insulin responsive tissues for the six key islet PDEs described above (Table 1). In addition to pancreatic islets, PDE1C, PDE10A and PDE11A were most significantly expressed in brain. Conversely, PDE3B, PDE4C and PDE8B were more broadly expressed in various rat tissues. Most notably, the mRNA levels of PDE3B, PDE4C and PDE8B in liver were all very high. PDE3B was also expressed, although to a lesser degree, in adipose tissue while PDE4C was high in skeletal muscle and heart.

3.3. *Effects of siRNA-mediated knockdown of various PDEs on insulin secretion in INS-1 (832/13) cell*

To assess the functional importance of each cAMP-specific PDE in the regulation of insulin secretion, a pool of 3 siRNA duplexes specific to each member of the rat PDE family were transfected into INS-1 (832/13) cell via electroporation. Insulin secretion at 2, 8 and 16 mM glucose was measured by static incubation performed 48 h after electroporation. As shown in Fig. 2A, the electroporated INS-1 (832/13) cells maintained a fourfold increase in insulin secretion in response to 8 or 16 mM glucose challenge over the basal insulin release at 2 mM glucose. The insulin secretory responses to glucose were significantly enhanced by approximately 2- to 3.5-fold in cells that had been transfected with si-PDE1C, si-PDE3B, si-PDE4C, si-PDE5A, or si-PDE8B duplex pools. Conversely, siRNA mediated knock down of glucokinase (GCK), the key glycolytic enzyme used as a functional control, suppressed GDIS by 40–60% in the same experiments (Fig. 2A).

Unmodified siRNA duplexes have been reported to cause false positive or false negative functional readouts, most of which can be potentially decreased using a second set of duplexes such as OnTarget*plus* oligos (OT-siRNA) purchased from Dharmacon [28]. We thus repeated the siRNA knockdown experiments using the OnTargetplus siRNA oligo against those six PDEs highly expressed in the INS-1 cell line (Fig. 1), as well as PDE5A since it was highlighted as a positive hit in our initial screen experiment using regular siRNA duplexes. Again we observed significant enhancement of GDIS in cells transfected with OT-siRNA against PDE1C, PDE3B, PDE4C, and PDE8B, but not with OT-siRNA for PDE5A, PDE10A and PDE11A (Fig. 2B). In this series of experiments, we achieved $\geq$60% knockdown of target mRNA levels as measured by TaqMan PCR analysis (Fig. 2C). Thus the increase of GDIS by the regular si-PDE5A observed in the previous series of experiments was most likely the result of some off-target activity of the siRNA duplexes.

3.4. *Effects of subtype selective inhibitors on GDIS in INS-1 (832/13) cells and rat islets*

In order to confirm and expand upon the RNAi data obtained, we tested several PDE inhibitors for their ability to stimulate glucose secretion in INS-1 (832/13) cells (Table 2). Although there are 11 PDE gene families, potent and/or selective inhibitors have only been identified for some of these gene families. The following isoform-selective PDE inhibitors were selected for testing in this study based on their potency for the intended targets, and selectivity over other PDE subfamilies [29]: erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) for PDE2 [30]; trequinsin for PDE3; roflumilast and L-826141 [31] for PDE4; sildenafil for PDE5; and papaverine for PDE10. IBMX was also tested as the pan PDE inhibitor [29]. The data in Table 2 and Fig. 3A demonstrate that the PDE3 inhibitor and the PDE4 inhibitors (along with the pan PDE inhibitor IBMX) resulted in an appreciable enhancement of GDIS in INS-1 cells, whereas inhibitors of PDE2 (EHNA), PDE5 (sildenafil) and PDE10 (papaverine) did not significantly effect GDIS in INS-1 cells. In this experiment, forskolin, which elevates cAMP through adenylate cyclase activation, resulted in the greatest glucose dependent insulin secretion.

The same set of PDE inhibitors were also tested for their effects on insulin secretion in isolated rat islets (Fig. 3B). Again, we observed significant enhancement of insulin secretion at 16 mM glucose in islets incubated with inhibitors of PDE3 (trequinsin) and PDE4 (roflumilast and L-826141), but not with inhibitors of PDE5 (sildenafil), PDE2 (EHNA) or PDE10 (papaverine).

While siRNA-mediated silencing of PDE8B significantly enhanced GDIS in INS-1 (832/13) cells in our hands, we do not know if inhibition of this enzyme will affect GDIS in isolated islets or insulin secretion *in vivo* because there is no selective inhibitor available for this enzyme.

The mechanism for GDIS through PDE inhibition is by an increase in intracellular cAMP. In order to confirm that the PDE3 and PDE4 inhibitor were working through this latter mechanism, INS-1 (832/13) cells were incubated with the

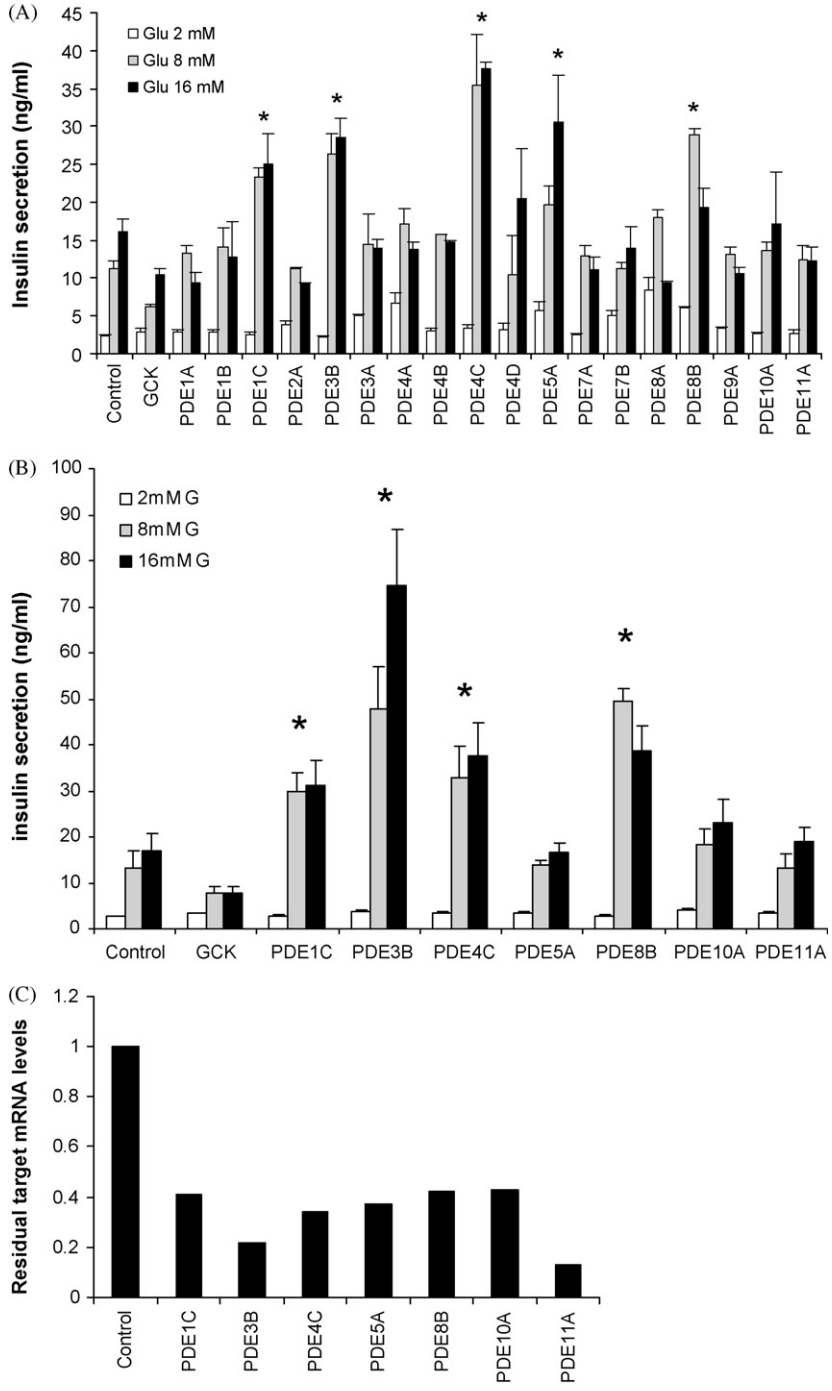


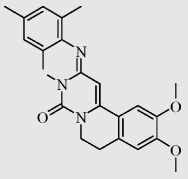
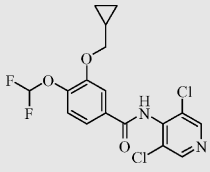
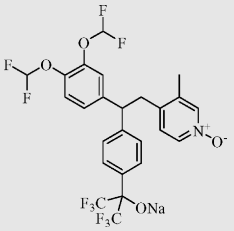
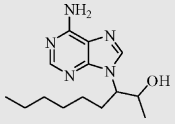
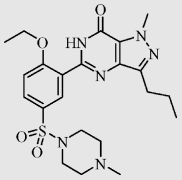
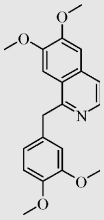
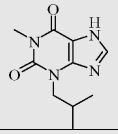
Fig. 2 – Effect of siRNA-mediated knockdown of PDE isoforms on insulin secretion in INS-1 (832/13) cells. A pool of three unmodified siRNA duplexes (A) or a pool of four chemically modified (OnTargetPlus™ Dharmacon) siRNA duplexes (B) to PDE isoforms were transfected via electroporation (Amaxa™) into INS-1 cells. Insulin secretion was measured by ELISA 48 h post-transfection following a 2 h stimulation with 2, 8 or 16 mM glucose. Data are mean ± S.E.M. of four independent experiments (*$P < 0.05$). Total RNA (C) was isolated 48 h post-transfection and analyzed by real time quantitative PCR. Relative gene levels were determined and reported as quantity remaining compared to control transfected cells which were arbitrarily set to 1.

compounds and intracellular cAMP levels were measured. Both trequinsin and roflumilast significantly elevated cAMP levels in the INS-1 cells (Fig. 4) which demonstrates that the GDIS observed was indeed achieved through direct PDE inhibition.

3.5. *Quantifying the total PDE component of GDIS in INS-1 cells*

We utilized the PDE3 and PDE4 selective inhibitors along with IBMX to estimate the proportions of PDE-cAMP induced GDIS

Table 2 – *In vitro* potencies and effect on GDIS of PDE inhibitors

PDE	Compound	Structure	In vitro IC_{50}	EC_{50} GDIS
PDE3	Trequinsin		0.05 nM	0.1 (0.5) nM
PDE4	Roflumilast		4A,B,D = 0. 1 nM; 4C = 0.6 nM	5 (5.6) nM
PDE4	L-826,141		4A,C = 1.3 nM; 4B,D = 0.4 nM	6 (1.1) nM
PDE2	EHNA		1 μM	>10 μm
PDE5	Sildenafil		0.3 nM	>1 μM
PDE10	Papaverine		50 nM	>1 μM
Pan PDE inhibitor	IBMX		PDE3 ~ 1200 nM; PDE4 ~ 30,000 nM	5200 (300) nM

IC_{50}s were determined in house, except for IBMX [29] and EHNA [30] against recombinant purified PDEs [35]. EC_{50} data obtained as described in Fig. 4.

that is attributable to these enzymes. We previously demonstrated that inhibition with IBMX corresponds to approximately 80% of the maximal GDIS signal obtained with forskolin (Fig. 3A). A complete dose–response curve with forskolin was conducted and 400 nM forskolin is at the plateau of the dose–response providing maximal insulin secretion (data not shown). We then titrated the PDE inhibitors which increased GDIS in the INS-1 cells (Fig. 5) in order to determine an EC_{50} for these compounds. The rank order of compounds from lowest EC_{50} to highest for stimulation of GDIS is trequinsin < L-826,141 = roflumilast ≪ IBMX. Trequinsin is the most potent PDE inhibitor identified for activation of GDIS with efficacy seen at very low doses with an EC_{50} of 0.1 nM (Fig. 5). A comparison of the intrinsic potency for inhibition of

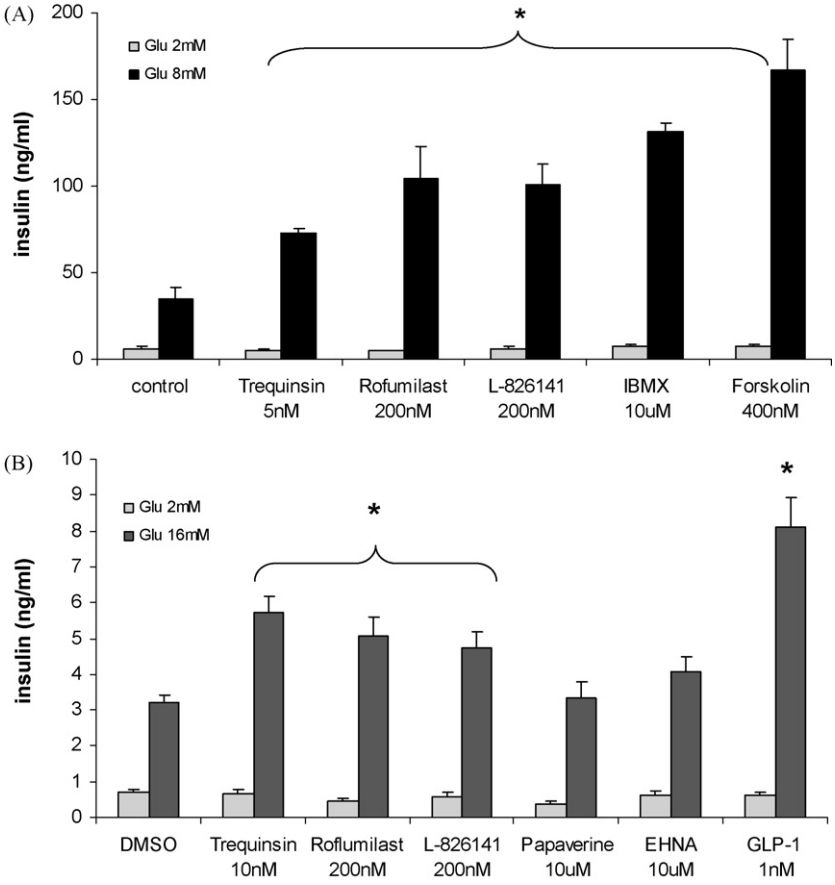


Fig. 3 – Effect of PDE inhibition on GDIS in INS-1 (832/13) cells and rat islets. INS-1 (832/13) cells (A) were stimulated for 2 h with KRB buffer supplemented with either 2 or 8 mM glucose in the presence or absence of PDE inhibitors. Inhibition of PDE3 (trequinsin) resulted in twofold increase in GDIS, compared to vehicle control (1% DMSO) while inhibition with PDE4 inhibitors (roflumilast and L-826141) produced a threefold increase in insulin secretion only in the presence of 8 mM glucose. Pancreatic islets (B) isolated from Sprague–Dawley rats were incubated for 60 min in KRB medium with 2 or 16 mM glucose in the presence of absence of PDE inhibitors. Inhibition of PDE3 and PDE4 resulted in twofold increase in GDIS compared to vehicle control (DMSO) at the 16 mM glucose level and no stimulation of insulin release with 2 mM glucose. Data are presented as the mean ± S.E.M. of three independent experiments, *P < 0.05 or less compared to vehicle control.

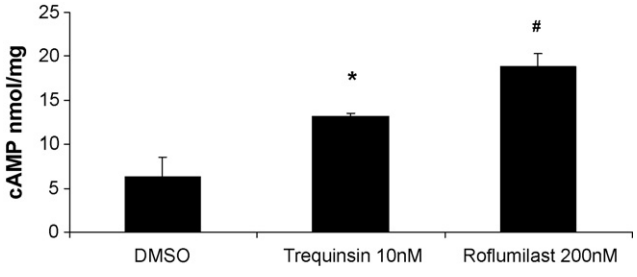


Fig. 4 – Inhibition of PDE3 and PDE4 increases intracellular cAMP levels in INS-1 (832/13) cells. INS-1 cells were treated with a PDE inhibitor or 1% DMSO (vehicle control) for 30 min in KRB medium supplemented with 8 mM glucose. Intracellular cAMP accumulated was determined in the cell lysate using BioTrak EIA (Amersham). Data are presented as the mean ± S.E.M. of three experiments, *P < 0.05 and #P < 0.01 compared to vehicle control.

PDE enzymes and the EC_{50} for activation of GDIS is presented in Table 2. The latter data demonstrates a direct correlation between rank order of potency for inhibition of the corresponding PDE enzymes and GDIS activation in INS-1 cells.

Secondly, we used trequinsin and roflumilast to reconstitute the total PDE activities (as determined by the pan PDE inhibitor, IBMX) in the context of GDIS. As shown in Fig. 6A, IBMX at 30 μM resulted in a fourfold increase in GDIS above the vehicle control (DMSO) in INS-1 (832/13) cells. Each of the PDE3 (trequinsin) and PDE4 (roflumilast) inhibitors resulted in a 1.5- to 2-fold increase in insulin secretion, independently. When the PDE3 and PDE4 inhibitor were combined, a similar level of insulin secretion was detected in the INS-1 cells as compared to IBMX Fig. 6A. A similar result was also obtained in isolated rat islets demonstrating the extrapolation to primary islets (Fig. 6B). Therefore, approximately 80% of the PDE component of glucose dependent insulin secretion is determined by PDE3 and 4 enzymes with the remaining contribution potentially regulated by PDE8B.

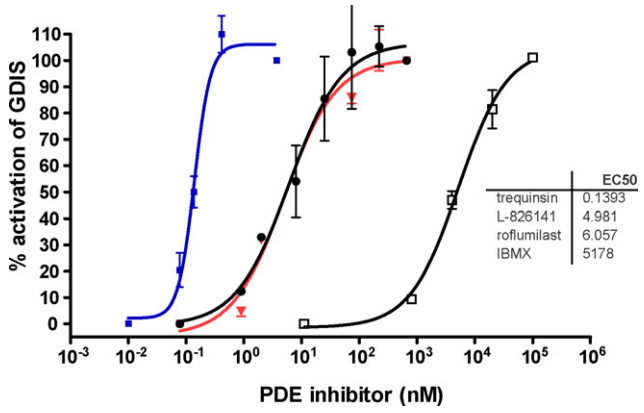


Fig. 5 – Dose–response of PDE inhibitors on stimulation of GDIS in INS-1 (832/13) cells. Titration curves of potentiation of GDIS induced by PDE3 (trequinsin), PDE4 (roflumilast and L-826141) and pan PDE inhibitor IBMX in INS-1 cells in the presence of 8 mM glucose. EC_{50} values were calculated from sigmoidal curve fitting using Prism software. Data are presented as the mean ± S.D. of two independent experiments.

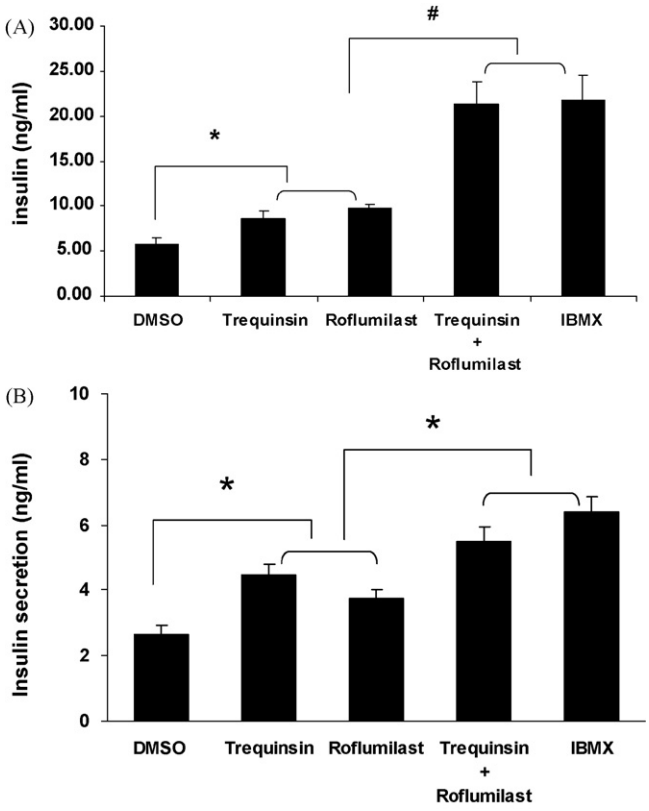


Fig. 6 – Additive effect of PDE3 and PDE4 inhibition on potentiation of GDIS in INS-1 cells and rat islets. The combined effect of PDE3 and PDE4 inhibitors on GDIS in INS-1 (832/13) cells (A) and rat islets (B) was determined by static incubations in KRB buffer with 8 mM (INS-1 cells) or 16 mM (islets) glucose. Dual inhibition of PDE3 (trequinsin 7 nM) and PDE4 (roflumilast 67 nM) resulted in fourfold increase in GDIS from INS-1 cells and 2.5-fold increase from rat islets similar to that obtained using IBMX (30 μM). Data are presented as the mean ± S.E.M. of $n = 4$ (INS-1 cells) and $n = 3$ (islets) experiments respectively.

4. Discussion

cAMP signaling in islets is well established to play a significant role in GDIS. Phosphodiesterases are the primary cAMP metabolizing enzymes which control this cAMP pool. PDE1, PDE3, and PDE4 have been previously reported to have a functional role in β cells. The presence of PDE3B in β-cells has been supported by the studies using islet β-cell lines and native β-cells by various methods [22,32]. Overexpression of PDE3B by adenovirus either in insulinoma cell lines or in islets greatly reduces glucose dependent insulin secretion [33]. Other PDEs, such as PDE4 [22] and PDE1C [34] were also shown to be present in the β-cells. However, most of these data were generated using various non-selective and relatively weak PDE inhibitors. Most importantly, it has not been established which isoform of the PDE family or family member is the major PDE species in islets and plays a dominant role in regulating GDIS.

In this study, we evaluated the relative mRNA level of 18 PDEs in rat islet and in INS-1 (832/13) cells by quantitative RT-PCR. PDE1C, PDE3B, PDE4C, PDE10A, and PDE11A were identified to be the most abundant PDEs in both rat islets and 832/13 cells.

Utilizing siRNA mediated knockdown, reduction of PDE1C, PDE3B, PDE4C, and PDE8B enhanced GDIS in INS-1 (832/13) cells. This data was confirmed using several modified siRNA duplexes in order to decrease the off target effects reported for siRNA duplexes. In order to extrapolate the data obtained in INS-1 (832/13) cells to primary islets, selective inhibitors were utilized. IBMX, the pan-PDE inhibitor, and forskolin which stimulates adenylate cyclase were utilized to determine the maximal PDE-cAMP component involved in GDIS. Inhibition with IBMX accounts for 80% of the GDIS detectable when stimulation is performed with forskolin. Utilizing this approach, and then combining the PDE3 and PDE4 selective inhibitors, we demonstrate that these compounds almost completely recapitulate the effect seen with IBMX. Therefore the majority of the PDE-cAMP metabolizing activity in both INS-1 (832/13) cells and primary rat islets is PDE3 and PDE4. We have also identified PDE8B as a negative regulator of GDIS in INS-1 (832/13) but due to lack of selective inhibitors, we could not compare its role relative to PDE3 and -4. A recent publication has also demonstrated a role for PDE8B [36] in stimulating insulin secretion in rat islets which confirms our data with INS-1 cells but comparison with effects of PDE3 and PDE4 knockdown will only be conclusive with selective PDE8B inhibitors.

Presently PDE4 and PDE3 inhibitors have been evaluated for several indications but due to mechanism based adverse events have been challenging to develop as novel therapeutics. The only clinically approved PDE3 inhibitor is cilostazol which is approved for the treatment of intermittent claudication [37]. This compound is a relatively weak PDE3 inhibitor and there have been no significant reports on its efficacy in an

insulin secretion paradigm in humans. Further development of these compounds may demonstrate if there is a therapeutic window for these inhibitors in the treatment of non-insulin dependent diabetics.

Conflict of interest

All authors are employees of Merck and Co.

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