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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 $P < 0.05$.