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A reporter gene assay for screening of PDE4 subtype selective inhibitors

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Abstract

Phosphodiesterase (PDE) constitutes a superfamily of enzymes that catalyze the hydrolysis of cAMP and cGMP into their corresponding monophosphates and play an important role in diverse physiological functions. The present study provides a process for identifying PDE4 subtypes selective inhibitors using a reporter gene assay. Stable recombinant HEK-293 cell lines expressing high levels of PDE4A4B, PDE4B2A, and PDE4D3 subtypes individually were generated. Transient transfection of pCRE-Luc plasmid, harboring luciferase reporter gene under the control of cAMP response element (CRE)-binding sequence, into these stable recombinant cell lines followed by treatment with PDE4 inhibitor, resulted in a dose dependent increase in luciferase activity. This methods provide a novel, simple and sensitive assay for high throughput screening of PDE4 subtype selective inhibitors for treatment of asthma and COPD.

Keywords: Phosphodiesterase; Stable cell lines; Cellular localization; Reporter gene assay

The phosphodiesterases (PDEs) constitute a large superfamily of isoenzymes that play an important role in regulating intracellular levels of cAMP and cGMP by catalyzing the hydrolysis of cyclic 3′,5′-adenosine monophosphate and guanosine monophosphate to the corresponding nucleotide 5′ monophosphates. Eleven PDE gene families have been identified and more than 50 splice variants have been detected in various human tissues [1–3]. They all share a highly conserved catalytic domain of about 270 amino acids fused to additional N- and C-terminal sequences that contain distinct domains unique to the members of a PDE family. These isoforms are differentially expressed and regulated in different cell types under various physiological conditions [1]. PDEs are therapeutic targets for a range of disorder such as retinal degeneration, congestive heart failure, depression, asthma, erectile dysfunction, and inflammation [4].

A high-affinity cAMP-specific isozyme known as PDE4 is predominantly found in immune and inflammatory cells and plays important roles in regulating intracellular levels of cAMP in these cell types [4]. PDE4 is encoded by four gene families (A, B, C, and D), each of which generates multiple splice variants [5]. In particular, PDE4A, 4B, and 4D subtypes are predominantly expressed in eosinophils and neutrophils, whereas PDE4C is expressed in brain and has not been detected in any inflammatory cells. cDNA for all the four subtypes of the human PDE4 family have been cloned. All the four subtypes of PDE4 contain a highly conserved catalytic domain of about 450 amino acids with more than 80% identity among the subtypes. Other short regions of conserved amino acid sequences are at the N-terminus of PDE4 proteins, designated as UCR1 and UCR2 for upstream conserved regions. The C-terminal regions of each of the four PDE4 subtypes are distinct [6–9]. PDE4 inhibitors suppress various functions of the inflammatory cells. *In vivo* anti-inflammatory effects of PDE4 inhibitors have been widely demonstrated. Therefore, there is significant interest in the potential utility

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of PDE4 inhibitors in the therapy of asthma, allergy, chronic obstructive pulmonary diseases (COPD), cognitive disorders including Alzheimer's disease and stroke [10–12].

Although PDE4 inhibitors have therapeutic potential for treatment of variety of inflammatory diseases, its clinical usefulness is limited by adverse effects, such as nausea and emesis, as observed in clinic, following administration of PDE4 inhibitors, including rolipram and tibenelast [13,14]. Moreover, non-specific PDE inhibition with theophylline, commonly used to treat asthma and COPD, have demonstrated increased mortality due to cardiac arrhythmias [15,16]. A recent finding by Lehnart et al. [17] have suggested that PDE4D3 inhibition could lead to cardiac dysfunction and arrhythmias. Therefore it is very important to develop a PDE4 subtype selective inhibitor to overcome these major side effects.

We report here the generation of recombinant stable cell lines, individually expressing high level of PDE4A4, PDE4B2, and PDE4D3 subtypes and development of simple and novel luciferase-based reporter gene assay for the high throughput screening of PDE4 subtype selective inhibitors.

Materials and methods

Materials. HEK-293 and U937 cells (American Type Culture Collection, Manassas, VA); Lipofectamine 2000 (Invitrogen, Carlsbad, CA); rabbit polyclonal PDE4A, PDE4B, PDE4D3 IgG, (FabGennix Inc., TX); donkey anti-rabbit polyclonal IgG HRP conjugated (Santa Cruz Biotechnology, Inc., Santacruz, CA); Alexa-conjugated secondary antibody (Molecular Probes, Eugene, Oregon); Chemiluminescence assay kit (Amersham Biosciences, Inc., Chicago, IL); Zeocin (Invitrogen, Carlsbad, CA); pcDNA3.1/Zeo expression vector (Invitrogen, Carlsbad, CA); pCRE-Luc vector (BD Biosciences Clontech, CA); rolipram (Sigma, St. Louis, MO); roflumilast was synthesized by the Department of Medicinal Chemistry, Ranbaxy Research Laboratories (Gurgaon, India); HitHunter cAMP assay kit (DiscoveRx Corp., CA); Steady-Glo Luciferase Assay system (Promega, Medison, WI).

Cloning of human PDE4 subtypes. Total RNA was extracted from U937 (for isolation of human PDE4A4B, PDE4B2A, and PDE4D3) cells using Trizol (Invitrogen). First strand cDNA was made using Superscript II reverse transcriptase (Invitrogen) with oligo dT/random primer. The full-length coding region of each gene was amplified by PCR using gene specific primers.

The full-length coding region of PDE4A4B subtype was amplified using forward primer: CGGGATCCATGGAACCCCCGACCGTCCCCTC and reverse primer: GCAAGCTTTCAGGTAGGGTCTCCACCTGA CCCCCCG. The full-length product was cloned in to the *Bam*HI and *Hin*dIII sites of plasmid pcDNA3.1/Zeo(−). The full-length coding region of PDE4B2A subtype was amplified using forward primer: GGGGTACC ATGAAGGAGCACGGGGG and reverse primer CCATCTAGATTAT GTATCCACGGGGGACTTG. The full-length product was cloned into *Kpn*I and *Xba*I sites of plasmid pcDNA3.1/Zeo(+). The full-length coding region of PDE4D3 subtype was amplified using forward primer: CCCAAGCTTATGATGCACGTGAATAATTTTCC and reverse primer CTAGTCTAGATTACGTGTCAGGAGAACGATC. The full-length product was cloned into *Hin*dIII and *Xba*I sites of plasmid pcDNA3.1/Zeo(+). Full-length cDNA of human PDE4A4B, PDE4B2A, and PDE4D3 subtypes were confirmed by restriction mapping and DNA sequencing.

Cell culture and transfections. U937 cells were propagated in RPMI medium 1640 supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% CO_2. HEK-293 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% CO_2. Stable cell lines were obtained by transfection of expression vector pcDNA3.1/Zeo containing the cDNA constructs of each of the human PDE4 subtypes into HEK-293 cells using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Stable clones were selected for resistance to Zeocin (1 mg/ml). Cells were harvested and lysate preparations were analyzed by Western blotting.

Western blotting. The cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.7, 0.2 mM EGTA, 10 mM $MgCl_2$, 0.5 % Triton X-100, protease inhibitor cocktail) and homogenized. The homogenate was centrifuged at 15,000*g* for 20 min at 4 °C and cell lysate was stored at −20 °C . The protein samples were separated on 7.5% SDS–PAGE and were transferred to nitrocellulose membranes. The membranes were incubated with rabbit polyclonal PDE4A IgG or PDE4B IgG or PDE4D3 IgG at 1:1000 dilution followed by incubation with secondary donkey anti-rabbit polyclonal IgG HRP conjugated at 1:2000 dilution. PDE4 subtype specific proteins were detected using chemiluminescence kit.

Immunocytochemistry. Stable HEK-293 cell lines expressing PDE4 subtypes were grown in 4-well Lab-Tek chamber slides for 24 h at 37 °C. The cells were fixed with 2% paraformaldehyde/0.1% Triton X-100 for 20 min at room temperature. The cells were blocked in 10% FBS for 20 min at room temperature. The cells were incubated with 1:100 dilution of appropriate primary antibody followed by incubation with 1:500 diluted Alexa-conjugated secondary antibody. Cells were analyzed under a fluorescent microscope TE 2000-E (Nikon Instech Co. Ltd., Japan).

PDE assays. Stable recombinant cells from culture flasks were detached in to 5 ml of DPBS pH 7.4 containing 5 mM EDTA. Cells were pelleted down by centrifugation at 2000 rpm for 5 min and resuspended in 4 ml of homogenizing buffer (50 mM Tris, pH 7.7, 0.2 mM EGTA, 10 mM $MgCl_2$, 0.5% Triton X-100) supplemented with protease inhibitors and homogenized. The homogenate was centrifuged at 15000*g* for 30 min at 4 °C and soluble fraction was collected. PDE4 activity was measured using HitHunter cAMP assay kit according to the manufacturer's instructions (DiscoveRx Corp., CA).

PDE4 reporter gene assay using U937 monocytic cells. U937 cells were seeded in a 6-well culture plate (1×10^6 cells per well) in 2 ml RPMI medium containing 10% FBS and 2 mM L-Glutamine and incubated at 37 °C and 5% CO_2 for 24 h. pCRE-Luc plasmid DNA(2 μg) was transiently transfected into the cells using Lipofectamine 2000. Cells were incubated at 37 °C and 5% CO_2 for 18–20 h. Cells were centrifuged at 1200 rpm/5 min. and resuspended in fresh RPMI medium containing FBS and 2 mM L-Glutamine. Cells were distributed (200 μl; 3×10^5 cells) in each well of 96-well plate. PDE4 inhibitor at various concentration was added in each well and cells were incubated at 37 °C and 5% CO_2 for 2–24 h. Cells were centrifuged at 1200 rpm for 5 min and lysed with 50 μl of steady-Glo lysis buffer. For each well, 50 μl of luciferin substrate (Steady-Glo Kit) was added and reaction mixture was transferred to a 96-well white bottom plate. Luminescence was measured in the luminometer. Analysis of inhibitor dose–response data and calculation of IC_{50} values were performed by using GraphPad Prism (GraphPad Software, Inc., CA).

Reporter gene assay using recombinant cell line expressing PDE4 subtypes. Recombinant stable cell line expressing PDE4 subtypes individually were seeded (1×10^7) in 10 cm^2 dish containing 10 ml DMEM and 10% FBS. Cells were incubated at 37 °C and 5% CO_2 incubator for 24 h. pCRE-Luc plasmid DNA (10 μg) was transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C in 5% CO_2 incubator for 18–20 h. Cells were detached and resuspended in 20 ml DMEM medium containing 10% FBS. Two hundred microliters of cells (3×10^5 cells) were added in each well of 96-well plate and the drug Rolipram at 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM concentration, was added and cells were incubated for 18–20 h at 37 °C and 5% CO_2. Cells were lysed with 50 μl of steady-Glo lysis buffer in each well. For each well, 50 μl of luciferin substrate (Steady-Glo Kit) was added and reaction mixture was transferred to a 96-well white bottom plate. Luminescence was measured in the luminometer. Analysis of inhibitor dose–response data and calculation of IC_{50} values were performed by using GraphPad Prism (GraphPad Software, Inc., CA).

Results and discussion

Cloning and generation of recombinant cell lines expressing PDE4 subtypes

Full-length cDNA encoding for PDE4A4, 4B2, and 4D3 were amplified from cDNA library prepared from U937 cells and cloned into mammalian expression vector pcDNA3.1/Zeo. cDNA sequences were confirmed by restriction mapping and DNA sequencing. DNA sequences of all the three subtypes of PDE4 were found to be same as per the published reports. HEK-293 cells were transfected with pcDNA3.1/Zeo vector harboring PDE4 subtype cDNA (i.e., PDE4A4, 4B2, and 4D3). For obtaining stable transfectants, several Zeocin resistant clones for each PDE4 subtype were isolated. These Zeocin resistant clones were screened by Western blot analysis using PDE4 subtype selective antibodies. These subtype selective antibodies recognize an epitope in the C-terminal region of a PDE4 subtype that is not common with the other three PDE4 subtypes [9,18]. Hence, these subtype selective antibodies recognizing an epitope in the C-terminal region of a PDE4 subtype, are important tool to detect the expression of a particular PDE4 subtype in a over-expressing recombinant cell line. Specific protein bands of 98, 63, and 74 kDa for PDE4A4, PDE4B2, and PDE4D3, respectively, were detected in the immunoblot as shown in Fig. 1A, B, and C. Recombinant clones with maximum expression levels for each PDE4 subtype were isolated and termed as HEK-PDE4A4, HEK-PDE4B2, and HEK-PDE4D3. All the three subtypes showed expected protein sizes with the subtype specific antibodies without any cross reactivity. No signal was detected in control HEK-293 cells.

Cellular localization of PDE4 subtypes

Cellular localization of PDE4A4, PDE4B2, and PDE4D3 was analyzed by immunocytochemical analysis using PDE4 subtype-selective antibodies. Expression of PDE4A4, PDE4B2, and PDE4D3 was mainly localized in cytoplasm as shown in Fig. 2B, D, and F, respectively. No signal was detected in the control cells (Fig. 2A, C, and E). It has been observed that recombinant PDE4A5 expressed in COS-7 was detected in both cytosolic (88%) and particulate (12%) fractions, and immunofluorescence studies further suggested its association with membrane structures such as cytoskeletal or cytoskeletal-associated proteins [19]. It had been shown by biochemical and histochemical studies that in cardiac tissues, besides their cytosolic distribution, PDE4 are associated to sarcolemma and nuclear envelope [20].

PDE4 reporter gene assay

PDE4 subtypes hydrolyze cAMP into AMP and inhibition of PDE4 activity by its selective inhibitors leads to elevated intracellular level of cAMP. The increase in cAMP levels within the cell can be monitored using pCRE-Luc plasmid. This plasmid is designed to detect the activation of cAMP response element-binding protein (CREB) and cAMP-mediated signal transduction pathways. Several signal transduction pathways are associated with the cAMP response element (CRE) and induction of these pathways enables endogenous transcription factor, CREB, to bind CRE and initiate the transcription of the downstream target genes [21]. pCRE-Luc plasmid vector contains the firefly luciferase gene under the control of CRE-binding sequences fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. Elevation of intracellular cAMP levels activates CREB to bind CRE, thus initiating transcription of the luciferase reporter gene.

pCRE-Luc plasmid was used to develop a reporter gene assay for screening of PDE4 subtype selective inhibitors. Reporter gene assay was initially optimized using U937 monocytic cells. U937 cells have been shown to express PDE4A4, PDE4B2, PDE4D3, and PDE4D5 isoenzymes [22]. These enzymes contribute a major fraction of cAMP-specific PDE activity in U937 cells. pCRE-Luc plasmid was transfected into U937 cells and cells were treated

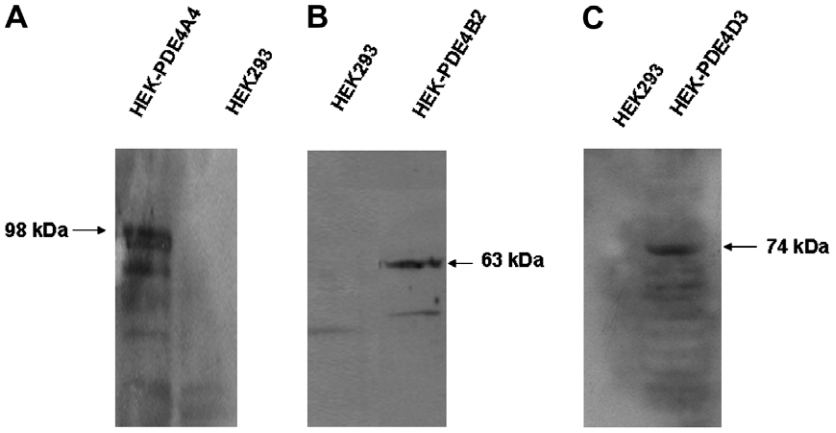

Fig. 1. Western blot analysis of PDE4 subtypes expressed in HEK-293 cells. Cell lysate from the cells expressing each subtype were run on SDS–PAGE, transferred to nitrocellulose membranes and Western blotted with rabbit polyclonal anti-PDE4A IgG (A) or PDE4B IgG (B) or PDE4D IgG (C).

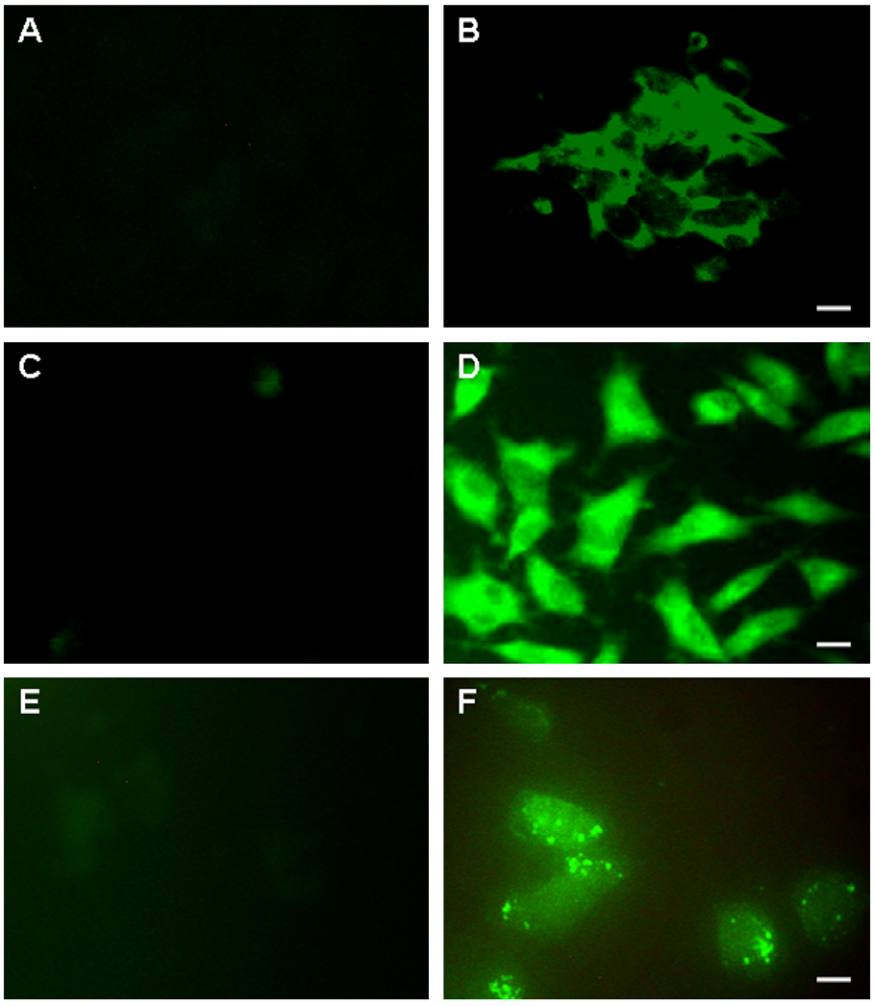

Fig. 2. Cellular localization of PDE4 subtypes in HEK-293 cells. HEK-293 cells, stably transfected with either pcDNA3.1/Zeo vector (A, C, and E) or PDE4A4 (B) or PDE4B2 (D) or PDE4D3 (F) cDNAs, were analyzed by immunofluorescence. Immunostaining was done with rabbit polyclonal anti-PDE4A IgG (A,B) or PDE4B IgG (C,D) or PDE4D IgG (E,F) (scale bar: 10 μm).

with various doses of PDE4 inhibitor, roflumilast, for varying time points. Transient transfection of pCRE-Luc plasmid into U937 cells followed by treatment with roflumilast, resulted in a dose and time dependent increase in luciferase activity as shown in Fig. 3. Maximum luciferase activity was observed at 24 h of drug treatment. In the absence of PDE4 inhibitor, there was very minimal basal level activity, indicating that increase in luciferase activity is only due to inhibition of PDE4 isoenzymes endogenously expressed in U937 with subsequent increase in inhibitor concentrations. Similar dose–response and time kinetics were observed with PDE4 inhibitor rolipram (data not shown). The IC_{50} for roflumilast was found to be 2.5 nM and for rolipram the IC_{50} value was 500 nM as determined by reporter gene assay using U937 cells expressing endogenous PDE4 isoenzymes. These studies indicate that U937 cells could be used for screening of PDE4 inhibitors using this reporter gene assay.

For screening PDE4 subtype selective inhibitors, we generated stable recombinant cell lines HEK-PDE4A4,

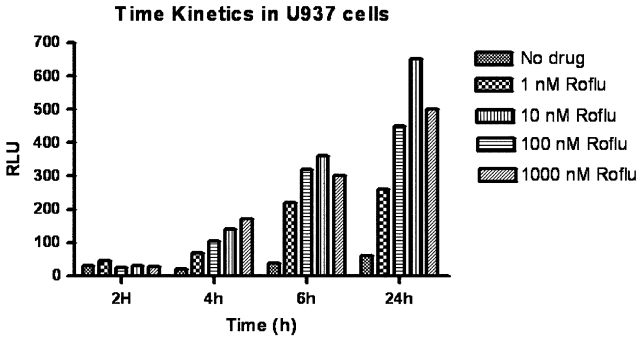

Fig. 3. PDE4 reporter gene assay using U937 cells, expressing PDE4 subtypes endogenously. U937 cells were transiently transfected with pCRE-Luc plasmid and treated with Roflumilast at 1 nM, 10 nM, 100 nM, and 1 μM concentrations for 2, 4, 6 and 24 h as described in Materials and methods. The luciferase activity was measured and plotted using GraphPad Prism 4.0.

HEK-PDE4B2, and HEK-PDE4D3, individually overexpressing PDE4A4, 4B2, and 4D3 subtypes, respectively, as mentioned above. PDE4 activity in these stable cell lines

was confirmed using HitHunter cAMP assay kit. Rolipram inhibited PDE4A4, 4B2, and 4D3 activity with IC_{50} value of 32, 54, and 69 nM, respectively. Reporter gene assay was developed using these stable recombinant cell lines, individually expressing a particular PDE4 subtypes. HEK-PDE4A4, HEK-PDE4B2, and HEK-PDE4D3 cell lines were transiently transfected with pCRE-Luc plasmid and cells were treated with various doses of PDE4 inhibitor rolipram. Transient transfection of pCRE-Luc plasmid into stable cell lines expressing a particular PDE4 subtype, followed by treatment with rolipram, resulted in a dose dependent increase in luciferase activity as shown in Fig. 4A–C). The PDE4A4 activity was inhibited by rolipram with IC_{50} value of 39 nM in HEK-PDE4A4 cell line. PDE4B2 activity was inhibited by rolipram with IC_{50} value of 32 nM in HEK-PDE4B2 cell line. Similarly PDE4D3 activity was inhibited by rolipram with IC_{50} value of 3 nM in HEK-PDE4D3 cell line. The IC_{50} values for rolipram were comparable to the reported values. In the control HEK-293 cells, there was very minimal luciferase activity even in presence of 10 μM of rolipram (Fig. 4A–C), suggesting that there is very low endogenous PDE4 activity in these cells and thus it is an ideal cell line for over-expressing PDE4 subtypes as there will be very low background while performing reporter gene assay. Moreover, in absence of drug (i.e., 0.1% DMSO), there was relatively low activity indicating that increase in the luciferase activity is only due to inhibition of PDE4 isoenzymes over-expressed in the stable cell lines with subsequent increase in rolipram concentrations. Rolipram is a PDE4 selective inhibitor and all the four subtypes of PDE4 are non-selectively inhibited by it. However, this reporter gene assay using stable cell lines, individually over-expressing a particular PDE4 subtype will be extremely useful to identify a PDE4 subtype selective inhibitor that can inhibit a particular PDE4 subtype more potently as compared to other PDE4 subtypes.

PDE4 inhibitors are being developed to treat common chronic diseases including asthma, COPD and Alzheimer's disease [10–12], but their clinical usefulness is limited by adverse effects such as nausea and emesis [13,14]. Clearly, a PDE4 inhibitor with a little or no emetogenicity is more desirable. Robichaud et al. [23] have demonstrated that PDE4D subtype is mainly responsible for the emetic side effect associated with the PDE4 inhibitors and PDE4B inhibition does not cause emesis. Recently Lehnart et al. [17] also showed that PDE4D3 inhibition is associated with heart failure and lethal cardiac arrhythmias. Their data indicated that PDE4D3 subtype plays a protective role in the heart against heart failure and arrhythmias. Hence non-specific inhibition of PDE4D subtypes could lead to increase in mortality due to cardiac arrhythmias. These studies clearly indicate that inhibition of PDE4D3 subtype could lead to adverse effects such as nausea, emesis, heart failure, and cardiac arrhythmias. On the contrary, PDE4B was shown to be essential for LPS-activated TNF-α responses, since in PDE4B deficient mice, lipopolysaccharide (LPS) stimulation failed to induce TNF-α secretion [24], indicating that a PDE4B inhibitor would be an anti-inflammatory drug without emetic and other side effects. Although the role of PDE4A and PDE4C subtypes in emesis and cardiac arrhythmias is not yet clear, a PDE4B subtype selective inhibitor with several fold selectivity over PDE4D3 would be ideal to overcome the adverse effects associated with the currently available PDE4 inhibitors. Thus, the cell-based reporter gene assay that we have developed could be very versatile tool for high throughput

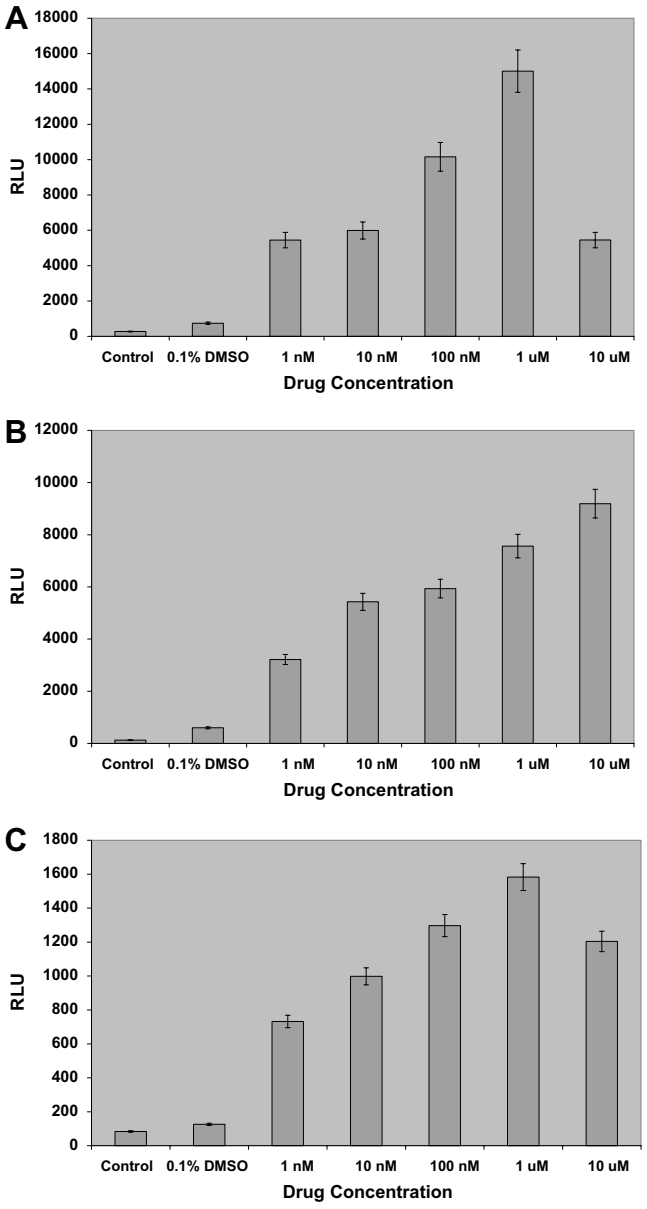

Fig. 4. PDE4 Reporter gene assay using recombinant stable cell lines expressing individual PDE4 subtype. HEK-PDE4A4 (A), HEK-PDE4B2 (B) and HEK-PDE4D3 (C) were transiently transfected with pCRE-Luc plasmid and treated with Rolipram at 1 nM, 10 nM, 100 nM, 1 μM and 10 μM concentrations as described in Materials and methods. The luciferase activity was measured and plotted using GraphPad Prism 4.0. The error bars indicate mean ± SEM of atleast three experiments performed in triplicate.

screening of a PDE4B subtype selective inhibitors with improved therapeutic index and without adverse effect such as emesis and cardiac arrhythmias.

In conclusion we have generated stable recombinant HEK-293 cell lines expressing high levels of PDE4A4B, PDE4B2A, and PDE4D3 subtypes individually and developed a simple and novel cell-based reporter gene assay for identifying PDE4 subtype selective inhibitors. Our data clearly indicate that reporter gene assay using stable recombinant cell lines, individually expressing high levels of different subtypes of PDE4 would facilitate the high throughput screening of subtype selective antagonists and development of a novel class of drugs with improved therapeutic index and better clinical profile.

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