

A Cell-Based PDE4 Assay in 1536-Well Plate Format for High-Throughput Screening

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The cyclic nucleotide phosphodiesterases (PDEs) are intracellular enzymes that catalyze the hydrolysis of 3',5'-cyclic nucleotides, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to their corresponding 5'-nucleotide monophosphates. These enzymes play an important role in controlling cellular concentrations of cyclic nucleotides and thus regulate a variety of cellular signaling events. PDEs are emerging as drug targets for several diseases, including asthma, cardiovascular disease, attention-deficit hyperactivity disorder, Parkinson's disease, and Alzheimer's disease. Although biochemical assays with purified recombinant PDE enzymes and cAMP or cGMP substrate are commonly used for compound screening, cell-based assays would provide a better assessment of compound activity in a more physiological context. The authors report the development and validation of a new cell-based PDE4 assay using a constitutively active G-protein-coupled receptor as a driving force for cAMP production and a cyclic nucleotide-gated cation channel as a biosensor in 1536-well plates. (*Journal of Biomolecular Screening* 2008:609-618)

Key words: phosphodiesterase, PDE4, cyclic nucleotide gated ion channels, cell-based assay, high-throughput screening

INTRODUCTION

CYCLIC NUCLEOTIDE PHOSPHODIESTERASES (PDEs) hydrolyze 3',5'-cyclic nucleotides, including cAMP and cGMP, to their corresponding 5'-nucleotide monophosphates AMP and GMP. Both cAMP and cGMP are important second messengers coupling to the G-protein-coupled receptors (GPCRs) and mediate the responses of a variety of hormones and neurotransmitters. PDEs are responsible for terminating cellular responses to hormones and neurotransmitters, which is critical for maintaining proper intracellular signaling events. There are 11 families of PDEs from 21 different genes.^{1,2} Each PDE family is distinguished functionally by unique enzymatic characteristics and pharmacological profile as well as distinct tissue distribution and cellular expression patterns.^{3,4} Because PDEs regulate a variety of cellular functions, they have become important drug targets for the treatment of several diseases including sexual dysfunction, asthma, chronic obstructive pulmonary disease, neurodegenerative

diseases (Parkinson's disease and Alzheimer's), diabetes, vascular diseases, osteoporosis, cancer, and rheumatoid arthritis.^{2,5,6} PDE4 isoenzymes specifically hydrolyze cAMP and are therapeutic targets for the treatment of several inflammatory disorders.

A number of biochemical assays are available for screening of PDEs that use purified recombinant enzymes and cAMP or cGMP as the substrate. However, a cell-free assay environment may not faithfully reproduce the physiological environment of the cell due to differences in buffer components, pH, and cofactors, among others. In addition, compounds active in enzyme assays are often inactive in disease models due to poor cell membrane permeability, intracellular metabolism, or active sites with highly polar groups.⁷ Therefore, inhibitors identified from enzyme assays usually need to be optimized in cell-based assays before proceeding to animal model studies. A cell-based PDE4 assay was described using the PDE4 gene transiently transfected into COS cells, and the PDE activity was measured with a radioimmunoassay kit in cell lysate.⁸ Another cell-based assay that examined PDE5 was reported using the rat fetal lung fibroblast (RFL-6) cells and the PDE activity was measured by the scintillation proximity assay (SPA) in cellular lysates.⁹ These cell-based PDE4 assays had complicated assay procedure and relatively limited screening throughput. Recently, a cell-based luciferase reporter gene assay using the cAMP responsive element (CRE) binding sequence was reported for the measurement of PDE4, PDE7, and PDE10 activities.¹⁰⁻¹² In general, reporter gene assays often cause shifts in measured compound activities and produce increased false positives in compound library screening due to a

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long cascade of signal transduction and reporter gene transcription/translation. Finally, a throughput-limited cell-based PDE4 assay using a cyclic nucleotide-gated (CNG) cation channel as a biosensor was reported. In this assay, PDE activity was detected using the calcium dye Fura-2 and electrophysiology (using the voltage-clamp method).¹³⁻¹⁵ We report here the development and validation of a new high-throughput-compatible PDE assay using a HEK293 cell line coexpressing a constitutively active GPCR receptor as a driving force for cAMP production together with a CNG channel as a cAMP sensor in 1536-well plate format.

MATERIALS AND METHODS

Materials

A PDE4 cell line (TSHR-CNG-HEK293), a parental cell line (CNG-HEK293), and the membrane potential dye kit were obtained from BD Biosciences (Rockville, MD). The DMEM medium, DPBS, Lipofectamine™ 2000, Geneticin®, trypsin-EDTA, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). The fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Puromycin and the pCMV-IRES-PURO vector were obtained from Clontech (Palo Alto, CA). Forskolin, isoproterenol, and PDE inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

Establishment of stably transfected PDE4 cell line and cell culture

The cell-based PDE4 assay uses a TSHR-CNG-HEK293 cell line (BD ACTOne™) coexpressing a thyroid-stimulating hormone receptor (TSHR) with a CNG cation channel stably expressed. This cell line was generated by transfecting 2 µg of a pCMV-TSHR-IRES-PURO plasmid with 6.25 µl of Lipofectamine™ 2000 to the BD ACTOne™ CNG-HEK293 cells. After 18 to 22 hr at 37 °C in 5% CO₂, the transfected cells were selected in DMEM media containing 10% FBS supplemented with 250 µg/ml Geneticin® and 2 µg/ml puromycin. Selection media was changed every 3 to 4 days, and colonies were observed 2 to 3 weeks after selection. Isolated colonies were selected and transferred into 24-well plates containing 1 ml of DMEM plus 10% FBS supplemented with 250 µg/ml Geneticin® and 1 µg/ml puromycin (as growth medium).

Cells were harvested when they reached 80% to 90% confluence. After detachment by trypsin-EDTA, cells were diluted in growth medium at 7×10^5 cells/ml and 100 µl/well of cell suspension was added to Biocoat 96-well poly-D-lysine-coated plates (BD Biosciences) and cultured for 16 to 24 hr. An equal volume of 1× ACTOne™ membrane potential dye solution was then added to each well and incubated for 1 hr. The known PDE4 inhibitor R020-1724 was added and the fluorescence intensity that reflected PDE4 activity was measured in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA).

The clones with a signal-to-basal ratio larger than 2-fold were selected as candidate cell lines for follow-up analysis. The final cell line was selected based on the IC₅₀ and concentration-response values of RO 20-1724 that were closest to the values reported previously.

The TSHR-CNG-HEK293 cells were maintained in the culture medium containing DMEM, 10% FBS, 250 µg/ml Geneticin®, 1 µg/ml puromycin, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37 °C under a humidified atmosphere containing 5% CO₂. The parental CNG-HEK293 cells (without TSH receptor) were maintained in the same medium without puromycin. Cells were passaged when they reached to 80% to 90% confluence.

Cell-based PDE4 assay

Cells were dispensed at a density of 1000 cells per well in black, clear-bottom, tissue culture-treated, 1536-well plates (Kalypsys, San Diego, CA) in 3 µl of assay medium containing DMEM, 2% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin and were incubated 24 hr at 37 °C with 5% CO₂ prior to compound screening; 3 µl/well of 1× membrane potential dye was added and incubated for 1 hr at room temperature. The library compounds in DMSO solution or the positive control, RO 20-1724, was added at 23 nl/well with a pin-tool station (Kalypsys). After a 60-min incubation with compounds at room temperature, the assay plate was measured in an EnVision™ fluorescence plate reader (PerkinElmer, Woburn, MA) in bottom reading mode with an excitation of 535 (±20) nm and emission of 590 (±20) nm. A flying reagent dispensing (FRD) workstation (Aurora Discovery, San Diego, CA) was used to dispense cells and reagents to 1536-well plates. The Kalypsys pin-tool station was used to transfer 23-nl compounds in DMSO solution to the 1536-well assay plate. The final DMSO concentration in the assay plates was under 0.5%. During library screening, all plate manipulations were done on an automated robotic system (Kalypsys).

PDE4 enzyme assay

The PDE4 A1A enzyme assay in IMAP format has been described previously.¹⁶ In brief, 2 µl per well of PDE4 A1A enzyme (BPS Bioscience, San Diego, CA) mixture (containing a final concentration of 0.05 ng/µl enzyme, 10 mM Tris, pH 7.2, 10 mM MgCl₂, 1 mM DTT, 0.05% NaN₃, and 0.15% BSA) was dispensed to 1536-well black solid-bottom plates. The compounds (diluted in DMSO) were added using the Kalypsys pin-tool station at 23 nl/well and then plates were spun at 1000 rpm for 15 sec in a tabletop centrifuge. After a 5-min incubation at room temperature, 2 µl/well of 200 nM cAMP was dispensed to each well and the plates were spun at 1000 rpm for 15 sec. The assay plates were incubated for 40 min at room temperature followed by an addition of 4 µl/well of IMAP detection reagent (Molecular Devices). The plates were measured in a ViewLux™ plate reader

(PerkinElmer) in fluorescence polarization detection format (excitation = 480 nm and emission = 540 nm) after 1 hr of incubation at room temperature.

Compound library

A collection of 2187 compounds including 1208 compounds of the Library of Pharmacologically Active Compounds (LOPAC, Sigma-Aldrich) and 979 compounds from Tocris (Bristol, UK) was screened against this cell-based PDE4 assay. The compounds were dissolved in DMSO at a concentration of 10 mM for all stock solutions. All compounds were serially diluted in DMSO at a ratio of the square root of 5 (1:2.236) to 15 concentrations in 384-well plates. Four sets of the interplate dilution plates were subsequently reformatted into 1 set of 1536-well plates with concentrations ranging from 0.13 μ M to 10 mM. After 23-nl compound addition, final compound concentrations in the 6- μ l assay volume were from 0.49 nM to 38.3 μ M.

Data analysis

The primary screening data and curve fitting were analyzed using custom software developed at the NIH Chemical Genomics Center. The maximal inhibition (100% activity) was determined by the response of 30 μ M RO 20-1724 and the basal response (0% activity) was measured by the DMSO control in both the enzyme and the cell-based PDE4 assays. In the counter-screen with the parental cell line, 10 μ M forskolin and 30 μ M RO 20-1724 were added to determine the maximal inhibition. The concentration responses of the compounds were analyzed using the methods described by Ingles and colleagues.¹⁷ In brief, concentration-response data were fitted to a 4-parameter Hill equation by minimizing the residual error between the modeled and observed responses. Concentration-response curves were divided into 4 classes: Class 1 contained complete concentration-response curves and r^2 values > 0.9. Class 2 contained incomplete concentration-response curves lacking the lower asymptote and show r^2 values > 0.9. Class 3 curves were of the lowest confidence as they were defined by a single concentration point for the inhibition. Finally, Class 4 contained all inactive compounds that did not show any concentration-response curves. Curves were further subdivided based on efficacy, either >80% inhibition compared with the control as class 1.1 or partial efficacy >50% inhibition as class 1.2. Clustering analysis of active compounds from the primary screening was performed with Leadscape® Hosted Client (Leadscape Inc., Columbus, OH). The results from other experiments were analyzed with Prism software (GraphPad, San Diego, CA).

RESULTS AND DISCUSSION

This cell-based PDE4 assay uses a stably transfected CNG cation channel¹⁴ as a biosensor whose signal can be detected by

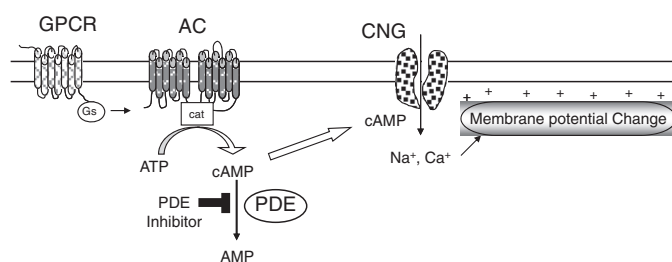


FIG. 1. Schematic diagram of the detection mechanism of the cell-based PDE4 assay. The thyroid-stimulating hormone (TSH) receptor was cotransfected in HEK293 cells with a modified cyclic nucleotide-gated (CNG) channel that serves as a biosensor of intracellular cAMP levels. This transfected TSH receptor has a low level of constitutive activity that stimulates adenylate cyclase (AC) to produce a small amount of cAMP. In the absence of a cyclic nucleotide phosphodiesterase (PDE) inhibitor, this small amount of cAMP is quickly hydrolyzed by PDE, preventing activation of the CNG cation channel. In the presence of a PDE inhibitor, the cAMP level rises, activating the CNG cation channel and resulting in membrane depolarization. A membrane potential dye is used to monitor the change in the membrane potential that is proportional to the inhibition of PDE by the PDE inhibitor.

a change in membrane potential (**Fig. 1**). The PDE substrate, cAMP, is produced by adenylate cyclase (AC), which is stimulated by the constitutive activity of stably transfected TSH receptors coupling through the $G_{\alpha s}$ subtype of the G-proteins.¹⁸ Previous studies have demonstrated that the TSH receptor exhibits a moderate level of constitutive activity in the absence of ligand when it is overexpressed in mammalian cells.^{19,20} The constitutive activity of transfected TSH receptors in the PDE4 cell line maintained a moderate level of cAMP production. The continual cAMP production provides the basis for the measurement of PDE activity without addition of a TSH receptor agonist. In the absence of a PDE inhibitor, the moderate level of cAMP in this PDE4 cell line is rapidly hydrolyzed by endogenous PDEs, predominantly PDE4 in the host HEK293,^{14,21} and is counted as a baseline. In the presence of a PDE4 inhibitor, cAMP accumulates and the increased levels of cAMP bind to and open the CNG cation channels, resulting in an influx of cations including Na^+ and Ca^{2+} . This cation influx triggers a cell membrane depolarization that can be quantified using a fluorescent membrane potential dye that does not require cell wash or medium change steps.²² Thus, the inhibition of PDE activity can be monitored homogeneously in live cells with a standard fluorescence plate reader.

There are several advantages of this cell-based PDE4 assay compared with other previously reported assays.^{9,10,23} First, this cell-based PDE4 assay does not require an addition of exogenous cAMP stimuli such as forskolin (an AC activator) or GPCR agonists, because the constitutive activity of cotransfected TSH receptor produces enough cAMP to generate a measurable signal in the presence of a PDE inhibitor. Second, detection with the membrane potential dye enables homogeneous measurement of PDE activity compared with the use of

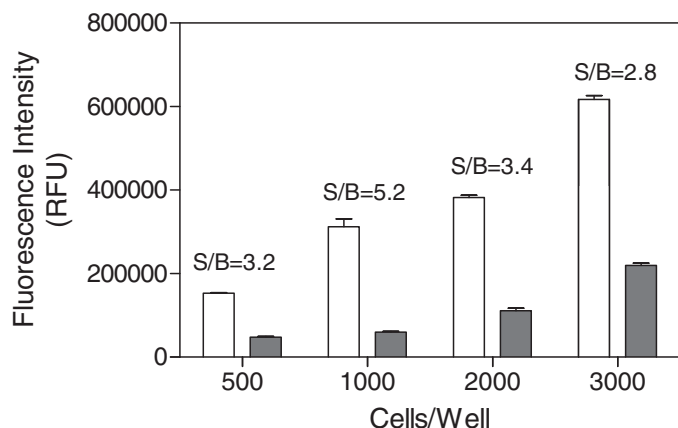


FIG. 2. The effects of cell density on the cell-based PDE4 assay. Cells were plated at 500, 1000, 2000, and 3000 cells per well in a 1536-well plate and incubated for 24 hr before the addition of membrane potential dye and a PDE4 inhibitor. The open bar represents the total fluorescence signal in the presence of 30 μ M RO 20-1724, and the closed bar is the basal fluorescence signal in the absence of RO 20-1724. The signal-to-background ratio (S/B) was calculated by dividing the average of stimulation (4 replicates) by the average of the untreated controls (4 replicates). RFU was the relative fluorescence unit measured at 590-nm emission.

Fura-2 dye or voltage-clamp methods, which have lower throughput and require a cell-wash step during the experiments. Third, the assay protocol is substantially simplified and standard fluorescence plate readers can be used, making this assay suitable for miniaturization and high-throughput screening.

Cell density optimization. All our experiments were carried out in the miniaturized 1536-well plate format. The effect of cell density was first tested in the presence or absence of 30 μ M (EC_{100}) RO 20-1724, a PDE4 inhibitor. Although the total fluorescence intensity in the presence of 30 μ M RO 20-1724 increased with the increase of cell number from 500 to 3000 cells per well, the basal signal also elevated as cell density increased. We found that 1000 cells per well produced a 5.2-fold signal-to-basal ratio, which was superior to other cell densities (**Fig. 2**). Thus, 1000 cells per well was chosen as an optimal cell density in 1536-well plate format for subsequent experiments. We also found that DMSO, the solvent for all the compounds in our screening collection, had no significant effect on this assay at concentrations up to 2%, indicating high tolerance to DMSO for this cell-based PDE4 assay (data not shown).

Time course for PDE4 inhibitors. The time course of 30 μ M RO 20-1724 treatment in the PDE4 cells was measured after compound addition every 10 min for 2 hr. We found that after the addition of RO 20-1724, the total fluorescence signal increased rapidly during the first 20 min followed by a slow

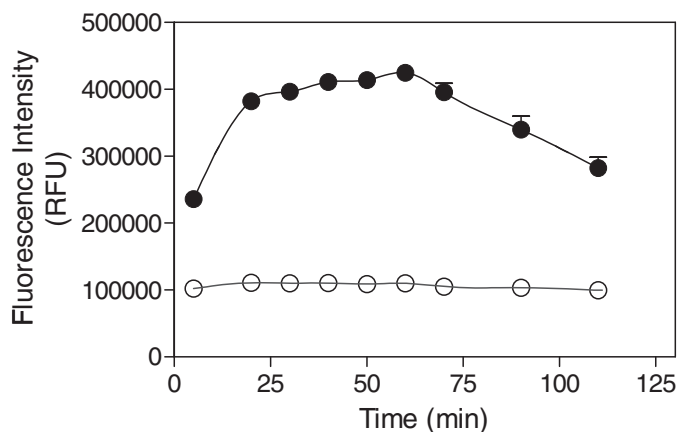


FIG. 3. Time course of the cyclic nucleotide phosphodiesterase inhibitor incubation. Cells were seeded at 1000 cells per well in 1536-well plates. After 24-hr incubation, the membrane potential dye and 30 μ M RO 20-1724 were added. The fluorescence signals of the membrane potential dye were measured every 10 min from 10 to 120 min after the addition of RO 20-1724 (●) or DMSO as a negative control (○).

increase to its peak at 60 min and a gradual decrease afterward. The basal signal did not change with incubation time (**Fig. 3**). This result suggested that cAMP pools began to accumulate in the cells after the addition of RO 20-1724 and the assay signal reached equilibrium at approximately 60 min. Other PDE4 inhibitors (rolipram, milrinone, trequinsin, and etazolate) were examined over a 70-min time frame, and the response of all compounds except trequinsin leveled off after 20 to 30 min. Trequinsin showed a gradual increase of activity over 70 min with no plateau; however, an acceptable signal-to-basal ratio was observed after 20 min of exposure (data not shown).

The decrease of the fluorescent signal after 60 min might be caused by the dye leaking out of cells and/or cells becoming less healthy 2 hr after the membrane potential dye treatment (60 min of dye loading and 60 min of stimulation). Thus, the response of PDE inhibition is stable and can be measured between 30 and 60 min after compound addition. This provides sufficient time for a measurement with a standard fluorescence plate reader without requiring a kinetic measurement.

TSH and forskolin effects on RO 20-1724 activity. In the cell-based PDE4 assay, a constitutively active GPCR (TSHR) is used to drive the production of cAMP, theoretically making the addition of exogenous GPCR agonists or forskolin unnecessary. However, before using this assay to detect novel PDE inhibitors, we tested it directly by examining whether the activity of a known PDE inhibitor was affected by a GPCR agonist or AC activator. The activity of RO 20-1724 in the presence or

absence of 10 nM (EC_{80}) TSH was measured in the PDE4 cell line as well as in the parental cell line stimulated by 200 nM forskolin (EC_{80}). In the PDE4 cell line, the IC_{50} values of RO 20-1724 in the presence and absence of TSH stimulation were 1.72 μ M and 2.39 μ M, respectively. The basal response in the TSH stimulated cells was high due to the constitutive TSH receptor activity, which reduced the assay window (**Fig. 4a**). Our results indicated that the potency of RO 20-1724 in the PDE4 cell line remained the same regardless of whether the TSH stimulation was added but that the basal signal was much lower without TSH stimulation (**Fig. 4a**). In the parental cell line, RO 20-1724 alone did not produce any measurable response (**Fig. 4b**), indicating that its activity in the PDE4 cell line was dependent on TSH receptor activity. The IC_{50} values of RO 20-1724 in the parental cell line with forskolin stimulation was 1.44 μ M (**Fig. 4c**), similar to that determined from the PDE4 cell line. These results demonstrated that the potency of RO 20-1724 determined in this cell-based PDE4 assay without the stimulation by an exogenous agonist was very similar to the potency determined in the experiments stimulated by the GPCR agonist or forskolin. The lack of requirement for a GPCR agonist or AC activator in this assay has several advantages, including cost and time saving in assay optimization, improved consistency, and less possibility of receptor desensitization, which occurs in many GPCRs including the TSH receptor,^{24,25} when exposed to high concentrations of agonists.

Compound library screening. A collection of 2187 compounds, most of which had a known pharmacological action, was screened in both the PDE and the parental cell lines. All compounds were titrated at 15 concentrations and the screen was performed in the quantitative high-throughput screening (qHTS) format¹⁷ As described in **Table 1**, 1000 cells per well (3- μ l cell suspension) were dispensed into 1536-well assay plates and incubated at 37 °C and 5% CO_2 in a cell culture incubator for 24 hr, followed by addition of 3 μ l of membrane potential dye per well and incubation at room temperature for 60 min to allow dye equilibration across the cell membrane, as recommended by the membrane potential dye manufacturer. The 15 compound dilutions were then added to the assay plates using a pin-tool at 23 nl/well, incubated for 60 min at room temperature, and fluorescence intensity was measured in a standard fluorescence plate reader at 530-nm excitation and 590-nm emission. The entire experimental process required reagent additions only, without the need for cell washing or medium aspiration, and was thus easily performed by both a robotic screening system and a semi-automated workstation.

Figure 5a shows the 1536-well format plate map; columns 1 to 4 are used for controls and columns 5 to 48 for experimental compounds. In a test screening with a DMSO plate, the signal-to-basal ratio was 3.9-fold, coefficient of variation (CV) was 10.5%, and Z' factor was 0.50 (**Fig. 5b**), indicating acceptable performance in 1536-well plate format. These assay statistics (signal-to-basal ratio, CV, and Z' factor) were very similar in the parental cell line

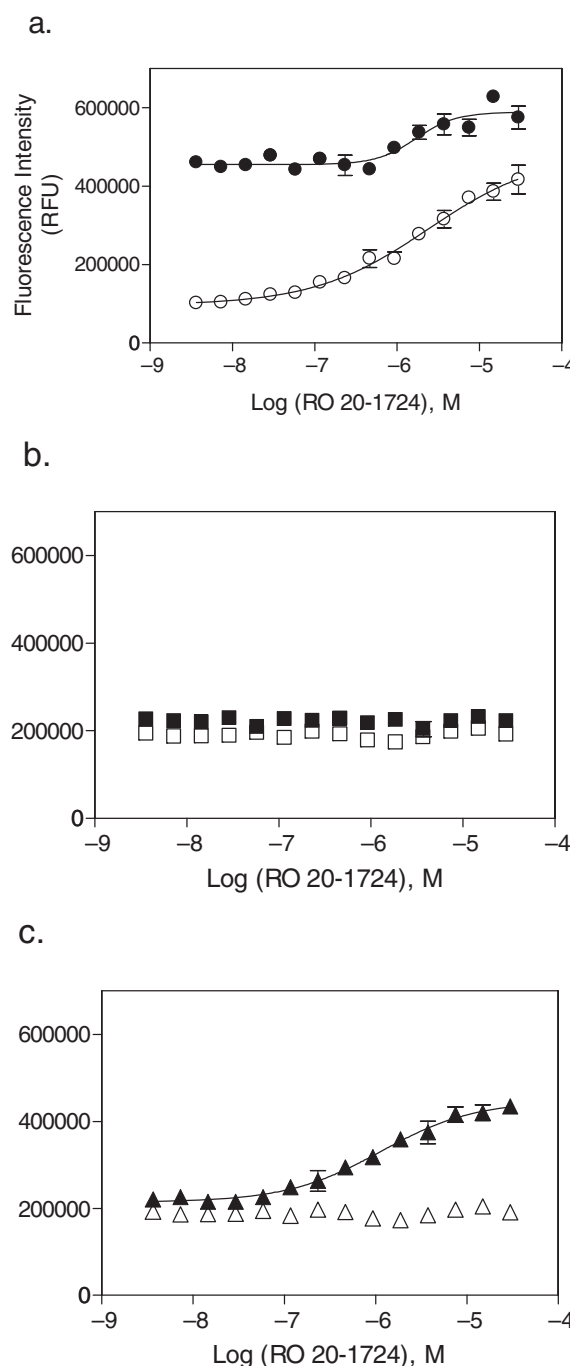


FIG. 4. Concentration responses of RO 20-1724 determined in the PDE4 cell line (TSHR-CNG-HEK293) and parental cell line (CNG-HEK293). Cells were plated at 1000 cells per well in 1536-well plates and incubated for 24 hr. **(a)** RO 20-1724 responses to thyroid-stimulating hormone (TSH) in the PDE4 cell line. The IC_{50} values of RO 20-1724 were 1.72 and 2.39 μ M in the presence (●) or absence (○) of 30 nM (IC_{80}) TSH stimulation, respectively. **(b)** RO 20-1724 responses to TSH in the parental cell line. Parental cells did not respond to TSH stimulation (with [■] or without [□] 30 nM TSH), respectively. **(c)** RO 20-1724 responses to forskolin in the parental cell line. The IC_{50} value of RO 20-1724 was 1.44 μ M (▲ in the presence of 0.2 μ M forskolin). In the absence of forskolin, RO 20-1724 did not produce any signal in this cell line (△).

Table 1. Cell-Based PDE4 Assay Protocol in 1536-Well Plate Format

Step	Parameter	Value	Description
1	Reagent	3 μ l	1000 cells per well
2	Incubation	24 hr	37 °C, 5% CO ₂
3	Reagent	3 μ l	Membrane potential dye
4	Incubation	60 min	Room temperature
5	Compound	23 nl	Columns 1-4 controls and columns 5-48 compounds
6	Incubation	60 min	Room temperature
7	Detector	Ex = 535 nm Em = 590 nm	Envision plate reader

Note: Ex, excitation; Em, emission.

(data not shown). We found that Z' factor was less than 0.5 (0.2–0.4) in some plates during the compound library screening. This might be due to the use of HEK293 cells, which exhibit reduced attachment on the assay plate after dye treatment and subsequently may have caused an increase in the variation between wells. Use of poly-D-lysine-coated plates might be able to improve the cell attachment and thus reduce the variation between wells; however, the 1536-well plates would need a customized coating of poly-D-lysine and this was not available at the time of screening. Recently, a similar HEK293 cell line assayed in non-poly-D-lysine-coated 1536-well plates was used in a successful qHTS campaign for GPCR agonist screening. The occasional low Z' factor numbers were also found in some assay plates; however, several novel agonists were identified and confirmed from the screen.²⁶

The screening results from the parental cell line were used to eliminate the nonspecific and false positive compounds including fluorescent compounds, cAMP pathway signal activators (such as endogenous GPCR ligands, forskolin, etc.). A total of 35 active compounds with a potency of less than 10 μ M and a curve class of 1.1 or 1.2¹⁷ with maximal inhibition greater than 50% were identified in the PDE4 cell line screen; of these, 17 were also active in the parental cell line. Many of the 17 compounds active in both cell lines have known actions in the cAMP signaling pathway (i.e., forskolin analogs, isoproterenol, etc.) whereas others are likely fluorescent compounds. Parallel screening of a parental cell line is therefore an effective method for eliminating the false positive compounds in this cell-based PDE4 assay.

Among the 18 remaining compounds, 11 were PDE inhibitors as defined by the compound providers (Table 2). The other 7 actives were reordered and tested in both PDE4 and parental cell lines, as well as in a PDE4 enzyme assay (Table 3). The activities of 4 of 7 compounds were confirmed in both PDE4 cell line and PDE4 enzyme assay, and they were not active in the parental cell line. Among these 4 confirmed compounds, arctigenin was an

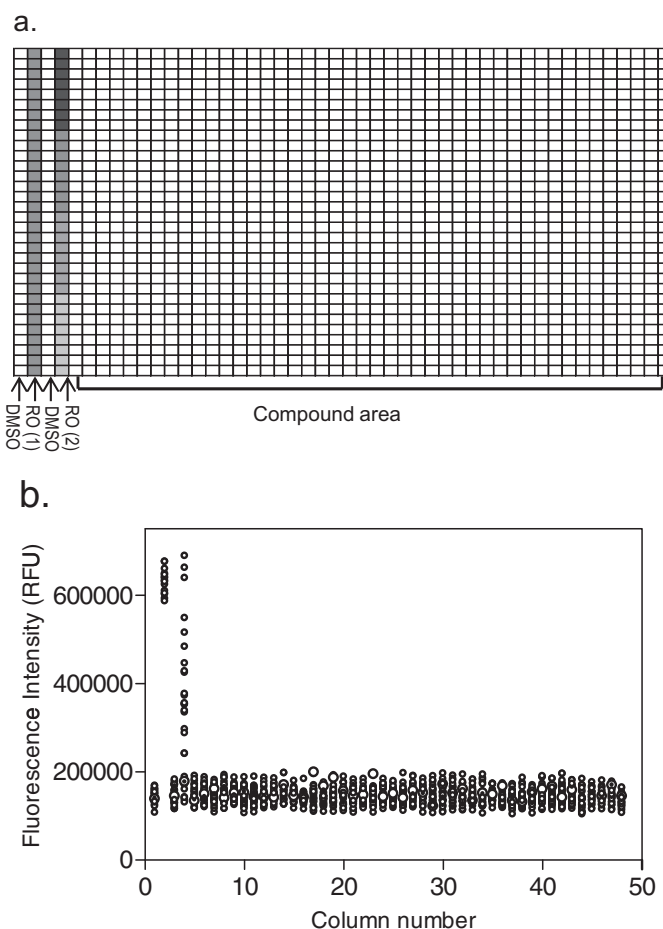


FIG. 5. Plate map and the results of a DMSO plate screen in 1536-well plate format. (a) In a 1536-well plate, 30 μ M RO 20–1724 (EC₁₀₀) was added to all the wells in column 2 as the positive control (RO (1)). The wells in columns 1 and 3 received DMSO solution as the negative control. The wells in column 4 received a titration of RO 20–1724 (1:2 dilution $\times$ 16 concentrations and $n = 2$) as a control concentration-response curve (RO (2)). A total of 1408 wells in column 5 through column 48 could be added with the compounds in DMSO solution (DMSO only for this test experiment). (b) Scatter plot of a DMSO plate screen in the PDE4 cell-based assay. The signal-to-background ratio was 3.9-fold, and the Z' factor was 0.46.

inhibitor of MKK1 and I κ Ba phosphorylation and was also previously reported as a putative PDE inhibitor.²⁷ The other 3 confirmed compounds were all xanthine derivatives that were reported as classical adenosine receptor antagonists as well as inhibiting PDE activity.^{28–31} Among the 3 unconfirmed compounds, CB 34 was a peripheral benzodiazepine receptor agonist that was also active in both PDE and parental cell lines in the retests, although in the primary screen it was not classified as active in the parental cell line due to its borderline activity. The

Table 2. Activities of Known PDE Inhibitors Determined from qHTS

Compound #	Compound Name	Specificity (PDE Isotype)	IC ₅₀ (μM) in PDE4 Cells	% Max. Resp. in PDE4 Cells	IC ₅₀ (μM) in Parental Cells	% Max. Resp. in Parental Cells	IC ₅₀ (μM, Reported)	Ref.
1	(S)-(–)-Rolipram (T-0905)	4	0.1	134	Inact		2.0	32
2	(R)-(-)-Rolipram (T-1349)	4	0.02	121	Inact		0.08	(Tocris)
3	Rolipram (LOPAC R-6250)	4	0.3	98	Inact		0.8	32
4	(S)-(+)-Rolipram (T-1350)	4	0.3	116	Inact		3.0	(Tocris)
5	Ro 20–1724	4	1.0	115	Inact		1.9	32
6	YM 976	4	0.01	92	Inact		2 nM	32
7	ICI 63197	4	0.2	68	Inact		35 nM	(Tocris)
8	Etazolate hydrochloride	4	9.9	105	Inact	18	2.0	(Tocris)
9	Trequinsin hydrochloride	3, (4)	3.2	140	15.8	14	3 nM, 1 μM	(Tocris)
10	Zardaverine	3, (4)	1.0	128	Inact		0.5, (0.8)	(Tocris)
11	Vinpocetine	1	Inact	21	Inact		20	(Tocris)
12	MMPX	1	Inact.		Inact		5.2	(Tocris)
13	8-Methoxymethyl IBMX	1	Inact		Inact		4.0	(Calbiochem)
14	Calmidazolium chloride	1	Inact		Inact		10 nM	(Calbiochem)
15	EHNA	2	Inact		Inact		0.8	(Calbiochem)
16	Imazodan	2	Inact		Inact		6.5	33
17	Cilostazol	3	11.9	59	20	22	0.2	(Tocris)
18	Milrinone	3	6.3	52	Inact		0.3	(Tocris), 34
19	Cilostamide	3	Inact		Inact		0.1	(Tocris)
20	Siguazodan	3	Inact		Inact		0.1	(Tocris)
21	Enoximone	3	Inact		Inact		1.0	32
22	Quazinone	3	Inact		Inact		0.6	35
23	T-1032	5	10	82	Inact		1 nM	36
24	T-0156 hydrochloride	5	Inact		Inact		0.2	(Tocris)
25	MY-5445	5	Inact		Inact		0.5	(Tocris)
26	T-0156	5	Inact		Inact		0.2	(Tocris)
27	Zaprinast	6, 5, 11, 9	Inact		Inact		0.2, 0.8, 12, 29	(Tocris)
28	Papaverine hydrochloride	Nonselect	15.8	155	Inact		0.4–20	32
29	Ibudilast	Nonselect	2.5	135	Inact		12.0	(Tocris)
30	IMBX	Nonselect	12.5	119	Inact		25.0	(Calbiochem)
31	1,3-Diethyl-8-phenylxanthine	Nonselect	2.0	113	Inact		10.0	31
32	Pentoxifylline	Nonselect	Inact		Inact		39.0	37

Note: Compound numbers in boldface represent the 11 cyclic nucleotide phosphodiesterase (PDE) inhibitors that passed screening cutoff criteria for data analysis. qHTS, quantitative high-throughput screening; % Max. Resp., % of maximal response normalized to the maximal response of 30 μM RO 20–1724; Nonselect, nonselective PDE inhibitor; Inact, inactive in the assay. Tocris, Tocris website (www.tocris.com); details in the Signal Transduction section; Calbiochem, Calbiochem website (www.merckbiosciences.co.uk/html/cbc/other_inhibitors_phosphodiesterase.htm).

activities of the 2 other hits were not confirmed in the retests (**Table 3**). Both of the compounds exhibited borderline activity in the primary screen with weak potency and low efficacy. None of these 3 compounds (CB 34, muscarine chloride, or terfenadine) exhibited PDE inhibitory activity in the purified PDE4 enzyme assay. Hence, 4 of 7 compounds were confirmed as PDE inhibitors in both the PDE4 cells and the PDE4 enzyme assay, whereas the activities of other 3 compounds were not confirmed due to the non-specific action in cells or marginal activity. All primary screen results can be viewed in the PubChem database (AID: 607, <http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=607>).

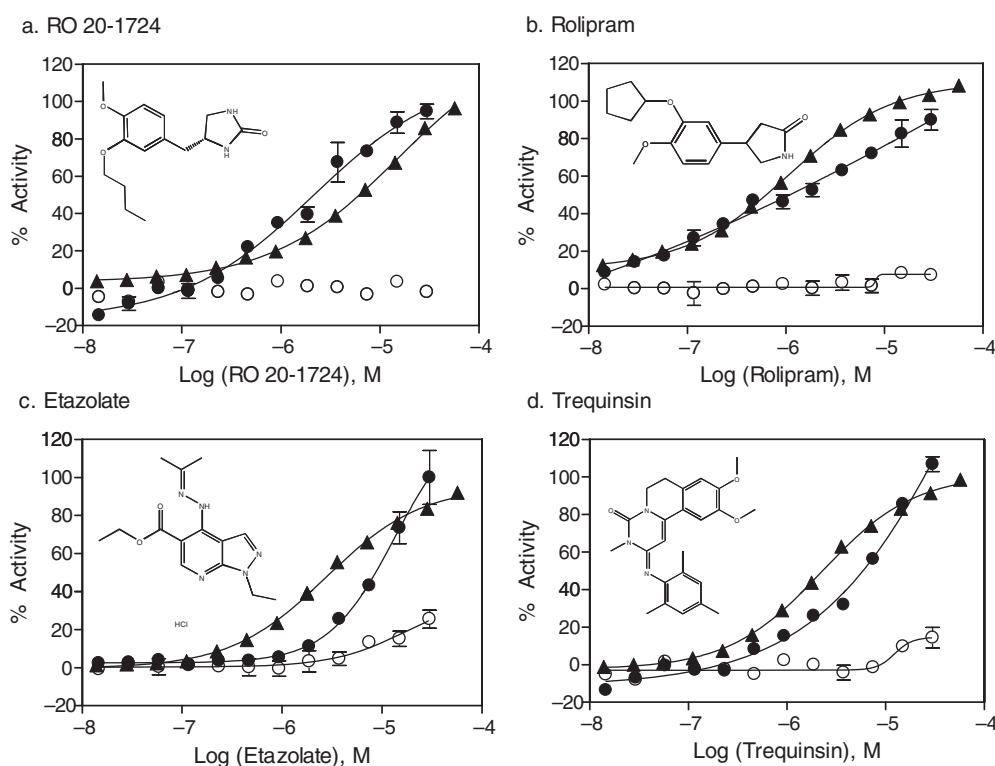
Activities of known PDE inhibitors. The selectivity and potency of the PDE inhibitors (as defined by the compound providers) from the cell-based PDE4 assay and counter-screen in the parental cell line are summarized in **Table 2**. All PDE4 specific inhibitors

showed IC₅₀ values in the cell-based PDE4 assay within a 10-fold window to those reported elsewhere. For example, the IC₅₀ values for RO 20–1724, (S)-(–)-rolipram, trequinsin, and etazolate were 1.0, 0.1, 3.2, and 9.9 μM, respectively, from this cell-based PDE4 assay, and they showed either no activity or very weak activity in the parental cell line (**Fig. 6**). Other compounds exhibited higher or lower potencies than reported values; this may be due to differences in the experimental conditions used in different laboratories (such as enzyme subtype, instrumentation, reagents, data normalization, etc). The activities of 5 nonselective PDE inhibitors including ibudilast, IMBX, 1,3-dipropyl-7-methylxanthine, and pentoxifylline in this assay were also within similar ranges of those reported in the literature (**Table 2**). The inhibitors specific to other PDE subtypes showed either no activity or very weak activity in this PDE assay. For example, vinpocetine, a PDE1 inhibitor with 20 μM reported IC₅₀, showed only a 20% response

Table 3. Activities of 7 Compounds Determined in the Follow-up Assays

Compound #	Compound Name	Compound Activity Description	IC ₅₀ (μM) PDE4 Cells	Max % Resp. PDE4 Cells	IC ₅₀ (μM) Parent Cells	% Max Resp. Parent Cells	IC ₅₀ (μM) PDE Enzyme	% Max Resp. PDE Enzyme
1	Arctigenin	Inhibitor of IκBα phosphorylation. Also inhibits MKK1	Bottom of Form 76.4	53	Inact.		8.8	94
2	DPCPX	Selective A1 adenosine receptor antagonist	8.1	52	Inact.		2.1	41
3	DPX	A1 Adenosine receptor antagonist	6.2	66	Inact.		0.4	93
4	FSCPX	Irreversible A1 adenosine receptor antagonist	1.1	58	Inact.		0.9	33
5	CB 34	Selective high affinity ligand at peripheral benzodiazepine receptors.	8.6	140	10.5	127	Inact.	
6	(+/-)-Muscarine chloride	Muscarinic acetylcholine receptor agonist	17.8	53	12.2	29	Inact.	
7	Terfenadine	Non-sedating H1 histamine receptor antagonist	4.1	20	8.4	35	Inact.	

Note: Compounds tested in cyclic nucleotide phosphodiesterase (PDE), parental cell line, and enzyme assay were described in Materials and Methods. Compound activity description was obtained from the compound provider. DPX, 1,3-diethyl-8-phenylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FSCPX, 8-cyclopentyl-N³-[3-(fluorosulfonyl)benzoyloxy]propyl]-N¹-propylxanthine; CB 34, N,N-dipropyl-2-(4-chlorophenyl)-6,8-dichloro-imidazo[1,2-a]pyridine-3-acetamide. % Max. Resp., % of maximal response normalized to the maximal response of 30 μM RO-20-1724 (PDE4 cells and enzyme) or 10 μM forskolin (parental cells).



(FIG. 6 continued)

(FIG. 6 continued)

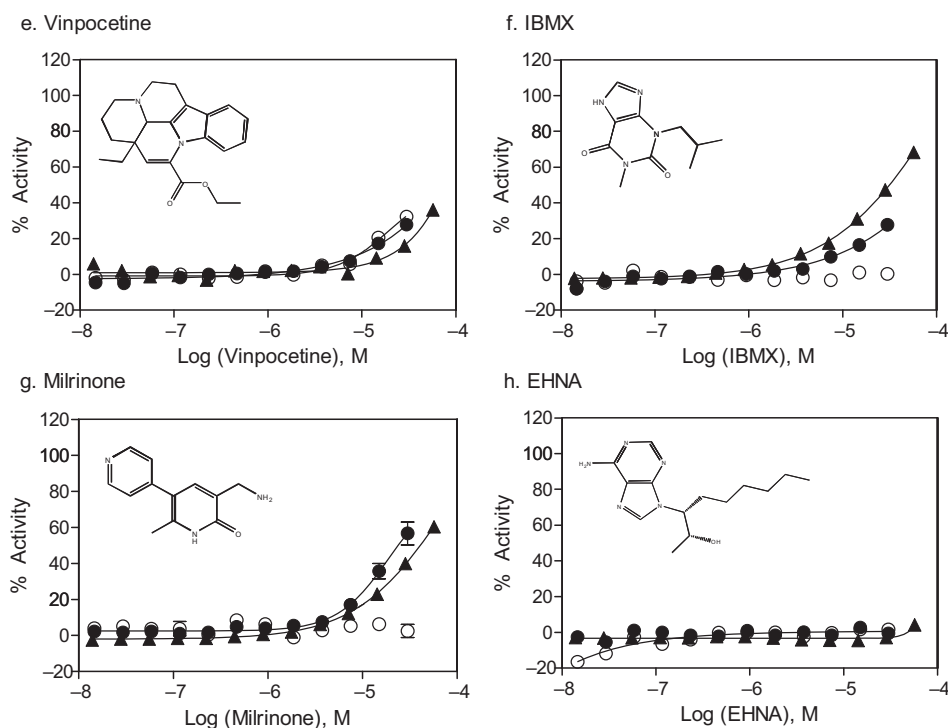


FIG. 6. Concentration-response curves of 8 representative known PDE4 inhibitors determined from the PDE4 cell-based assay. The experiments were carried out in the PDE4 cell line (●) or purified PDE4 A1A enzyme (▲) for the measurement of cyclic nucleotide phosphodiesterase (PDE) inhibition or in the parental cell line (○) as a control. (a) RO 20-1724. (b) Rolipram, a PDE4 inhibitor. (c) Etazolate, a PDE4 inhibitor. (d) Trequinsin, a PDE3,4 inhibitor. (e) Vinpocetine, a PDE1 inhibitor. (f) IBMX, a nonselective PDE inhibitor. (g) Milrinone, a PDE3 inhibitor. (h) EHNA, a PDE2 inhibitor. Data were calculated from 4 samples per point.

at 33.3 μ M in both the PDE and the parental cell lines. These results suggest that PDE4 is the predominant PDE enzyme type present in HEK293 cells, consistent with literature reports.^{14,21}

CONCLUSION

We have developed and validated a cell-based PDE4 assay in a 1536-well plate format. Cell-based PDE4 activity can be determined with a membrane potential dye in a standard fluorescence plate reader; most false positives are easily eliminated by a parallel screen or a counter-screen of the parental cell line. The simplicity of this assay indicates that it is suitable for high-throughput screening of large compound libraries in a 1536-well format to identify novel cell-permeable PDE4 inhibitors. Preliminary results also suggest that this cell-based PDE4 assay strategy can be extended to other PDEs types, either by heterologous expression of other PDEs in the TSHR-CNG-HEK293 cell line, or by utilizing this biosensor in cell lines expressing high levels of other PDE family members.

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