Development of a Novel PDE10A Cell Based Assay

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Abstract

The cyclic nucleotide phosphodiesterases (PDEs) are proteins that catalyze 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 important roles in controlling cellular concentrations of cyclic nucleotides and have central roles in a variety of intracellular signaling events.

Currently, most PDE assays are performed biochemically using pure substrates (cAMP or cGMP) and purified recombinant PDE enzymes. There are no commercially available cell-based screening assays for PDE inhibitors. As with other biochemical screenings on intracellular targets, false positives and false negatives may result from issues involving cell membrane permeability, cellular metabolism, and the like. Cell-based assays would provide better assessment of compound activity in a more physiological context.

Here we report the development of a novel PDE10A cell-based assay. The assay was based on the ACTOne™ technology which utilizes a modified cyclic nucleotide-gated channel (CNG) to monitor intracellular cAMP/cGMP changes in live cells. The influx of cations through the CNG channel can be quantified using ACTOne membrane potential (MP) dye. On that, we developed a PDE10A cell line by over-expressing the gene in the system. We obtained very specific response from a specific PDE10A inhibitor, PF-2545920. To further improve the system, we knocked down the endogenous PDE4 activities by introducing shRNAs against PDE4D and PDE4B. With this improved system, we screened two libraries: 1) LOPAC Library from Sigma; 2) a selective library from Chembridge. Afterwards, two novel PDE10A specific inhibitors have been discovered and confirmed.

Introduction

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 that 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].

PDE10A has been compellingly validated for schizophrenia. Remarkably, high levels of PDE10A mRNA and protein are expressed specifically in the medium spiny projection neurons of the mammalian striatum [7]. The striatum is the largest component of the basal ganglia circuit that regulates motor and cognitive functions. Dysfunctions in the basal ganglia circuits have been linked to various CNS disorders, including schizophrenia [8]. Thus, the specific expression of PDE10A in these neurons raises hopes that PDE10A-targeted drugs could potentially treat schizophrenia with improved efficacy and better tolerability. Administration of potent and selective inhibitors to rats increase both cGMP and cAMP levels in the striatum [9]. As a result, phosphorylation of the downstream effectors CREB and ERK, two markers for enhanced neuronal activation, are increased, suggesting PDE10A inhibitors could treat diseases characterized by a reduced activation of neurons, such as schizophrenia. Deletion of the PDE10A gene blunts locomotor response when challenged with *N*-methyl-D-aspartate (NMDA) receptor antagonist phencyclidine (PCP) and significantly delays the acquisition of conditioned avoidance, a behavioural response predictive of clinical antipsychotic activity [10]. Consistent with these genetic studies, PDE10A inhibitors are found to attenuate conditioned avoidance, and to inhibit PCP- and amphetamine (dopamine-releasing agent)-stimulated locomotor activity in rats and mice. Furthermore, they antagonize apomorphine-induced climbing in mice, block NMDA antagonist-induced deficits in prepulse inhibition of acoustic startle response in rats, while improving baseline sensory gating in mice. Particularly encouraging results have been obtained in animal models of the negative and cognitive symptoms of the psychosis, where these compounds increase sociality in BALB/cJ mice, enhance social odor recognition in mice, and improve novel object recognition in rats, suggesting targeting PDE10A could potentially treat all positive, negative, and cognitive [11].

Codex BioSolutions developed a cell-based HTS assay that measures PDE10A activity using proprietary ACTOne™ technology (Fig. 1). The technology utilizes a permanently-expressed modified cyclic nucleotide-gated channel (CNG) to monitor intracellular cAMP changes in live cells. To assay PDE10A activity, a Gs-coupled GPCR is over-expressed to constitutively activate membrane bound adenylyl cyclases, leading to cAMP synthesis. The produced cAMP is hydrolyzed by PDE10A so the steady cAMP is kept at low level. Upon PDE activity inhibition, the cellular cAMP level rises quickly and is measured by CNG biosensor. The assay will sensitively and reliably identify PDE10A inhibitors in high-throughput campaigns. As the first commercial cell-based PDE10A assay available to the pharmaceutical and biotech industry, it will greatly accelerate the drug discovery efforts for schizophrenia disease, one of the leading public health challenges

Summary

A first commercial cell based PDE10A assay has been developed with a modified cyclic nucleotide-gated ion channel as the biosensor.

The assay sensitivity is comparable to the biochemical assay and can be used for HTS.

LOPAC Library from Sigma and a selective library from Chembridge were screened with the newly developed PDE10A cell based assay.

Two novel PDE10A specific inhibitors have been discovered and confirmed.

References

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Fig. 1 Phosphodiesterase and ACT*One* Biosensor

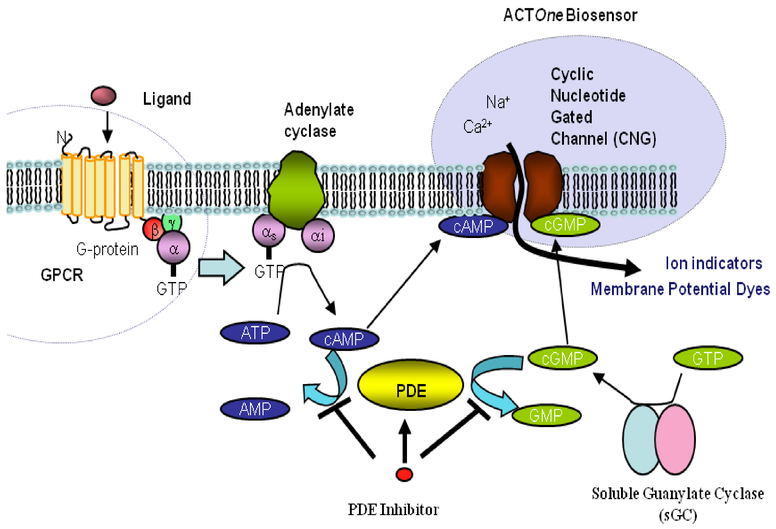
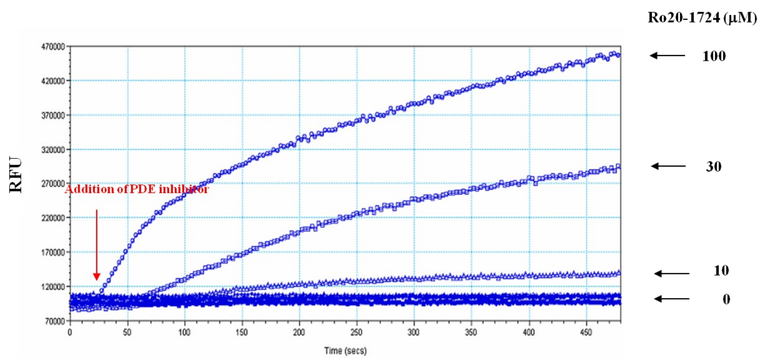
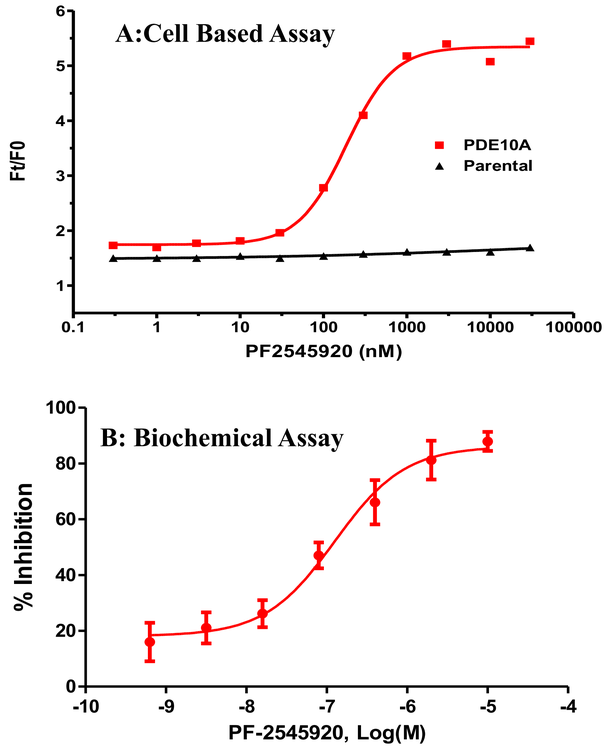


Fig. 2 Kinetic Curves of ACT*One* PDE4 Cells in Response to Ro20-1724 (cAMP Change)



Kinetic response of ACTOne cells optimized for PDE4 assay to Ro20-1724 using ACTOne Membrane Potential Dye Kit. ACTOne cells optimized for PDE4 assay were plated overnight in 100 µl culture medium on a 96 well Biocoat poly-D lysine coated plate. The next day, cells were dye-loaded with 100 µl/well of ACTOne Membrane Potential Dye. After 2 hour of incubation at room temperature, dilutions of Ro20-1724 were added to the plate by a FlexStation (Molecular Devices), and the data was recorded simultaneously.

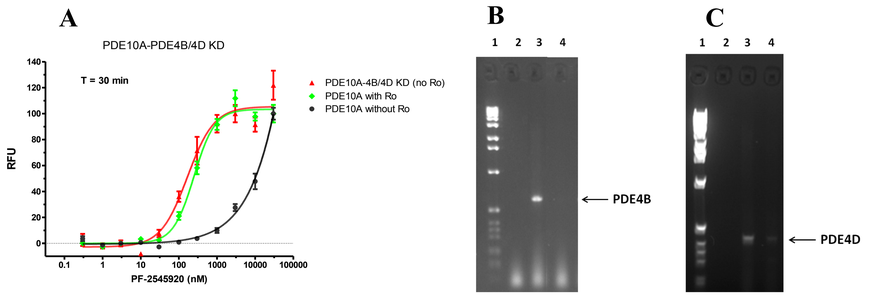
Fig. 3 Cell Based Assay Vs Biochemical Assay for PDE10A (cAMP)



A) ACTOne PDE10A (cAMP) or the parental cells were plated overnight in 20 µl culture medium on a 384-well plate. The next day, cells were loaded with 20 µl/well of ACTOne Membrane Potential Dye with 50 µM Ro20-1724 and incubated at room temperature for 2hr. Different concentrations of PF2545920 were prepared in DPBS.10 µl of compound solution was added into each well and the data was recorded on a Hamamatsu's FDSS 6000.

B) 75 ng of PDE10A enzyme (from BPS Bioscience) was pre-treated with different concentrations of PF2545920 at RT for 20 min. Then the assay buffer containing 10 mM Tris (pH7.4), 10 mM MgCl2, 0.05% Tween-20, 200 mM substrate (cAMP) and 5 kU nucleotidase was added. The total volume for is 50 µl. The reaction was kept at 37oC for 20 min. The OD was measured at 620 nM on a plate reader.

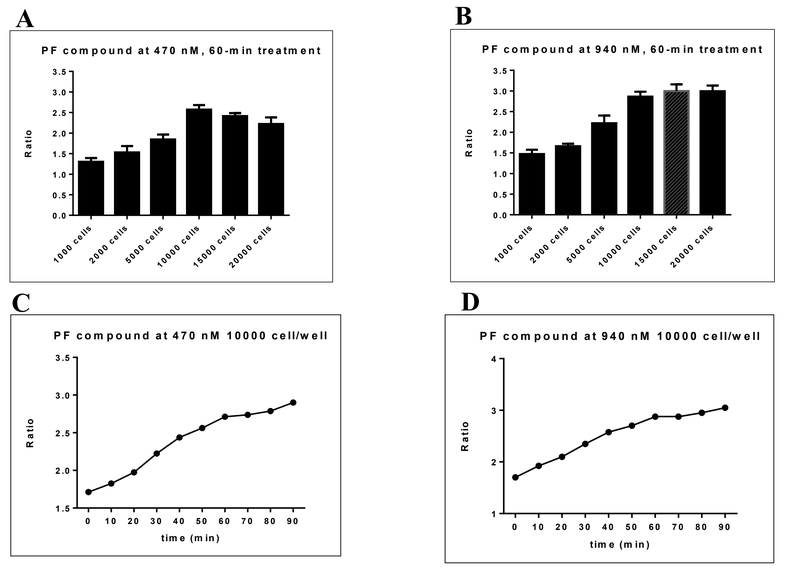
Fig. 4 Endogenous PDE4B/4D Knock Down with shRNA



(A) ACTOne PDE10A cells with endogenous PDE4B/4D knock down or ACTOne PDE10A cells (parental) were plated overnight in 20 µl culture medium on a 384-well plate. The next day, cells were loaded with 20 µl/well of ACTOne Membrane Potential Dye and incubated at room temperature for 2hr. Different concentrations of PF-2545920 solutions were prepared in the presence or absence of 10 µM Ro 20-1724 and added into each well. The data was recorded on FlexStation 30 min after the compound addition.

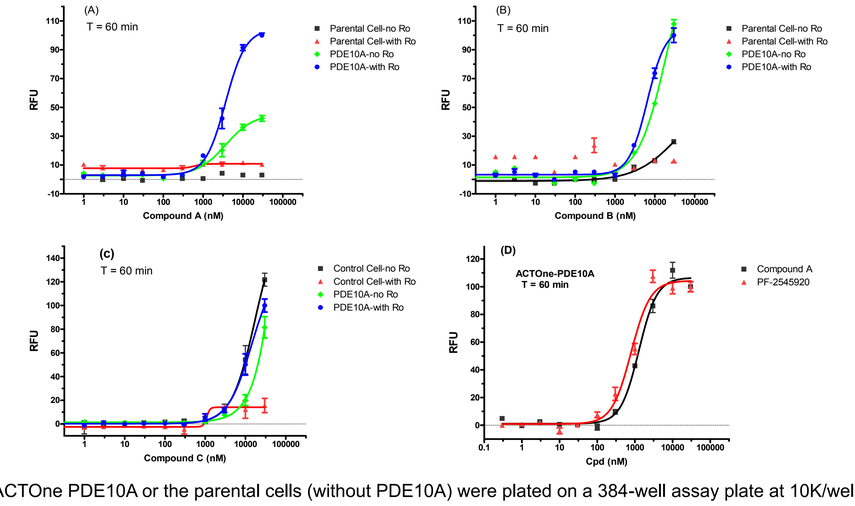
Total RNAs from PDE4B/4D shRNA knock down cells and the ACTOne PDE10A cells were extracted with Qiagen RNeasy Mini Kit. RT-PCRs were performed with Invitrogen's Super Script™ One Step RT-PCR with Platinum® Taq System. (B) RT-PCR with PDE4B specific primers. (C) RT-PCR with PDE4D specific primers. 1) 1kb DNA ladder; 2) Negative control; 3) ACTOne-PDE10A; 4) PDE10A-4B/4D knock down.

Fig. 5 Assay Conditions for Compound Screening on PDE10A Cell Line with PDE4B/4D Knock Down



(A) and (B): Different numbers of the PDE10A cells with PDE4B/4D knocked down in 20 µl of culture medium were plated into each well of a 384-well plate. On the 2nd day, an equal volume of 1X ACTOne membrane potential dye was added into each well. The cell plate (with dye) was incubated at room temperature in the dark for 2 hours. The cell plate was placed on a Hamamatsu FDSS 6000 and the baselines (F0) were recorded. Two different concentrations of PF-2545920 were added into the wells (without Ro 20-1724). The plate was recorded on a Hamamatsu FDSS 6000 again 60 min later. (C) and (D): PDE10A cells (PDE4B/4D KD) were plated into each well of a 384-well plate at a density of 10,000 cells/well in 20 µl culture medium. On the 2nd day, an equal volume (20 ml) of 1X ACTOne membrane potential dye was added into each well. The cell plate (with dye) was incubated at room temperature in the dark for 2 hours. Two different concentrations of PF-2545920 were added into the wells (without Ro 20-1724). The plate was recorded on a Hamamatsu FDSS 6000 every 10 min.

Fig. 6 Confirmation of PDE10A Specific Hits



ACTOne PDE10A or the parental cells (without PDE10A) were plated on a 384-well assay plate at 10K/well. On the 2nd day, an equal volume of 1X ACTOne membrane potential dye with or without 20 µM Ro20-1724 was added into each well. After two hours incubation, different concentrations (5X of the final concentrations) of the compounds (A-C) were prepared in 1X DPBS with 2.5% DMSO.10 µl of above solutions was added into each well. The cell plate was read on a FlexStation after 60 min of compounds addition. (D) Dose response curves of compound A and PF-2545920 on ACTOne-PDE10A cells in the presence of 10 µM Ro 20-1724

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