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A Novel Cell Based Assay for PDE Inhibitor Screenings

Jianming Lu[1], Steve Titus[2], Wei Zheng[2] and Xiao Li[1]
1. BD Biosciences, 15010 Broschart Road, Rockville, Maryland 20850, USA
2. NIH Chemical Genomics Center, 9800 Medical Center Drive, Rockville, Maryland 20850, USA

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 an important role in controlling cellular concentrations of cyclic nucleotides and have a central role in a variety of intracellular signaling events. cAMP PDEs are emerging as a promising class of drug targets in asthma and cardiovascular disease therapeutic areas.

There are no commercially available cell-based screening assays for PDE inhibitors: currently biochemical assays are done using pure substrates (cAMP or cGMP) and purified recombinant PDE enzymes. As with other biochemical screens on intracellular targets, false positives and false negatives may result from issues involving cell membrane permeability, cellular metabolism, and the like. Here we report the development of a cell-based screening assay for cAMP phosphodiesterase (PDE) inhibitors. The assay is based on the BD ACT*One*™ technology which utilizes a modified cyclic nucleotide-gated channel (CNG) to monitor intracellular cAMP changes in live cells. The influx of cations through the CNG channel can be quantified using BD™ PBX Calcium Assay Kit or membrane potential (MP) dyes (including the ACT*One*™ MP dye). We report here modifications to the BD ACT*One*™ technology that allow the detection of PDE inhibitors. As proof of principal, we performed an inhibitor screen for PDE-IV, which is expressed endogenously in HEK293 cells.

Introduction

PDEs form a superfamily of enzymes that are subdivided into 11 major families.[1] Each PDE family is distinguished functionally by unique enzymatic characteristics and pharmacological profiles. In addition, each family exhibits distinct tissue, cellular, and subcellular expression patterns. By administering a compound that selectively regulates the activity of one family or subfamily of PDE enzymes, it is possible to regulate cAMP and/or cGMP signal transduction pathways in a cell- or tissue-specific manner.

Cyclic nucleotide-gated (CNG) channels of vertebrates are cation channels controlled by the cytosolic concentration of cGMP and cAMP[2]. These channels conduct cation currents, carried by mixed ions-Na^+, K^+ and Ca^{2+}-and serve to couple both electrical excitation and Ca^{2+} signaling to changes of intracellular cyclic nucleotide concentration.

BD ACT*One*™ cAMP assay was originally developed to monitor real time intracellular cAMP changes triggered by GPCR activation in a high throughput format without requiring a cell lysis step. A modified CNG channel is used as a reporter, and the channel is activated by elevated intracellular levels of cAMP, resulting in ion flux and cell membrane depolarization.

However, the original ACT*One* cell lines can not be used to detect intracellular cAMP accumulation caused by PDE inhibition without additional manipulation due to the sensitivity range of the CNG channel. External stimulation of forskolin or agonists of endogenous Gs coupled receptor to increase intracellular cAMP is required to detect PDE inhibitor activity by CNG activation.[3] The optimal amount of stimulating compound for a particular PDE assay must be empirically determined, and the complexity of cell-based assays that use an externally provided stimulator of intracellular cAMP production make such assays undesirable for applications such as a high and medium throughput screening.

We report here an improved cell-based assay for PDE inhibitor screening using cyclic nucleotide-gated channel as intracellular cAMP sensor. The assay can be carried out without the use of an externally provided stimulator of intracellular cAMP production.

Conclusion

We have developed a cell line for cell-based high-throughput cAMP PDE inhibitor screening using a modified cyclic nucleotide-gated ion channel as the biosensor.

The assay is in homogeneous format, and does not require addition of external compound to stimulate intracellular cAMP production.

The specificity of the assay has been verified by known PDE inhibitors. Only PDE4 and pan-PDE inhibitors showed positive signals in the cell line that was optimized for PDE4 inhibitor screening.

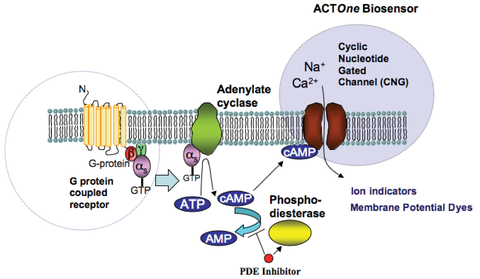
In collaboration with NCGC, the assay has been automated and miniaturized to 1536-well format. A small scale compound screening has been done, and all known PDE4 inhibitors have been identified from the screening.

False positives resulted from the screening are agonists of endogenous Gs coupled receptors, activators of adenylate cyclases and other compounds that stimulate intracellular cAMP production.

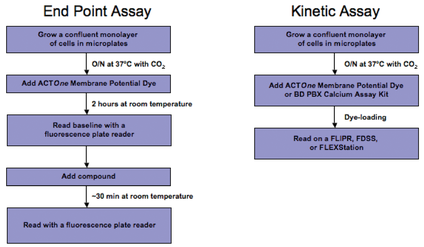
References

1. Beavo, *Physiol. Rev.* 75: 725-48, 1995
2. Kaupp, *Curr. Opin. Neurobiol.* 5:434-442, 1995
3. Rich *et al. J. Gen. Physiol.* 118, 63-67, 2001

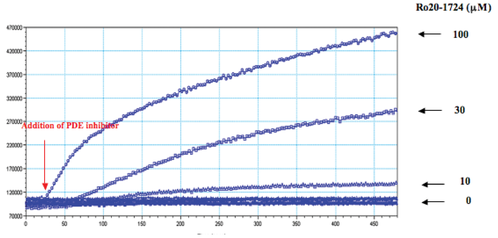
1 Phosphodiesterase and ACT*One* Biosensor



2 ACT*One* PDE Assay Protocol

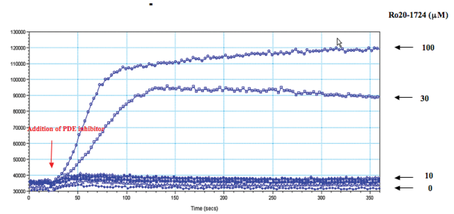


3 Membrane Potential Changes of ACT*One* PDE4 Cells in Response to Ro20-1724



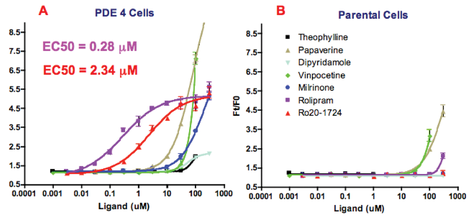
Kinetic response of ACT*One* cells optimized for PDE4 assay to Ro20-1724 using ACTOne Membrane Potential Dye Kit. ACT*One* cells optimized for PDE4 assay were plated overnight in 100 µl culture medium on a 96 well Biocoat poly-D lysine coated plate. The next day, cells were dye-loaded with 100 µl/well 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.

4 Calcium Influx of ACT*One* PDE4 Cells in Response to Ro20-1724



Kinetic response of ACT*One* cells optimized for PDE4 assay to Ro20-1724 using BD™ PBX Calcium Assay Kit. ACT*One* 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 BD™ PBX Calcium Assay Kit. After 1 hour of incubation at 37°C, dilutions of Ro20-1724 were added to the plate by a FlexStation (Molecular Devices), and the data was recorded simultaneously.

5 Dose Response Curves of Various PDE Inhibitors



Response of cells optimized for PDE4 assay and parental cells to different PDE inhibitors. Cells were plated overnight in 20 µl culture medium on a 384 well Biocoat plate. The next day, cells were dye-loaded with 20 µl/well of ACT*One* membrane potential dye. After 2 hour of incubation at room temperature, baseline was recorded using a FlexStation (Molecular Devices). 10 µl of PDE inhibitors at various concentrations were added to the cell plate, and the data was recorded 30 minutes after drug addition. Dose response curves were generated by Prism (Graphpad). A. Cells optimized for PDE4 assay. B. Parental cells.

6 PDE Inhibitors Tested on PDE4 Cell Line

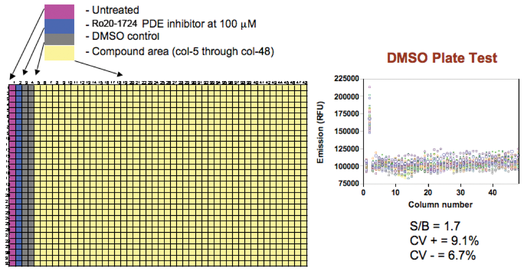
Isozymes	Inhibitors	IC50 (µM)
PDE1	Vinpocetine 8-MM IBMX	>10 >100
PDE2	EHNA	>100
PDE3	Milrinone	>10
PDE4	Ro20-1724 Rolipram Etazolate	2.34 0.28 42.11
PDE5	Dipyridamole Zaprinast	>100 >100
PDE6	Dipyridamole Zaprinast	>100 >100
PDE7	Dipyridamole	>100
PDE8	Dipyridamole	>100
PDE9	Dipyridamole Zaprinast	>100 >100
PDE10	Dipyridamole	>100
PDE11	Dipyridamole Zaprinast	>100 >100
Pan PDE	IBMX Papaverine	46.67 >10

7 Miniaturization of ACT*One* PDE Assay in 1536-well Format

BD PDE inhibitor screen (Optimized and Conducted by NCGC)			
Sequence	**Parameter**	**Value**	**Description**
1	Cells	3 µL	1000 cells/well 1536 TC treated black wall/clear bottom plate
2	Time	12 hours	37°C 5% CO_2
3	Compound (for positive control only)	0.5 µL	Kalypsys dispense RO compound 100 µM final [A] Column 1-2
4	Reagent	3 µL	BD membrane potential dye
5	Time	1 hour	Room temperature
6	Compound	23 nL	Libraries
7	Time	1 hour	Room temperature
8	Detector	See below [B]	Envision (Perkin Elmer)

A. The stock 2 mM RO 20-1274 PDE inhibitor was made in 10 % DMSO. Parental cell line received 10 µM forskolin + 100 µM RO.
B. Envsion settings: Bottom Excitation 535/20 nm top emission 590/20, gain of 150 and 5 flashes per well.

8 Screening Template – 1536-well Plate Format



Screening Result

- 9205 compounds were screened in 7 or 15 concentrations in 1536-well plate format.
- One third of the compounds are from LOPAC (Sigma), Tocris and Prestwick libraries containing known compounds. The rest are compounds with unknown functions.
- 53 compounds showed PDE cell line specific signaling in a titration dependent manner. Of the 53 positive compounds, 11 are known PDE inhibitors.
- All known PDE4 inhibitors have been identified from the screening.