A Stable, Sensitive Fluorescence-Based Method for Detecting Cyclic AMP

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Abstract

Adenosine 3′, 5′-cyclic monophosphate (cyclic AMP, cAMP) is a universal secondary messenger that connects changes in the extracellular environment, as detected by cell surface receptors, to transcriptional changes in the nucleus. Since cAMP-mediated signal transduction plays a role in critical cell functions and human diseases, monitoring its activity can aid in understanding these responses and the process of drug discovery. This report examines the performance of a fluorescence-based competitive immunoassay in 384-well microplate format. Using purified cAMP as a competitor, the estimated detection limit was determined to be 0.1 pmol/mL and Z′ factor was greater than 0.83, which indicates that the assay is of high quality and one of the most sensitive assays currently on the market. Of note, the results obtained were similar whether the reaction was allowed to proceed for 10 minutes or up to 60 minutes. Next, HEK 293 cells were treated with the promiscuous adenylate cyclase activator, forskolin, and the -adrenoceptor agonist, isoproterenol. The resultant average EC_{50} values were 11 nmol/mL and 123 pmol/mL, respectively, which correspond to those found in the literature. Together, these results demonstrate that this assay is a fast, accurate, non-radioactive method that is ideal for high-throughput screening.

Introduction

Cyclic AMP is involved in intra- and inter-cellular signaling in organisms as diverse as bacteria, slime mold, fruit flies and humans (4, 5, 8, 9). Cyclic AMP is produced from ATP by the enzyme adenylate cyclase (AC) and is hydrolyzed by cAMP phosphodiesterases. Regulation of these enzymes allows for tight control over the levels of cAMP inside the cell. In mammals, extracellular ligands, such as peptide hormones and neurotransmitters, interact with transmembrane proteins called G-coupled receptors. Ligand binding leads to a conformational change in the receptor that allows its association with a GTP-binding regulatory protein (G-protein). G-coupled receptors activate AC when bound to a stimulatory form of G-protein, G_s, and inhibit the enzyme when bound to an inhibitory form of G-protein, G_i. Activation of G_s-coupled receptors results in increased production of cAMP, which in turn, activates cyclic AMP-dependent protein kinase or PKA. This enzyme phosphorylates a variety of proteins, which eventually leads to regulation of transcription factor activity and effects on gene expression. Targets for PKA include other enzymes, ion channels and transcriptional regulators. In addition, cAMP plays a direct role in transcription by binding and augmenting the activity of the transcription factor, cAMP-responsive element-binding protein or CREB. This protein plays a role in modulating the expression of cAMP-inducible genes (6). Further complicating this pathway is the role that cAMP plays in directly regulating the activity of cation channels (10).

Depending on the cell type, this signal transduction pathway may be responsible for neural and immune responses, metabolism, mitogenesis oocyte maturation, as well as many other critical functions (1–5). Therefore, an accurate method for quantitative measurement of intracellular cAMP is critical for basic research and drug discovery purposes. Current methods for quantitating cAMP include radioactive immunoassays (RIA), scintillation proximity assays (SPA), and fluorescence polarization- and luminescence-based assays. RIA and SPA methods, which use radioactive isotopes, are cumbersome, include indirect costs for waste storage and removal and entail long incubation times. Fluorescence polarization methods also require long incubation times. On the other hand, luminescence-based assays have short incubation times, but the signal is not stable. The new fluorescence intensity-based assay described here is unique because the signal is stable from 10 minutes to 24 hours, the overall time to process the assay is less than three hours, it includes only one wash step and is one of the most sensitive assays currently available on the market. The stable signal makes this assay ideal for high throughput screening purposes. In this report, we illustrate these advantages as well as the high quality of the assay.

Materials

Cells: HEK 293 cells (human embryonic kidney cells that stably overexpress the Adenovirus E1A protein) were grown in DMEM with 10% FBS at 37 °C in 5% CO_2.

Reagents: CatchPoint™ cyclic AMP fluorescent assay kit (Molecular Devices, cat #R8044, Tel: 800-635-5577), DMSO, low water content (Sigma, cat #D2650, Tel: 800-325-3010), Krebs-Ringer Bicarbonate Buffer (KRBG, Sigma, cat #K4002). sodium bicarbonate (Sigma, cat #S5761), cAMP agonists including forskolin (Sigma, cat #F6886) and isoproterenol (Sigma, cat #15627), phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine or IBMX (Sigma, cat #17018) and 30% hydrogen peroxide (H_2O_2) solution (Sigma, cat #H0904).

Methods

Cell preparation: HEK 293 cells were grown to approximately 80% confluence at 37 °C. Cells were detached using 0.526 mM EDTA in PBS and washed once with KRBG. Cells were resuspended at a concentration of 1 x 10^6 cells/mL in stimulation buffer (KRBG buffer that contains 0.75 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor). Next, cells were seeded in a volume of 7.5 µL at a concentration of 0.75 x 10^4 cells/well of a 384-well microplate. The microplate was incubated for 10 minutes at room temperature.

Cell stimulation: In order to stimulate cells to produce cAMP, 15 µL doses of activator and agonist were diluted in PBS (forskolin and isoproterenol, Sigma), or for unstimulated cell samples, PBS alone was added. The range of agonist final concentrations used was 0 to 1 mM. In addition, 15 µL samples of cAMP calibrator at final concentrations ranging from 0 to 3333 pmol/mL were added to the wells in order to generate a calibration curve. Each of the above conditions was performed in replicates of eight. The microplate was gently mixed and incubated at 37 °C for 15 minutes at 5% CO_2. In order to lyse the cells, 7.5 µL of cell lysis buffer (supplied with kit) was added to each well, and the plate was agitated for 10 minutes at room temperature using a plate shaker. The final volume in each well was 30 µL.

Immunoassay: The immunoassay procedure was started by transferring 20 µL of each sample to the 384-well solid black assay microplate (supplied with kit). Then the samples were treated with 20 µL of rabbit anti-cAMP antibody (supplied with the kit), and the microplate was gently agitated on a plate shaker for 5 minutes to ensure proper mixing. Next, 20 µL of HRP-cAMP conjugate (supplied with kit) was added to all wells, the plate was agitated and incubated for 2 hours at room temperature. The assay microplate was washed four times with 80 µL/well of wash buffer (supplied with kit). Then, 50 µL of Stoplight Red solution (supplied with kit) was added to every well. The microplate was covered to protect it from light and incubated for up to one hour at room temperature. The resulting fluorescence intensity was read at 10 minutes and 60 minutes using a Gemini XS and Analyst™ AD (Molecular Devices Corporation). The instrument settings used for the Gemini XS were excitation wavelength of 530 nm, emission wavelength of 590 nm, emission cutoff filter of 570 nm, 6 readings per well with the photomultiplier tube set to automatic. The instrument settings used for the Analyst AD are shown below:

Parameter	Settings
Mode	Fluorescence Intensity
Excitation Filters	Rhodamine 530–25 nm
Emission Filters	Rhodamine 590–20 nm
Dichroic Mirror	50/50 Beamsplitter
Z-height	3 mm
Attenuator	Medium
Integration Time	50,000 msec
Lamp	Continuous
Readings Per Well	One
PMT Setup	Smart Read+ (sensitivity = 2)
Units	Counts/sec

Assay Performance

Analysis of the data obtained for samples containing cAMP calibrator resulted in the plots shown in Figure 1. These data were obtained after 10 minutes and 60 minutes incubation with Stoplight Red substrate. In this case, the C value (2.4 pmol/mL for 10 minutes [Fig 1A] and 3.7 pmol/mL for 60 minutes [Fig 1B]) is the midpoint, and hence the EC_{50} value the curve. The EC_{50} values read at 10 minutes and 60 minutes were similar, which demonstrated the relative stability of the fluorescent signal over time. The signal is also stable for 24 hours (data not shown). The average EC_{50} value obtained when the assay was performed on different days was 3.55 ± 0.2 pmol/mL. This value is in agreement with the average EC_{50} value of 3.4 ± 0.6 pmol/mL, which was obtained using the Analyst AD.

For the experiments shown in Figure 1, the signal-to-background (no cAMP calibrator) ratio was 240, and the limit of detection (LOD, defined as three standard deviations difference from the zero cAMP calibrator control) was 0.1 pmol/mL cAMP for both fluorescence microplate readers. The optimal performance range of the assay falls between 0.14–33 pmol/mL. These results are in accordance with those obtained using the Analyst AD (0.1–80 pmol/mL, data not shown). In addition, the Z′ factor for the assay was calculated. The concentration of cAMP calibrator that achieved maximal inhibition (33 pmol/mL) was used as the positive control, and the concentration of cAMP calibrator that achieved the minimal inhibition (0.14 pmol/mL) was used as the negative control (8). A value between 0.5 and 1.0 indicates a high quality, robust assay appropriate for screening purposes (8). The Z′ factors for the assay read on the Gemini XS and Analyst AD were 0.84 and 0.90, respectively.

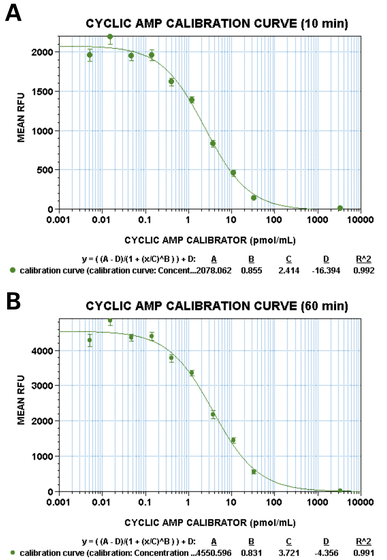

Figure 1. Dose response curves for cAMP calibrator using Gemini XS. Dose responses are shown for (A) 10 minutes and (B) 60 minutes after addition of Stoplight Red substrate. Each point on the dose response curves represents the average of eight replicate samples. The error bars denote the standard deviation from the mean. The R^2 value is 0.99 for a 4-parameter curve fit. RFU denotes relative fluorescence units.

Response of HEK 293 Cells to Forskolin and Isoproterenol

Next, the response of HEK 293 cells to forskolin and isoproterenol was examined. Forskolin is a natural diterpene, which is a promiscuous activator of all adenylate cyclase isoforms, while isoproterenol is a -adrenoceptor agonist (9, 10). HEK 293 cells are a suitable model system with which to study the regulation of cAMP because of their low endogenous level of adenylate cyclase activity (9). The results of the experiments are shown in Figures 2 and 3. The EC_{50} values obtained for forskolin were 12.7 nmol/mL and 9.3 nmol/mL using Gemini XS and Analyst AD, respectively (Figure 2). These results are in accordance with values obtained using other products ([FP]2™ kit from NEN/Perkin Elmer, see web site product information).

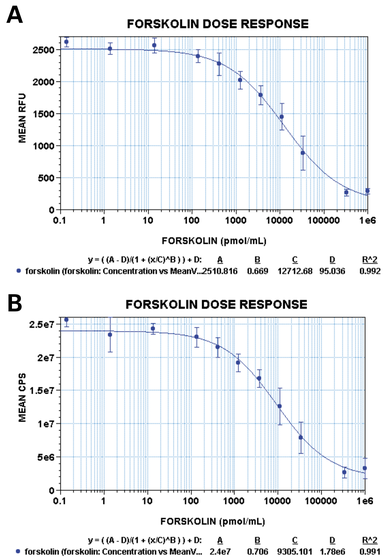

Figure 2. Dose response curves for forskolin treated HEK 293 cells. Each point on the dose response curves represents the average RFU of eight replicate samples for one concentration of the activator. The plate was incubated with Stoplight Red substrate for 60 minutes before reading this data set. The error bars denote the standard deviation from the mean. The R^2 value for the experiment was 0.99 using a 4-parameter curve fit. The curve shown in (A) was obtained using Gemini XS and the curve shown in (B) was obtained using Analyst AD. The EC_{50} values were (A) 12.7 nmol/mL and (B) 9.3 nmol/mL for Gemini XS and Analyst AD, respectively.

The EC_{50} values obtained for isoproterenol were 123 pmol/mL using both the Gemini XS and Analyst AD, respectively (Figure 3). The cAMP values shown on the Y-axis were obtained by interpolation of the original data. The graphs in Figure 3 were shown to demonstrate the ability of the software supplied with both fluorescence microplate readers to convert RFU to cAMP values. The EC_{50} values for isoproterenol are in accordance with values obtained using another cell line (10).

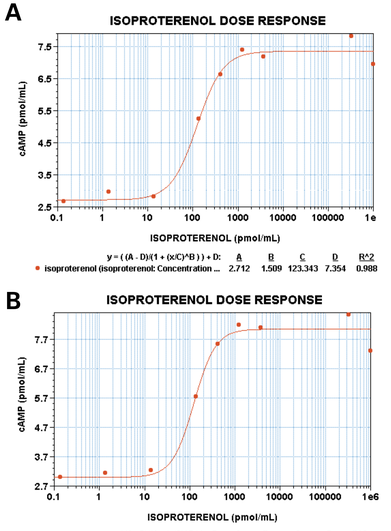

Figure 3. Dose response curves for isoproterenol treated HEK 293 cells. Each point on the dose response curves represents the average cAMP of eight replicate samples for one concentration of the agonist. The error bars denote the percent coefficient of variation. The R^2 value for both experiments was 0.98 for a 4-parameter curve fit. The dose response curves shown were obtained after 60 minutes incubation of samples with Stoplight Red substrate. The curve shown in (A) was obtained using Gemini XS and the curve shown in (B) was obtained using Analyst AD. The cAMP values shown on the Y-axis were obtained by interpolation of the original data. The EC_{50} values were (A) 123 pmol/mL and (B) 123 pmol/mL for Gemini XS and Analyst AD, respectively.

Discussion

Compared to radioactive methods for cAMP detection such as scintillation proximity assays (SPA) and radioimmunoassays (RIA), the CatchPoint cAMP fluorescent assay is fast (well under three hours to process), requires no costly waste clean up and has at least ten-fold higher sensitivity. The typical sensitivity for a SPA assay is 2 pmol/mL for the non-acetylated form of cAMP, while the LOD obtained with CatchPoint is 0.1 pmol/mL. In addition, CatchPoint is more sensitive than fluorescence polarization methods of measuring cAMP (1.25 pmol/mL) by approximately ten-fold.

With respect to speed of assay and sensitivity, one luminescence-based kit is similar to CatchPoint but requires special instrumentation for detection of the signal. Finally, the short incubation time required to reach a stable signal (10 minutes to 24 hours) makes the CatchPoint assay kit ideal for high throughput screening. This point is confirmed by comparison of the results after 10 minutes and 60 minutes incubation with Stoplight Red substrate. The LOD and EC_{50} values for the cAMP calibrator were very similar at the two time points. Furthermore, this fluorescent assay provides a high quality screening assay as demonstrated by the Z′ factor and similar results obtained on different days and with different fluorescence microplate readers. In conclusion, CatchPoint cyclic AMP fluorescent assay allows simple, convenient and highly sensitive quantitative measurement of intracellular cAMP.

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