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Research Article

A CE assay for the detection of agonist-stimulated adenylyl cyclase activity

A CE assay was developed for the detection of adenylyl cyclase (AC) activity stimulated at the AC and G protein-coupled receptor (GPCR) level. In the assay, cell membranes overexpressing GPCR and/or AC were incubated with modulators and substrate ATP to produce cAMP in a dose-dependent manner. In both the CE-UV and a radiochemical assay, the addition of forskolin (FSK) resulted in a two- to three-fold maximum increase in AC activity with EC_{50}s of 4.2 ± 0.7 and 2.4 ± 0.7 μM, respectively, demonstrating that similar results were obtained by both assays. GPCR activation was also detected using cell membranes overexpressing AC and the β_2-adrenergic receptor (β_2AR) fused to the stimulatory G protein. Terbutaline (β_2AR agonist) increased the basal rate of cAMP formation 1.7 ± 0.1-fold resulting in an EC_{50} of 62 ± 10 nM. The assay's ability to detect antagonists is demonstrated by the expected right-shifted EC_{50} of terbutaline by the β_2AR antagonist propranolol. The CE-UV assay offers advantages over the traditional radioactivity assay in terms of safety and labor.

Keywords:
Adenylyl cyclase / CE / Enzyme assay / G protein-coupled receptor
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1 Introduction

The G protein signaling cascade is initiated by an extracellular ligand (or drug) binding to a membrane-bound G protein-coupled receptor (GPCR). The ligand-binding event causes a conformational change in the associated intracellular G proteins that further activate downstream effectors such as adenylyl cyclase (AC). Over 50% of current drugs target GPCRs [1], and GPCRs are implicated in many conditions including addiction [2], heart failure [3], and infection by the human immunodeficiency virus [4, 5]. More recently, AC has also been viewed as a drug target based on its involvement in Alzheimer's [6], diabetes [7], and addiction [8, 9]. Novel, rapid, and robust assays are required for ligand screening and drug discovery at both of these signal transduction targets.

AC converts ATP to the second messenger cAMP, and therefore ligands directly targeting AC can be studied by monitoring this enzymatic reaction. A common approach to detect GPCR activation is to monitor a downstream biochemical reaction that results from binding of ligands to the GPCR. For example, GPCR activation may be detected by monitoring the activation of AC by stimulatory G proteins ($G\alpha_s$) coupled to the GPCR [10–14]. An advantage of monitoring AC activity for GPCR studies is that it is often more sensitive than assays upstream of the biochemical pathway, such as [^{35}S]GTPγS binding, due to signal amplification [12, 15]. Therefore, the same AC assay can be used to monitor ligands binding to GPCRs or AC.

The most common method for detecting AC activity uses radioactive substrate as a tracer and ion-exchange chromatography to separate the product from substrate and side-product nucleotides (AMP and ADP) [16, 17]. Although sensitive and reliable, this technique is slow, labor intensive, and uses radioactivity, which adds danger and cost to the assay. Desire for a safer, nonradioactive assay led to the development and commercialization of enzyme immunoassays (EIAs) for cAMP [18–20] to probe AC activity, but the limited dynamic range, expense, and time associated with the EIA make it impractical for everyday use. Several other assay platforms have also been commercialized and have been recently reviewed, including the use of radio- or fluorescently labeled cAMP to compete with cAMP formed in the assay in a competitive immunoassay format [12, 21, 22]. Drawbacks of commercially available assays include requirements for special reagents (antibodies and/or labeled cAMP), time-

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Abbreviations: AC, adenylyl cyclase; **β_2AR,** β_2 adrenergic receptor; **EIA,** enzyme immunoassay; **FSK,** forskolin; **GPCR,** G protein-coupled receptor; **PDE,** phosphodiesterase

consuming filtering steps, or custom endpoint detectors, all of which add expense to the assay. An ideal assay would eliminate the requirements for radioactivity and expensive reagents with limited shelf lives. One possibility would be to use native ATP as the substrate and a high-resolution separation technique, such as CE, to resolve the substrate from product (cAMP).

CE has emerged as a valuable tool for measuring the enzymatic activity due to low volume sample requirements, rapid analysis times, high-resolution separations, and ease-of-use (for reviews, see [23–25]). Many variations of the CE enzyme assay have been reported [23], but the simplest involves off-line incubation of substrate–enzyme mixture followed by injection and separation. The different charge-to-size ratios of cAMP and ATP suggest that CE could potentially be a valuable addition to the armamentarium of techniques used to measure AC activity.

In this work, CE was used to measure AC activation in membranes from *Spodoptera frugiperda* (fall armyworm, *Sf9*) cells expressing AC or coexpressing the β_2-adrenergic receptor (β_2AR) fused to $G\alpha s_L$ (a long splice variant of $G\alpha_s$) and AC. CE is used to detect the conversion of ATP to cAMP following the addition of drugs that act either at the GPCR or directly at AC. The technique offers a more rapid and automatable approach to detect AC activity, and has promise to be scaled up for use in high-throughput screening.

2 Materials and methods

2.1 Materials

Sf9 cells were purchased from Invitrogen (Carlsbad, CA). Tris-HCl, sodium phosphate monobasic monohydrate, and $MgCl_2 \cdot 6H_2O$ were purchased from Fisher (Fair Lawn, NJ, USA). Forskolin (FSK; from *Coleus forskohlii*) was purchased from Calbiochem (San Diego, CA, USA) and Ro 20-1724 was purchased from A.G. Scientific (San Diego, CA, USA). [α-^{32}P]ATP and [^{3}H]cAMP were purchased form Perkin-Elmer. (±)-Propranolol, (−)-isoproterenol, terbutaline, and all the other materials were purchased from Sigma (St. Louis, MO, USA). Buffers were made in deionized water purified by E-Pure water systems (Barnstead International, Dubuque, IA, USA).

2.2 Protein expression and purification

Cytosolic AC domains VC_1 (isoform V, first cytosolic domain) and ArgC-IIC_2 (IIC_2, isoform II, second cytosolic domain) were expressed and purified as previously described [26, 27] and stored at −80°C until use.

2.3 Membrane preparations

Sf9 cells (cell density of $1.6–1.8 \times 10^6$ cells/mL) were infected with viruses containing the cDNAs for AC2 alone (AC) or a fusion of β_2AR and $G\alpha s_L$ (β_2ARGαs_L) [28] and AC2 (β_2ARGαs_L-AC) and incubated at 27°C under rotation. The AC2 virus was a kind gift from A. G. Gilman (University of Texas Southwestern Medical Center). Cells were harvested 48–72 h post infection in buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and protease inhibitors (32 μg/mL each of *N*-tosyl-L-phenylalanine chloromethyl ketone and *N*-α-*P*-tosyl-L-lysine chloromethyl ketone, 35 mg/mL phenylmethylsulfonylfluoride, and 3.2 μg/mL each of leupeptin and soybean trypsin inhibitor) and lysed by nitrogen cavitation using a Parr bomb (600 psi, 30 min). Unlysed cells were removed by a low speed spin ($2000 \times g$, 10 min), and membranes were collected following ultracentrifugation ($100\,000 \times g$, 35 min). Membranes were diluted in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, and protease inhibitors (same as above) and protein concentrations were determined by the Bradford Assay (BioRad Laboratories, Hercules, CA, USA). With the exception of the cell infection, all the steps were performed at 4°C. Aliquots were snap frozen in liquid nitrogen and stored at −80°C. Thawed samples were homogenized (Kimble Kontes, Vineland, NJ, USA) prior to use.

2.4 Adenylyl cyclase assays for purified AC

Samples containing 1 μM AC (1 μM VC_1 and 7.6 μM IIC_2) and 1 mM ATP were incubated with or without 100 μM FSK in buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT. Samples were maintained at 25°C and were not prepared in a regeneration system because endogenous ATPase and phosphodiesterases (PDEs) were absent.

2.5 Adenylyl cyclase assays for membrane-bound AC

2.5.1 Radioactivity assay

AC assays were performed as previously described [16, 26, 28, 29]. Briefly, 25–100 μg of membrane was incubated with modulators in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 5 mM $MgCl_2$ for 30 min on ice with a final volume of 50 μL. The reaction was initiated by the addition of 50 μL of activation buffer (20 mM Tris-HCl pH 8.0, 3 mM potassium phosphoenolpyruvate, 100 μM Ro 20-1724, 0.6 mM EDTA, 5 mM $MgCl_2$, 50 μg/mL pyruvate kinase, 2 mM ATP, and $\sim 10^6$ cpm [α-^{32}P]ATP), and membranes were incubated for 30 min at 30°C, unless otherwise indicated. The reaction was quenched with buffer containing 0.25% SDS, 5 mM ATP, and 0.175 mM cAMP and nucleotides were separated with Dowex and alumina columns. [^{3}H]-cAMP was used as the internal standard to account for column recovery variation.

2.5.2 CE assay

Samples were prepared the same as above with the exceptions of the elimination of radiolabeled ATP and reactions being quenched with 2 μL of 500 mM EDTA ($[EDTA]_{final}$ = 10 mM). The autosampler was at 30°C when

kinetic studies were performed on-line. Bovine $G\alpha_s$ was expressed in *Escherichia coli*, purified and preactivated with GTPγS as previously described [30]. Thymidine was used as the internal standard.

2.6 CE with UV detection

A PACE/MDQ CE unit (Beckman Coulter, Fullerton, CA, USA) was used to separate nucleotides using a 75 μm inner diameter, 360 μm outer diameter fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an effective length of 20 cm and a total length of 30 cm. Absorbance was measured at 254 nm. The autosampler was maintained at 4°C for all the experiments unless otherwise noted. Data acquisition (16 Hz) and control were performed using P/ACE 32 Karat Software Version 5.0 (Beckman) for Windows 2000 on a 2.0 GHz IBM personal computer.

For experiments using purified cytosolic domains, separation buffer was 20 mM sodium phosphate, pH 8.8. For experiments using membrane-bound AC, separation buffer was 20 mM sodium phosphate, 10 mM EDTA pH 10.3. At the beginning of each day, capillary was rinsed with 0.1 M NaOH, H_2O, and separation buffer for 5 min each. Between separations, the capillary was rinsed with 0.1 M NaOH and electrophoresis buffer for 1 min each prior to injection. Unfiltered samples were injected for 3 s at 0.5 psi and separation was at 15 kV (500 V/cm) and 10 kV (333 V/cm) for experiments using purified and membrane-bound AC, respectively.

2.7 Data analysis

CE data were analyzed with Cutter 7, software written in-house [31]. The amount of cAMP produced in the CE assay was quantified using a calibration curve of cAMP standards. For the radioactivity assay, cAMP was quantified using scintillation counting as described elsewhere [17]. EC_{50} values were determined by fitting dose–response data using GraphPad Prism (Version 3.0, GraphPad Software, San Diego, CA, USA) to $y = \text{Bottom} + [(\text{Top-Bottom})/(1 + 10^{(\text{LogEC50-}x)})]$ where x is the logarithmic concentration of the activator in M and y is the rate of cAMP formation. Each dose–response experiment was performed in quadruplicate and similar EC_{50} values were obtained. Error is reported as ± S.E.M.

3 Results and discussion

Our goal was to develop a radioactive-free, rapid, and robust CE assay for the detection of AC activity. The assay was initially demonstrated with purified AC before being extended to cell membranes overexpressing AC to better mimic AC's physiological environment. After the assay was optimized for cell membranes, AC drugs were detected using membranes overexpressing AC, then GPCR drugs were detected using membranes overexpressing GPCR and AC.

3.1 Detection of AC activity using purified AC

Initial experiments used purified cytosolic domains of AC and monitored the conversion of ATP to cAMP under basal and FSK-stimulated conditions. The cytosolic domains contain the active site, and previous studies have used the cytosolic domains to model membrane-bound AC [27, 32]. When cytosolic domains of AC were incubated with ATP and the resulting mixture separated by CE, peaks corresponding to product (cAMP, $t = 1.9$ min) and substrate (ATP, $t = 3.7$ min) were observed (Fig. 1A), validating the assay's ability to detect AC activity. (The identity of the cAMP and ATP peaks was confirmed by spiking in standards.) The small sample volumes associated with CE allowed the same sample to be serially injected for measuring the rate of cAMP formation. Figure 1B demonstrates that linear rates can be monitored under basal conditions (inset). When AC was stimulated with FSK, the rate of cAMP formation increased as expected, but rapidly deviated from linearity (Fig. 1B). When product and substrate were monitored with time under these conditions (Fig. 1C), it became apparent that the deviation from linearity was due to rapid substrate depletion. It was also noted that the amount of cAMP formed plus ATP remaining was not constant throughout the 100 min reaction (Fig. 1C). This was not likely due to the formation of side products as none were detected. It is partially due to a lower extinction coefficient for cAMP (12.3×10^3/M/cm) [33] than ATP (15.4×10^3/M/cm) [34], resulting in a decrease in the total peak area as the amount of cAMP formed increased. In view of the narrower peaks for cAMP than ATP, the decrease in total area may also be partially due to using a concentration-sensitive detector, which can yield smaller peak areas for a given mass injected if the peak is narrower.

3.2 Assay optimization for detecting AC activity in cell membranes

When measuring AC activity in cell membranes, modifications to the assay must be made to account for endogenous ATPase and PDE activities [17]. ATPases convert ATP to ADP and AMP resulting in substrate depletion and nonlinear rates of product formation. PDEs convert cAMP to AMP making the detection of cAMP difficult if not impossible. To compensate for ATPase activity, ATP-regeneration systems (such as pyruvate kinase/phosphoenol pyruvate) are commonly used to regenerate ATP from ADP and AMP, ensuring a constant substrate supply [35, 36]. PDE activity is prevented by the addition of PDE inhibitors such as Ro 20-1724, allowing accumulation and detection of cAMP.

All nucleotides must be well resolved to ensure proper functioning of the regeneration system and PDE inhibitors. The separation conditions used for the experiments involving purified AC cytosolic domains were sufficient for the separation of cAMP and ATP (samples lacked endogenous ATPases and PDEs and therefore separating ADP from ATP was not a concern). A change in separation conditions was

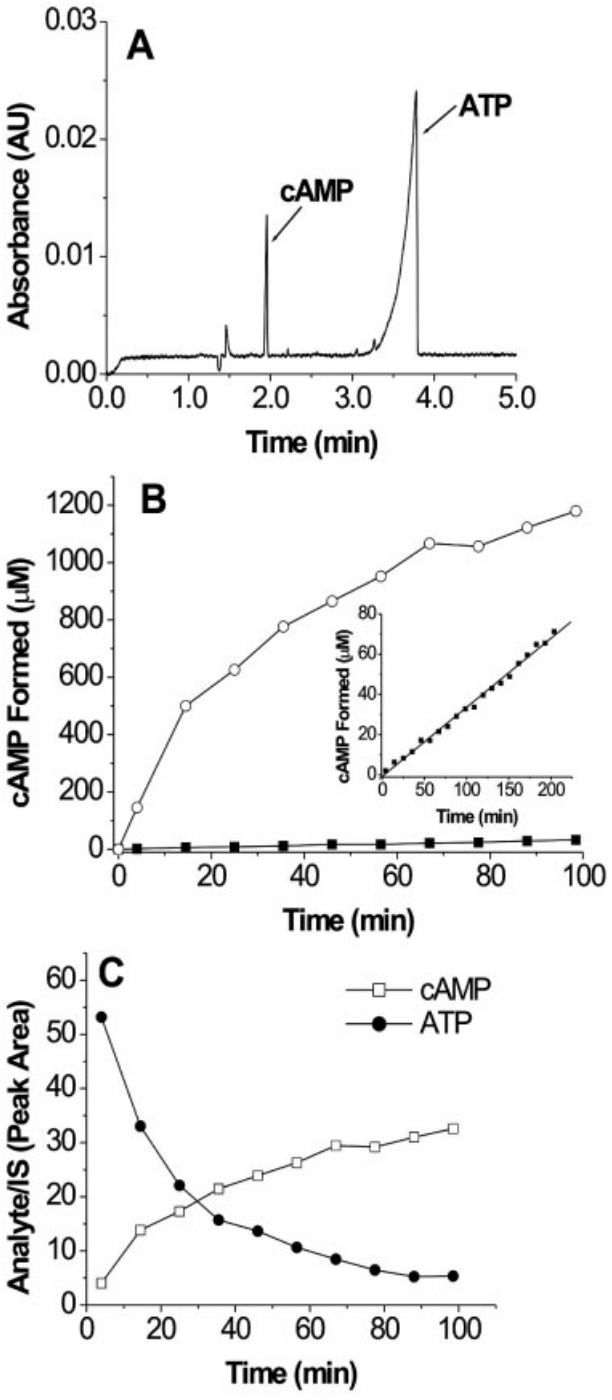

Figure 1. Purified cytosolic domains validate the use of the CE-UV assay to monitor AC activity. All the samples contained 1 μM AC and 1 mM ATP. (A) Electropherogram of sample containing AC, ATP, and 100 μM forskolin after 4 min of reaction. (B) Samples containing AC, and 0 μM (■) or 100 μM forskolin (○) were serially injected and cAMP was monitored with time. The inset is a blowup of the linear cAMP formation under non-stimulated (0 μM forskolin) conditions. (C) ATP and cAMP were monitored with time in sample containing AC, ATP, and 100 μM forskolin. The *y*-axis represents the peak area of ATP or cAMP divided by the peak area of internal standard (IS).

required for analysis of AC activity in membranes because ADP was not well resolved from ATP using the separation conditions for purified AC. Addition of EDTA to the separation buffer was found to improve the resolution between the nucleotides; however, this also increased the current resulting in slower EOF and longer migration times. To alleviate some of the heating associated with the increased current, the electric field was decreased to 333 V/cm, further contributing to longer migration times.

3.3 Detection of basal AC activity in cell membranes

Using the improved sample and separation conditions, detection of AC activity in cell membranes was attempted using CE. *Sf*9 membranes containing overexpressed AC were incubated with ATP (see Section 2) and cAMP was produced, migrating at 3.3 min by CE, as shown in Fig. 2A. After 30 min of reaction, ~5 μM cAMP (or 133 fmol cAMP) was formed. The identity of cAMP peak was confirmed by spiking with standards. ATP migrated after cAMP at $t = 9.0$ min. Both cAMP and ATP migrated at longer times than in Fig. 1A because the separation buffer was changed to better resolve the nucleotides (see above). After ~100 min of reaction time (depending on the amount of protein used in the assay), AMP and ADP were detected in the electropherogram migrating between cAMP and ATP (data not shown). The presence of these nucleotides indicated depletion of the PDE inhibitor and regeneration system. Occasionally, spikes of irreproducible migration time (denoted by asterisks in Fig. 2A) were observed in electropherograms and were attributed to the injection of membrane fragments or other particulates from the unfiltered samples.

An important experiment when detecting enzyme activity is to ensure that the amount of product formed increases linearly with the amount of protein in the sample. To test the linearity of cAMP formation as a function of protein amount, substrate was added to various amounts of AC membrane and the amount of cAMP formed after a 30 min reaction was measured. As expected, the amount of cAMP produced increased linearly as a function of protein amount (Fig. 2B). However, increasing protein amounts to above 75 μg/tube also led to irreproducible migration times, making quantification more difficult by increasing the peak area RSD from 4% (25 μg/tube) to 17% (100 μg/tube). For most of the experiments, 25–75 μg protein/tube was used to achieve a balance between signal strength and quantification reproducibility.

To determine the assay's utility for kinetic experiments, sample containing AC membrane and ATP was serially injected and cAMP formation monitored (Fig. 2C). The rapid separation of substrate from enzyme in the capillary is used to quench the reaction of the aliquot, allowing the sample reaction to continue in the autosampler. In this way, the sample can be repeatedly injected for the acquisition of kinetic information, drastically reducing sample consumption compared to kinetic studies using the radiochemical

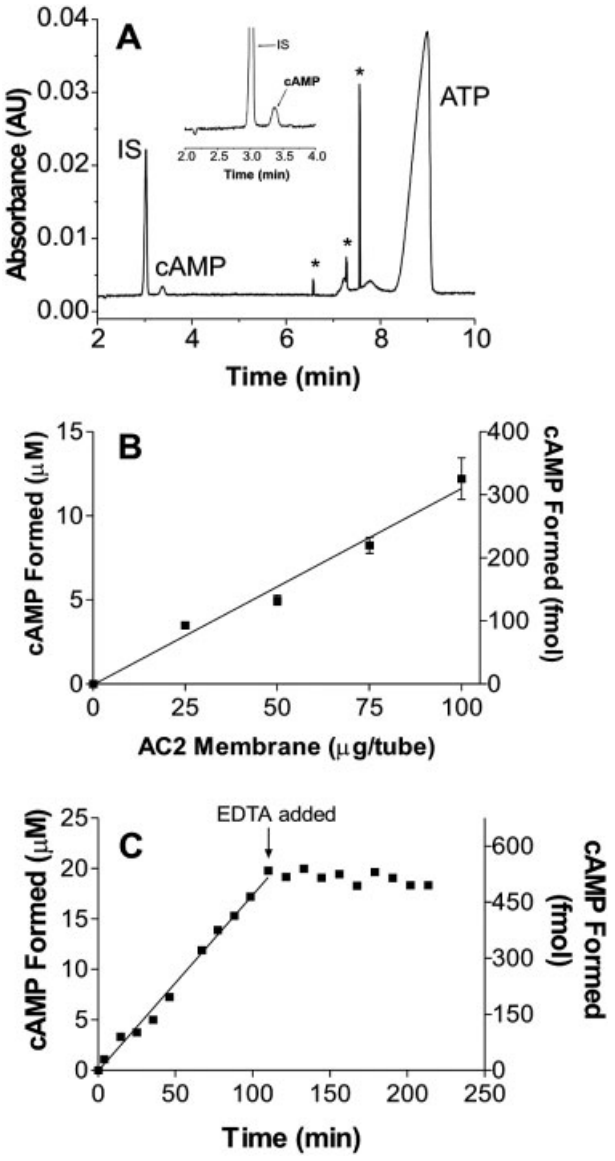

Figure 2. ATP is converted to cAMP upon interaction with AC. (A) Electropherogram of sample containing AC membrane and ATP. Spikes in the electropherogram (denoted by *) were the result of membranes being injected. Thymidine was used as the internal standard (IS) to account for injection volume variation. The inset more clearly depicts the cAMP peak. (B) cAMP formation was measured in samples containing 0–100 μg AC membrane. (C) Sample containing AC and ATP was serially injected and cAMP measured with time. At t = 100 min, 10 mM EDTA was added and sample storage set to 4°C to quench the reaction. All samples contained 50 μg AC membrane and were incubated for 30 min at 30°C unless otherwise indicated. CE conditions are described in Section 2.

assay. Using serial injections, cAMP formation was monitored with time and a linear increase in cAMP formation was observed up to 100 min. To ensure cAMP rates were not skewed by substrate depletion or depletion of the PDE inhibitor and/or regeneration system, reaction rates for all the experiments were determined at 30 min unless otherwise noted.

Some applications require only one measurement of cAMP formation and quenching is required to stop the reaction at a specified time. For example, in a drug screening experiment with numerous samples, the reaction is allowed to proceed for a fixed time before being quenched; the cAMP formed in each sample is then compared against basal. AC is a Mg^{2+}-dependent enzyme and the addition of excess EDTA is expected to quench cAMP formation. Figure 2C shows that when 10 mM EDTA was added to the sample after 100 min, the amount of cAMP present in the sample remained constant with an RSD of 3%, confirming the validity of quenching with EDTA. The consistency and stability of the cAMP signal with time further validates preparing numerous samples simultaneously and analyzing one-by-one in an automated system over the course of several hours.

3.4 Detection of AC stimulation and inhibition

Recently the use of AC as a therapeutic target has been proposed [37–40], and therefore one of the goals was to detect the modulation of AC activity with various drugs directly targeting AC. These experiments were performed using membrane-bound AC instead of purified AC to better mimic AC's physiological environment. To test if this assay could be used to quantify the activation of AC in membranes, the rate of cAMP formation was measured with and without 10 μM FSK, an exogenous AC activator [41, 42]. Figure 3A shows that FSK increased the rate of cAMP formation two-fold from 0.2 to 0.4 nmol/mg/min. To compare the results obtained by the CE-UV and radioactivity techniques, parallel assays were performed to measure the rate of cAMP formation in membranes with increasing amounts of FSK. Fitting the CE-UV data to a dose–response curve resulted in an $EC_{50} = 4.2 \pm 0.7$ μM ($n = 4$) as shown in Fig. 3B. This EC_{50} value was similar to that observed using the radioactivity assay (Fig. 3B, $EC_{50} = 2.4 \pm 0.7$ μM, $n = 4$). The EC_{50} values obtained by both techniques agree well with the literature value of 5–10 μM [43]. Furthermore, a similar two- to three-fold maximal increase in activity was observed by both techniques. Although similar EC_{50}s and maximal increases were obtained for the two techniques, differences in basal rates were observed. Discrepancies in basal rates could be due to variations in the experimental procedure (*e.g.*, incubation temperature variations, protein lability, or variation in substrate/internal standard addition), or differences in the method of cAMP quantification. (The CE-UV assay quantified using a cAMP calibration curve, whereas the radioactivity assay used here multiplied [^{32}P]cAMP formed by the unlabeled/labeled ATP dilution factor to quantify the cAMP produced [17]. Dilution error and/or detector differences could attribute to differences in cAMP quantification.) Comparing the two assays, it appears that the CE-UV assay has

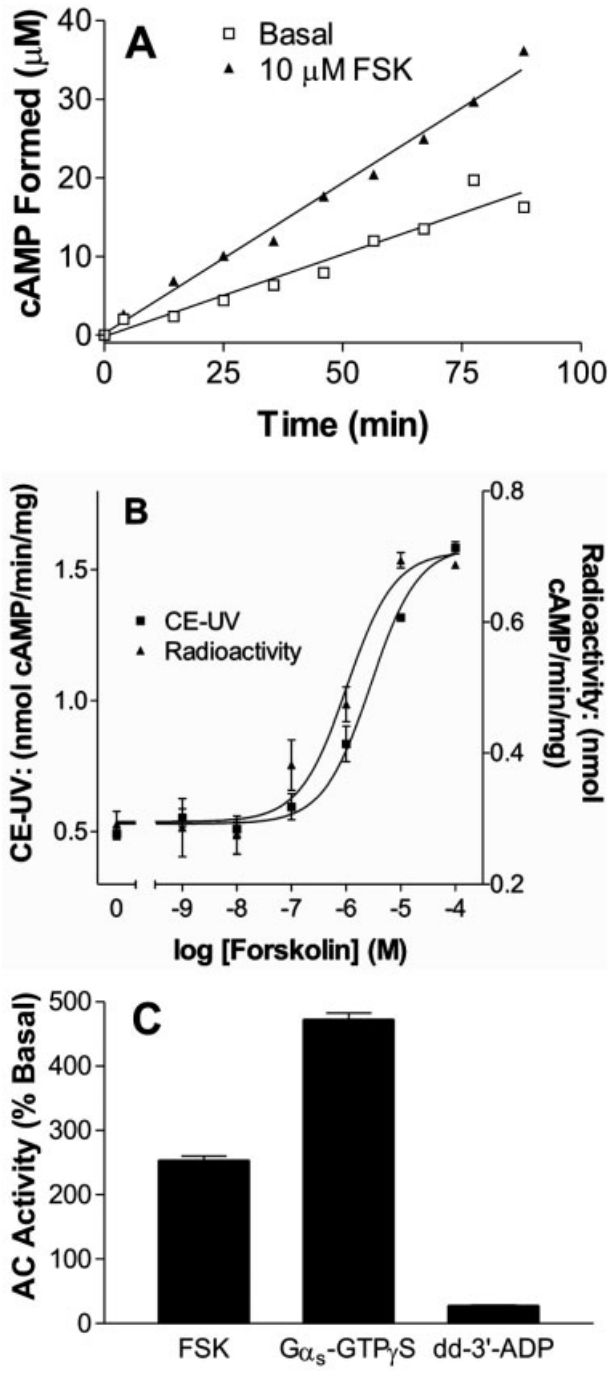

Figure 3. Rates of cAMP formation can be modulated with AC drugs. (A) Addition of 10 μM forskolin (FSK, ▲) to 50 μg AC membrane increased the basal (□) rate of cAMP formation. (B) AC activity was determined in samples containing 75 μg AC (CE-UV) or 25 μg AC (radioactivity), ATP, and 0–100 μM forskolin. Results are representative of four independent experiments for each type of assay. (C) Samples containing 75 μg AC membranes were incubated with ATP and 10 μM forskolin (FSK), 5 nM $G_{\alpha s}$-GTPγS ($G\alpha_s$-GTPγS), or 25 μM 2′,5′-dd-3′-ADP (dd-3′-ADP) and the rate of cAMP formation was compared to basal. All samples were incubated for 30 min at 30°C unless otherwise indicated. CE and radioactivity assay conditions are described in Section 2.

greater precision than the radioactivity assay; the largest peak area RSD for the radioactivity assay is approximately double (22%) compared to the CE-UV assay (12%).

In a drug screen-type experiment, the CE-UV assay may be used by mixing the membranes with drugs, and then quenching the reaction for readout by CE. To test this mode of operation, AC membranes were incubated with various modulators and the amount of cAMP formed after 30 min was measured using an automated system (Fig. 3C). FSK and $G\alpha_s$-GTPγS increased the amount of cAMP formed by 253 ± 7 and 472 ± 10%, respectively, compared to basal ($n = 4$), yielding Z' values [44] of 0.5 and 0.7, respectively. The Z' value measures the quality of a high-throughput assay, and a value above 0.5 is considered an excellent assay [44]. Therefore, the CE-UV assay has sufficient reproducibility and S/N for high-throughput screening. The P-site inhibitor 2′,5′-dd-3′-ADP only produced 28 ± 1% of the basal signal ($Z' = 0.5$, $n = 4$), also demonstrating the ability of the CE-UV assay to detect AC inhibition. DMSO (0.5%; vehicle for FSK) had no effect on basal cAMP rate formation (data not shown).

3.5 GPCR drug screening using the CE-UV assay

GPCRs are more customary drug targets than AC, so this assay was tested for its ability to detect agonist-activation at the GPCR level using membranes coexpressing β_2ARGαs_L and AC (β_2ARGαs_L-AC). Addition of β_2AR agonists was expected to increase the rate of cAMP formation as previously demonstrated [15, 45–47]. Figure 4 shows incubating β_2ARGαs_L-AC membranes with substrate and increasing amounts of terbutaline (a specific β_2AR agonist) resulted in a 1.7 ± 0.1-fold maximal increase in the rate of cAMP formation and revealed a sigmoidal dose–response curve with an EC_{50} of 62 ± 10 nM ($n = 4$).

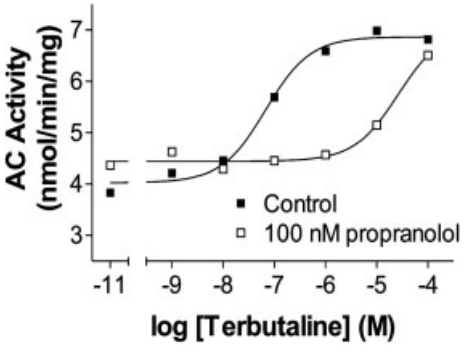

Figure 4. Rates of cAMP formation can be modulated with GPCR drugs. Samples containing 30 μg β_2ARGαs_L-AC membrane, ATP, 25 μM GTP, and 0–100 μM terbutaline were incubated in the presence (□) or absence (■) of 100 nM propranolol. EC_{50}s were determined by fitting dose–response data. Error bars are smaller than the data points. Results are representative of at least two experiments. Sample preparation and CE details are described in Section 2.

The assay was next tested for its ability to detect antagonists binding to the receptor. Addition of a fixed amount of antagonist should shift the agonist dose–response curve to the right in cases where agonist and antagonists compete for the same binding site. Figure 4 shows that the terbutaline dose–response curve was right-shifted in the presence of 100 nM propranolol (β_2AR antagonist), resulting in EC_{50} of 27 ± 1 μM ($n = 2$). As expected, addition of 50 nM propranolol resulted in a smaller right shift with and EC_{50} of 17 ± 2 μM ($n = 2$, data not shown). These results demonstrate that this assay can be used to screen both agonists and antagonists.

In the same manner as modulators for AC were screened (Fig. 3C), agonists for various GPCRs were incubated with β_2ARGαs_L-AC membranes and cAMP formation was measured. The amount of cAMP formed after 30 min was accelerated in the presence of β_2AR agonists isoproterenol (165 ± 5%, $Z' = 0.4$) and terbutaline (171 ± 8%, $Z' = 0.5$). As negative controls, the effects of α_2-adrenergic receptor agonist UK 14 304 ($n = 4$) and dopamine 3 receptor agonist PD 128 907 ($n = 4$) were tested and found to have no effects (98 ± 2 and 102 ± 2%, respectively) as expected.

3.6 Advantages and limitations of CE-UV assay

The CE-UV assay addresses many of the disadvantages associated with the radioactivity, RIA, and EIA techniques for measuring AC activity. Coupling CE with UV detection allows nucleotides to be separated and detected, eliminating the need for radioactive substrate used in the radiochemical assay. Furthermore, the cost of the assay is reduced because the CE-UV assay does not require special reagents or filtering steps. Eliminating the filtering step also reduces assay time (by removing washing steps), allows acquisition of kinetic information (same sample can be serially injected), and makes the assay more amenable to robotic operation. Similar to the radioactivity assay, the linear dynamic range of the CE-UV assay is several orders of magnitude larger than that of the RIA or EIA, and is limited by enzyme kinetics (*i.e.*, substrate depletion) rather than the assay itself. The results presented herein indicate that the CE-UV assay has greater precision than the radioactivity assay. Furthermore, the combined sample preparation and analysis involved in the CE-UV assay is less labor intensive than more traditional techniques.

Although multiplexed CE systems were initially designed for DNA sequencing [48–50], they have recently been used for increasing throughput in CE drug screening assays [51]. For example, He and Yeung [51] used a 48-capillary array system to improve throughput for the determination of six IC_{50} values (screening three inhibitors *vs.* two enzymes). Transferring the AC assay described herein to a capillary 96-array system would drastically improve throughput for screening both AC and GPCR modulators. Furthermore, if a hit is present in a screen, it should be readily identified because the assay has Z' values in the range of 0.4–0.7.

Use of microgram quantities of membranes overexpressing AC is common in radioactivity assays [36, 40], and the CE-UV assay provided sufficient sensitivity to detect AC activity under these conditions. Detection difficulties could arise in samples expressing physiological levels of AC. Using an S/N of 3, the LOD was ~1 μM cAMP (~30 fmol of cAMP). For tissue samples with low AC activity, sample stacking (oncolumn concentration) techniques could be implemented to increase the cAMP signal [52–54], or assay conditions could be altered (longer reaction times, more protein/tube) to increase cAMP production and allow reliable cAMP detection and quantification.

In recent work, we have used a fluorescent substrate and CE with LIF to detect the activity of purified AC [55]. This work differs substantially in that we do not require a special fluorescent substrate and in that we use the assay with enzymes still bound to the membrane. This allows us to detect GPCR activity that is coupled to the AC enzyme. These modifications make the assay of greater interest for potential drug screening.

4 Concluding remarks

CE separation coupled with UV detection allowed the rapid and automatable detection of AC activity in cell membranes. CE eliminates the need for manual column chromatography and washing steps, and UV detection allows the use of native, radioactive-free substrate alleviating safety and cost concerns surrounding the radioactivity assay. When the CE-UV and radioactivity assays were directly compared, similar EC_{50} values and stimulation increases were obtained demonstrating the validity of the CE-UV assay. Detection of agonist-mediated GPCR activation suggests that the assay has promise in drug screening applications.

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