Adenovirus encoded cyclic nucleotide-gated channels: a new methodology for monitoring cAMP in living cells

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Abstract The current, static methodologies for measuring cyclic AMP (cAMP) may underestimate its regulatory properties. Here, we have exploited the Ca^{2+}-conducting properties of cyclic nucleotide-gated (CNG) channels to measure cAMP in live cells, in response to various stimuli. We placed a mutated CNG channel with high sensitivity to cAMP in adenovirus to maximize and render facile its expression in numerous cell types. The ready, continuous nature of the readout contrasted with the traditional approach, which yielded similar static information, but lacked any continuous or interactive qualities. It seems fair to predict that this readily adopted approach will broaden the perception of cAMP signaling.

Key words: Adenylyl cyclase; Cyclic AMP; Cyclic nucleotide-gated channel; Adenovirus

1. Introduction

Cyclic AMP (cAMP) is the prototypical second messenger, which has established the model of second messenger cascades comprised of kinases, phosphatases and, more recently, anchoring proteins. Nevertheless, this messenger may possess important regulatory features that have eluded us so far. For instance, it has been formally proposed that cAMP levels – like cytosolic calcium concentration ($[Ca^{2+}]_i$) – can oscillate [1,2]. As with oscillations in $[Ca^{2+}]_i$, similar changes in cAMP might also suggest hitherto unexpected signaling modes for cAMP. However, the observation of dynamic changes in cAMP – necessarily in living cells – is precluded by available methodologies. The pioneering studies of Sutherland and colleagues applied a bioassay for cell extracts of cAMP by the activation of glycogenolysis [3]. The uncertainties and complexities surrounding this approach were circumvented by the application of a protein binding assay [4,5] and radioimmunoassays of extracted cAMP. Later the activation of cAMP-dependent protein kinase (PKA) was used as a measure of cAMP levels [6]. All of these approaches suffer from the measurement of static accumulated levels of cAMP, which precludes the possibility of seeing dynamic and continuous changes. A step towards a continuous method has been described that involves changes in fluorescence resonance energy transfer (FRET) between the two subunits of fluorescently tagged PKA upon the elevation of cellular cAMP [7]. This method initially required the microinjection of labeled subunits into the cytosol of single cells. The problems associated with microinjection into cells have now been addressed [8] by heterologously expressing genetically encoded subunits of PKA fused with green fluorescent protein (GFP) constructs. However, the modest signal-to-noise associated with the change in FRET upon binding cAMP, coupled with slow reassociation of the subunits and association of GFP-labeled subunits with endogenous PKA subunits limit the utility of this approach. We recently developed a method for measuring cAMP using the electrical properties of the olfactory cyclic nucleotide-gated (CNG) channel encoded in an adenovirus vector to maximize its expression. We first showed that the native channel could measure cAMP in the domain of the plasma membrane, in which it was synthesized by using electrophysiological measurements of the channel activity in response to the activation of adenylyl cyclase or to exogenous cyclic nucleotides [9]. However, the CNG channel is also a Ca^{2+} channel [10] and therefore the increase in $[Ca^{2+}]_i$ could be followed fluorometrically, as a measure of the activation of adenylyl cyclase. In the present study we have adopted a published mutation that increases the channel's sensitivity to cAMP over cGMP, and used this version encoded in adenovirus to measure cAMP in populations and single cells. We show that the method allows the facile detection of a β_2-adrenergic receptor coupled to adenylyl cyclase in C6-2B cells with appropriate pharmacology. Adenylyl cyclase activity can also be seen to rapidly respond to receptor stimulation, followed by antagonism, or inhibition, succeeded by re-stimulation by forskolin. A continuous readout of cAMP accumulation in the domain where it is synthesized is possible in either populations of cells in a spectrofluorimeter or potentially in single cells by fluorescence imaging. This ability to monitor cAMP continuously provides a dramatic opportunity to readily assess adenylyl cyclase activity in situ in response to changing stimuli, which promises to have wide-ranging application.

2. Materials and methods

2.1. Materials

Thapsigargin and forskolin were from Calbiochem (La Jolla, CA, USA). [2-^{3}H]Adenine, [^{3}H]cAMP, and [α-^{32}P]ATP were obtained from Amersham Corp. (Arlington Heights, IL, USA). Fura-2/AM

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Abbreviations: $[Ca^{2+}]_i$, cytosolic calcium concentration; CNG channel, cyclic nucleotide-gated channel; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; ddA, 2′,5′-dideoxyadenosine; 2MeSATP, 2-methylthioadenosine 5′-triphosphate

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and pluronic F-127 were from Molecular Probes, Inc. (Eugene, OR, USA). Other reagents were from Sigma (St. Louis, MO, USA).

2.2. Construction of CNG channel-encoding adenovirus

The E583M mutation to the wild type (WT) olfactory CNG channel was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The E583M channel-encoding adenovirus was constructed as described previously for the WT channel [11].

2.3. Tissue culture and channel expression

C6-2B glioma cells were maintained in culture and infected with adenovirus as described previously [11].

2.4. Measurement of cAMP accumulation

Static cAMP accumulation in intact cells was measured as described previously [12] with some modifications. C6-2B cells on 100 mm dishes were incubated in F-10 medium (90 min at 37°C) with [2-^{3}H]adenine (20 μCi/dish) to label the ATP pool. The cells were then washed once, detached with phosphate-buffered saline containing EDTA (0.03%), and resuspended in a nominally Ca^{2+}-free Krebs buffer containing 120 mM NaCl, 4.75 mM KCl, 1.44 mM $MgSO_4$, 11 mM glucose, 25 mM HEPES, and 0.1% bovine serum albumin (fraction V), adjusted to pH 7.4 with 2 M Tris base. Experiments were conducted in the presence of the phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 100 μM), and Ca^{2+} (2 mM), which was preincubated with the cells for 5 min prior to assay at room temperature. Aliquots (900 μl) were removed every 10 s and immediately added to trichloroacetic acid (TCA; 100 μl; 100%; w/v final) to terminate the reaction. After pelleting, the [^{3}H]ATP and [^{3}H]cAMP contents of the supernatant were quantified as described previously [13]. Accumulation of cAMP is expressed as the conversion of [^{3}H]ATP into [^{3}H]cAMP.

2.5. $[Ca^{2+}]_i$ measurements

Detached cells (10×10^6/ml) were loaded with fura-2/AM (2 μM) plus 0.02% pluronic acid in F-10 media (without serum, containing HEPES 20 mM, 0.1% bovine serum albumin, pH 7.4) for 25 min at room temperature, and $[Ca^{2+}]_i$ measurements were made as previously described [11]. Cells were maintained in Krebs media (as above, without bovine serum albumin) containing Ca^{2+} (2 mM) throughout, unless stated otherwise. The results are expressed as the ratio of the emissions from 340/380 nm excitation. The data presented are representative of at least three similar experiments.

3. Results

The ability of CNG channels to conduct Ca^{2+} [10] provides a potentially simple and rapid means to estimate endogenously synthesized or exogenously applied cyclic nucleotides by monitoring fura-2 fluorescence. Indeed, we had shown earlier that the WT olfactory CNG channel expressed in C6-2B cells responded with a modest rise in $[Ca^{2+}]_i$ following exposure to high concentrations of the cell permeant cGMP analog, 8-*p*-chlorophenylthio-cGMP [11]. In addition, we had shown that in cells stably expressing high activities of adenylyl cyclase type 8, sufficient cAMP was produced in response to forskolin that a rise in $[Ca^{2+}]_i$ was detected [9]. However, there are two drawbacks to the use of WT CNG channels to detect increases in cAMP. First, WT channels have a low apparent affinity for cAMP, which curtails their ability to detect the low cAMP concentrations that activate PKA [14,15]. Second, these channels are activated more readily by cGMP than cAMP [16], which might generate ambiguities in some situations. To overcome these problems, we modified the WT CNG channel to increase its affinity for cAMP and decrease its affinity for cGMP. Mutation of the highly homologous, rod CNG channel at D604 to methionine was previously shown to both increase the cAMP affinity and decrease the sensitivity to cGMP [17]. Methionine is naturally found at this position in several cAMP binding domains, such as that of the bovine cAMP-dependent kinase IIa domain B and the rat olfactory CNG channel subunit 2 [18,19]. Therefore, we modified the analogous residue in the WT olfactory CNG channel α subunit, E583, to methionine.

The rise in $[Ca^{2+}]_i$ was compared in populations of C6-2B cells, a rat glioma cell line which is non-excitable, infected with either the WT or E583M CNG channel adenovirus, in response to increasing concentrations of the β-adrenergic receptor agonist, isoproterenol, added at 300 s (Fig. 1). The WT CNG channel is modestly stimulated by 100 nM isoproterenol (black trace) and only weakly stimulated by 1 nM isoproterenol (yellow trace, Fig. 1A). In contrast, much more rapid responses are yielded by the E583M CNG channel when exposed to the same isoproterenol concentrations (Fig. 1B). The $[Ca^{2+}]_i$ rises generated by the two CNG channels at a given isoproterenol concentration are quite different with respect to both the slope of the rising phase and the time to half-maximal stimulation (t_{50}). In the case of 10 nM isoproterenol, the WT CNG channel reaches a half-maximal value in 140 s, while the E583M CNG channel reaches a half-maximal value in 68 s. Similarly, the slope of the rising phase after treating the cells with 10 nM isoproterenol is 0.0058 for the WT CNG channel, compared with 0.0191 for the E583M CNG channel. Therefore, the E583M CNG channel generates a $[Ca^{2+}]_i$ rise that is both faster and more robust than the WT CNG channel, which is consistent with the greater sensitivity of the mutant channel to cAMP. Under conditions of maximal adenylyl cyclase stimulation (10 μM isoproterenol, 10 μM forskolin), the differences in the kinetics of the response in cells expressing the WT CNG channel (black trace) or the E583M CNG channel (red trace) are readily evident (Fig. 1C). Furthermore, with maximal adenylyl cyclase activation, both the WT- and E583M CNG channel-expressing cells elicit similar maximal responses, indicating that the extent of channel expression was similar. Control, uninfected cells showed no response to either isoproterenol or IBMX, nor did infected cells in the absence

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Fig. 1. Adenovirus-mediated expression of WT and E583M CNG channels in C6-2B cells. C6-2B cells were infected with the indicated CNG channel construct at a multiplicity of infection of 100, 48 h prior to $[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ was determined in aliquots of 4×10^6 fura-2-loaded C6-2B cells, as described in Section 2. Cells expressing either the WT (A) or E583M (B) CNG channels were maintained in Krebs medium containing 2 mM Ca^{2+}. The cells were pretreated with IBMX (100 μM) 5 min prior to the addition of varying isoproterenol concentrations at 300 s (1 nM, yellow trace; 3 nM, green trace; 10 nM, red trace; 100 nM, black trace), along with forskolin (1 μM) to increase the signal, particularly in the case of the WT channel. The time to half-maximal stimulation (t_{50}) following stimulation with either 1 nM, 3 nM, 10 nM, or 30 nM isoproterenol was 186 s, 176 s, 140 s, and 113 s, respectively, for the WT channel and 107 s, 86 s, 68 s, and 56 s, respectively, for the E583M mutant channel. Insets: plot of the slope of the rising phase ($\times100$) versus the isoproterenol concentration in cells infected with either the WT channel (A, inset) or the E583M mutant channel (B, inset). With maximal adenylyl cyclase stimulation (10 μM isoproterenol, 10 μM forskolin; added at 300 s), the peak of the $[Ca^{2+}]_i$ rise generated by both CNG channels (E583M, red trace; WT, black trace) is similar, although again the rate of the rise is much faster with E583M CNG channel, indicating that the two CNG channels are expressed at similar levels (C). Uninfected cells showed no response to isoproterenol (not shown). The resultant $[Ca^{2+}]_i$ rise is depicted as an increase in the ratio of the emissions following 340/380 excitation.

of extracellular Ca^{2+} (data not shown; i.e. none of these agents promotes Ca^{2+} release from intracellular stores). The E583M channel was used in all further experiments.

We next wished to test the application of the E583M CNG channel as a sensor for changes in $[cAMP]_i$ in a simulated receptor screening exercise. We compared the magnitude of the $[Ca^{2+}]_i$ rise produced by varying concentrations of three β-adrenergic receptor agonists, which stimulate adenylyl cyclase via G_s. Populations of C6-2B cells were treated with increasing concentrations of either isoproterenol, epinephrine, or norepinephrine, ranging from 1 to 1000 nM (Fig. 2). Isoproterenol was the most effective, giving a slight increase of 340/380 fluorescence at 1 nM, a very robust stimulation at 10 nM, and a maximal stimulation at 100 nM. Epinephrine was slightly less effective, with the fluorescence traces shifted to the right. Norepinephrine was very impotent in eliciting any increase in 340/380 fluorescence, even at the highest concentration. This agonist potency order of isoproterenol > epinephrine ≫ norepinephrine is the classic order expected for β_2-adrenergic receptors [20], which are expressed in C6-2B cells [21]. Control, uninfected cells did not yield a response to any of the β-adrenergic agonists used (data not shown). This result shows not only the ability of the E583M CNG channel to act as a sensor for cAMP, but also that the sensor can be used to identify receptor subtypes in a particular cell, and thereby allow screening of potential receptor agonists.

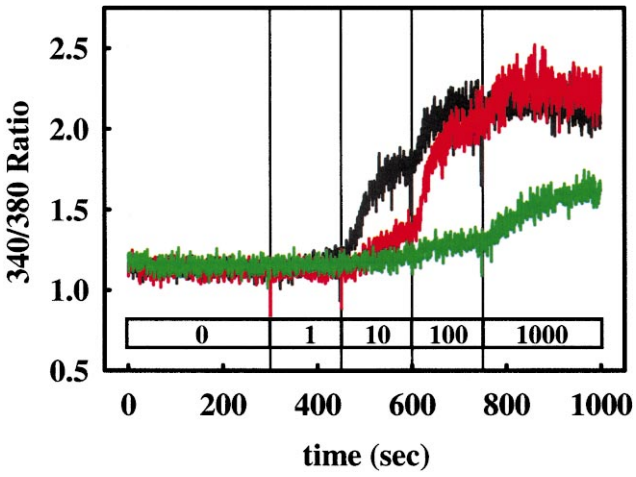
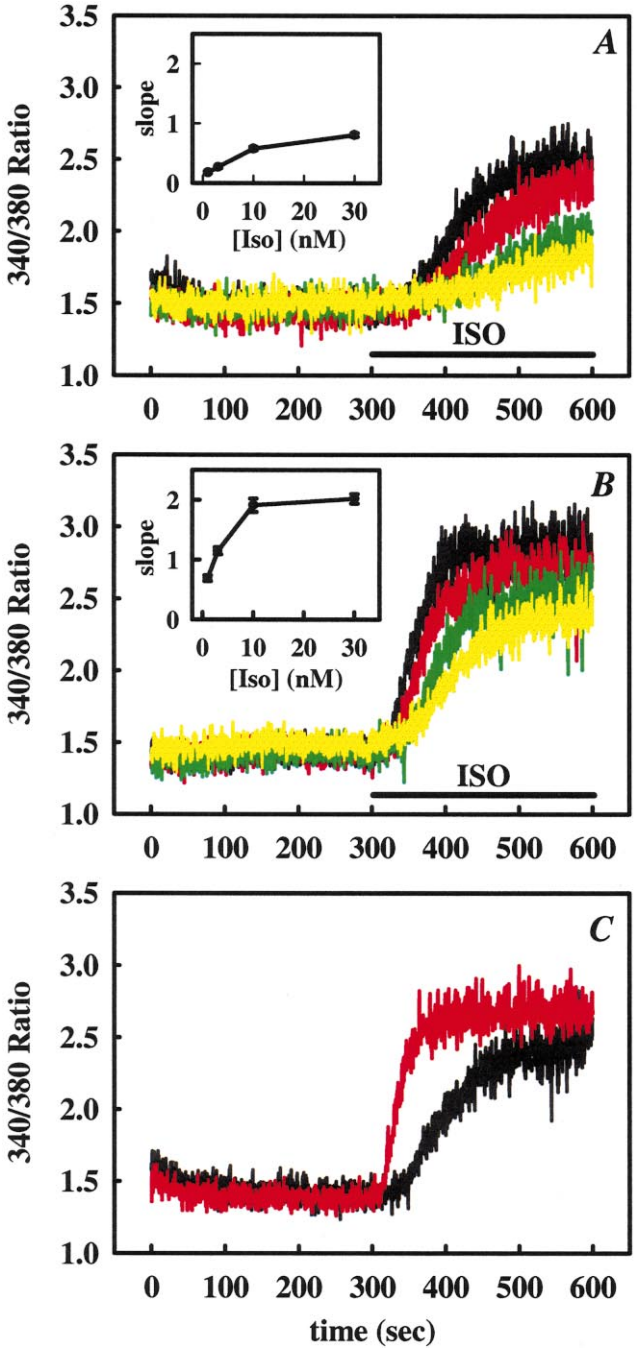


Fig. 2. Comparison of the efficacy of three β-adrenergic receptor agonists using the E583M CNG channel. Fura-2-loaded C6-2B cells infected with the E583M CNG channel construct were incubated in Krebs medium containing 2 mM Ca^{2+} and pretreated with IBMX (100 μM) 5 min prior to the addition of the β-adrenergic receptor agonists. Increasing concentrations of either isoproterenol (black trace), epinephrine (red trace), or norepinephrine (green trace) were added as follows: 300 s, 1 nM; 450 s, 10 nM; 600 s, 100 nM; 750 s, 1000 nM. None of these agents produced a response in control, uninfected cells (not shown).



If the CNG channel could also respond to decreases in cAMP, this would permit the study of G-protein-coupled receptor antagonists, as well as inhibitors of adenylyl cyclase. To determine whether the sensor could reflect decreases in cAMP, populations of C6-2B cells were first stimulated with isoproterenol, followed by the β-adrenergic receptor antagonist, propranolol (Fig. 3A). Addition of propranolol (100 μM) 30 s after isoproterenol (1 μM) showed a rapid decrease in the 340/380 fluorescence (Fig. 3C), as compared to isoproterenol alone (Fig. 3A). G_i-mediated inhibition of isoproterenol stimulation was also examined. 2-Methylthioadenosine 5′-triphosphate (2MeSATP; 100 nM) which is a P_{2Y}-purinergic receptor agonist that only couples to G_i in C6-2B cells [22], significantly decreased 340/380 fluorescence compared to the control (Fig. 3B). Neither propranolol nor 2MeSATP produced a response in control, uninfected cells or in cells which were not stimulated with isoproterenol (data not shown). This experiment was conducted in the presence of the PDE inhibitor, IBMX, to maximize the signal and to more accurately reflect changes in adenylyl cyclase activity. However, to examine 'net' cAMP changes, which would be the sum of cAMP synthesis by adenylyl cyclase and cAMP degradation by PDE, and to accentuate the inhibitory response [23], the ability of the E583M CNG channel to report changes in cAMP in the absence of PDE inhibitors was also examined. In the same set

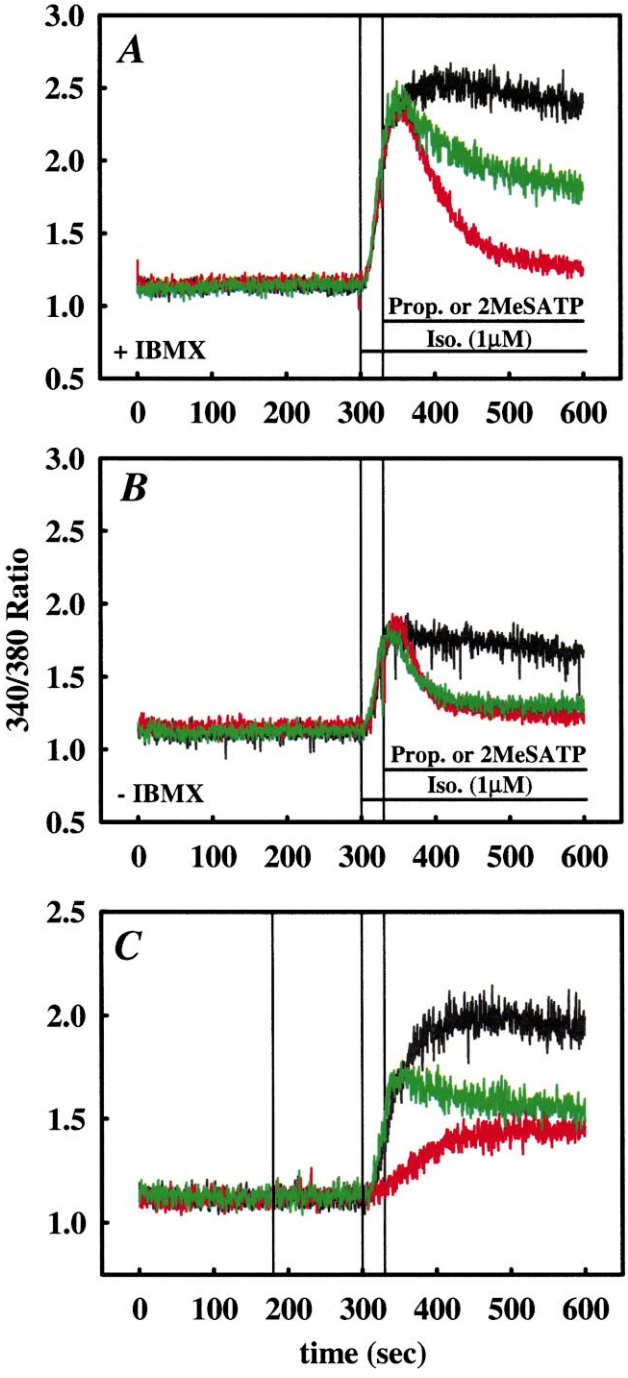


Fig. 3. Detection of decreases in $[cAMP]_i$ using the E583M CNG channel following receptor antagonism or adenylyl cyclase inhibition. Fura-2-loaded C6-2B cells infected with the E583M CNG channel were incubated in Krebs medium containing 2 mM Ca^{2+}. A,B: Cells were pretreated with either IBMX (A, 100 μM) or vehicle alone (B, DMSO) 5 min prior to the addition of isoproterenol (1 μM; 300 s), followed by the addition of either vehicle alone (water; black trace), 2MeSATP (100 nM; green trace), or propranolol (100 μM; red trace) at 330 s. C: Cells were pretreated with IBMX (100 μM) 5 min prior to the addition of isoproterenol (1 μM; 300 s; black trace). The adenylyl cyclase inhibitor ddA (100 μM) was added either prior to (180 s; red trace) or following (330 s; green trace) the isoproterenol addition.

of cells as in Fig. 3A, in the absence of IBMX, isoproterenol gave a robust increase in 340/380 fluorescence (Fig. 3B), although the magnitude was smaller than in the presence of IBMX (cf. Fig. 3A). However, under these conditions, both propranolol and 2MeSATP rapidly eliminated the signal elicited by isoproterenol. This more rapid inhibitory effect in the absence of PDE inhibitors would be expected, given that background PDE can have profound effects on cAMP levels [23]. The observed rapidity of elimination of the cAMP signal speaks for the utility of this assay in physiological conditions.

As a further measure of the ability of this sensor to respond to changes in cAMP, the action of the adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine (ddA) was examined (Fig. 3C). Populations of C6-2B cells were treated with isoproterenol (1 μM) at 300 s, resulting in a rapid rise in 340/380 fluorescence. Addition of ddA (100 μM) either prior to (180 s) or after (330 s) isoproterenol addition resulted in a greatly diminished signal, with the prior treatment being more effective. It is interesting to note that in in vitro adenylyl cyclase assays, ddA is extremely effective at inhibiting adenylyl cyclase activity [24], while in vivo, the inhibitory effects of ddA are limited by its ability to cross the plasma membrane. This conclusion is supported in the present experiment showing that ddA is more effective at longer incubation times and generates only partial inhibition of adenylyl cyclase at these times. Consequently, this assay could be used to quickly optimize the time of preincubation of a slowly permeating agent.

The traditional cAMP accumulation assay was compared with the continuous assay developed here using the CNG channel under the same experimental conditions as those applied in Fig. 3 (Fig. 4). Populations of C6-2B cells in Krebs medium containing 2 mM Ca^{2+} were pretreated with the PDE inhibitor, IBMX. Aliquots were removed every 10 s and immediately placed into TCA, followed by determination of the

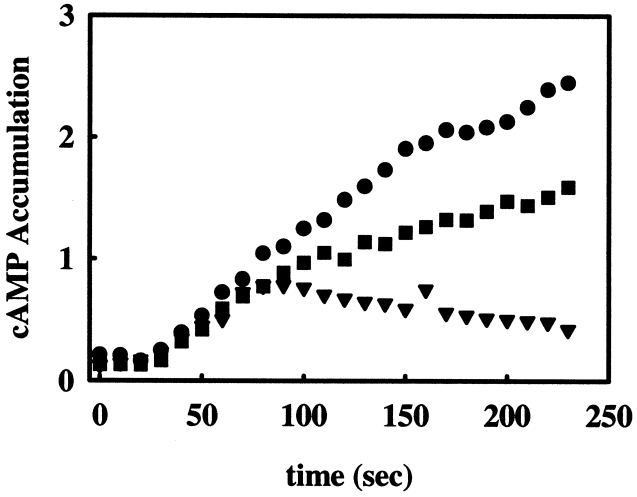


Fig. 4. Measurement of adenylyl cyclase activity by a conventional, static method. C6-2B cells were incubated with [^{3}H]adenine to label the ATP pool, washed and resuspended in Krebs medium containing 2 mM Ca^{2+}, as described in Section 2. Populations of C6-2B cells were pretreated with the PDE inhibitor IBMX 5 min prior to assay. Aliquots were removed every 10 s and immediately placed into TCA, followed by determination of the [^{3}H]cAMP and [^{3}H]ATP. At 25 s, isoproterenol (1 μM) was added, followed by either no addition (circles), propranolol (100 μM; triangles), or 2MeSATP (100 nM; squares) at 55 s. The results are expressed as the percent conversion of [^{3}H]ATP to [^{3}H]cAMP.

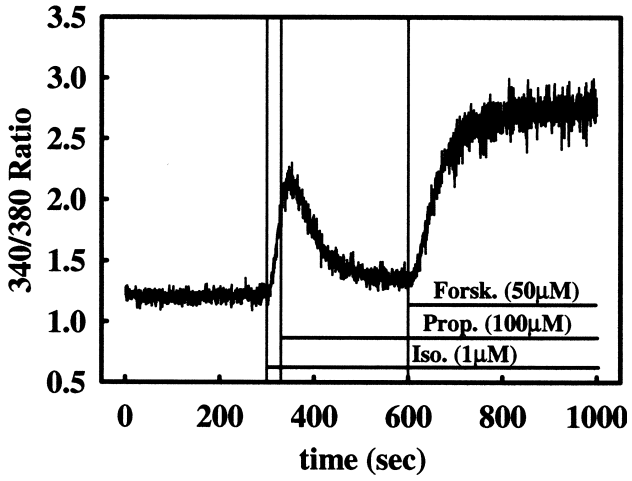


Fig. 5. Continuous measurement of sequential activation and inhibition of adenylyl cyclase activity. Fura-2-loaded C6-2B cells infected with the E583M CNG channel were incubated in Ca^{2+}-containing (2 mM) Krebs medium and pretreated with IBMX (100 μM) 5 min prior to the sequential additions of isoproterenol (1 μM; 300 s), propranolol (100 μM; 330 s), and forskolin (50 μM; 600 s).

[^{3}H]cAMP and [^{3}H]ATP contents [12,13]. At 25 s, isoproterenol (1 μM) was added, followed either by no addition, propranolol (100 μM), or 2MeSATP (100 nM) at 55 s. Propranolol was the most effective, while 2MeSATP, acting via G_i, was intermediate in its ability to decrease the amount of cAMP accumulated. These results mirrored the finding using the E583M channel as a sensor for cAMP accumulation, and thereby validate the use of that assay. However, the continuous readout from the CNG channel assay and the facility of obtaining those data contrast with the more laborious, traditional method.

A particularly useful attribute of this sensor over more standard means of determining cAMP accumulation is its ability to generate a 'continuous' readout. This is exemplified by the sequential addition of a series of agonists and antagonists. In Fig. 5, C6-2B cells were initially stimulated with isoproterenol (1 μM) at 300 s, followed by the addition of the β-adrenergic antagonist, propranolol (100 μM), at 330 s and finally, the adenylyl cyclase activator, forskolin (50 μM), at 600 s. The cells responded to this series of agents by showing an increase in 340/380 fluorescence with isoproterenol, which was subsequently decreased by propranolol and then increased again in response to forskolin. Therefore, the impact of a series of agents that affect cAMP accumulation can be monitored sequentially, which provides a great improvement over static modes of studying cAMP[1].

4. Discussion

The use of the Ca^{2+}-conducting properties of a modified CNG channel as a sensor for changes in cAMP concentration provides the first readily applicable and continuous methodology for monitoring cAMP levels in living cells. We have shown that the application of the CNG channel as a sensor is made extremely easy due to its insertion in adenovirus, which allows high and easily controlled expression in a wide variety of cell types, coupled with the fact that the readout is in the form of a $[Ca^{2+}]_i$ rise, which is a widely available, real-time measurement. Furthermore, expression of the CNG channel does not interfere with the cell's Ca^{2+} or cAMP homeostasis; Ca^{2+} release and capacitative Ca^{2+} entry are the same in uninfected and infected cells, as is the production of cAMP, as judged by traditional cAMP assays (not shown). Of course, because changes in $[Ca^{2+}]_i$ are used as the readout and many factors can impinge on Ca^{2+} homeostasis, the measurements are qualitative and caution must be exercised in applying mechanistic conclusions. For instance, depolarization of excitable cells would be accompanied by a robust $[Ca^{2+}]_i$ rise, which could overwhelm any signal originating from the CNG channel. However, as with any other methodology, awareness of the basis of the method should minimize any misleading interpretations. The mutant channel allows the ready detection of the modest amounts of cAMP formed in response to very low concentrations of isoproterenol. Adrenergic receptor subtypes, or others that might be coupled to adenylyl cyclase in other cell types, are rapidly identifiable. The localization of the cAMP sensor at the plasma membrane is a major advantage, which allows the measurement of cAMP concentrations in the domain of the cell, where many of the actions of cAMP take place, for instance, the activation of PKA bound to A-kinase-anchoring proteins near the plasma membrane. Because cAMP is measured locally, alterations in cAMP accumulation brought about by local PDE activity or receptor desensitization, for example, could be monitored continuously and compared with global measurements, using traditional cAMP determinations. The ability to detect changes in cAMP concentration continuously in living cells provides a significant improvement over the traditional, static methods for measuring cAMP accumulation. As with current methodologies for assaying cAMP accumulation, the present methodology does not provide absolute values for cAMP. However, this approach provides a simple system for applications such as screening potential receptor agonists and antagonists that are coupled to adenylyl cyclase, determining factors that influence PDE activity and assessing the cell permeability of agents that act on the cAMP system, such as P-site inhibitors and forskolin. Another possible application of this cAMP sensor is the ability to measure cAMP in single cells. Polarized cells, like epithelial cells, or those with elaborate morphologies, such as neurons, or identifiable sub-populations of cells, may show localized changes in cAMP concentration in response to selective agents. It is already well documented that apparently homogeneous populations of cells respond heterogeneously to agents that are coupled to rises in $InsP_3$. For instance, single cell Ca^{2+} imaging experiments reveal heterogeneity in the $[Ca^{2+}]_i$ response in individual cells promoted by various PLC-coupled receptor agonists [25,26]. The same situation may apply to agents coupled to the stimulation or inhibition of adenylyl cyclase. Furthermore, it must be stressed that oscillations in cAMP, like oscillations in $[Ca^{2+}]_i$, can only be discerned at the single cell level. Unless oscillations are coordinated, individual responses are averaged in a population. These and other issues that could not readily be addressed by static methodologies can now be approached.

In signaling, conceptual developments are dictated by the information content of the methodologies for measuring the

[1] These experiments were performed on cells in suspension in a stirred cuvette. Thus, none of the reagents was removed. However, the experiment could be readily adopted to cells attached to a coverslip with agents continually exchanging by perfusion of the cuvette.

second messengers. Thus, the dynamic, single living cell methods that were developed for the study of $[Ca^{2+}]_i$ allowed the discovery of $[Ca^{2+}]_i$ oscillations, the profusion of theories for their origins, along with searches for how they might be used in signaling. In contrast, due to the static and cell-sacrificing methodologies for studying cAMP, the possibility that cAMP levels might change dynamically, although theoretically supported, could not be realistically considered. Thus, it is possible that fundamental aspects of cAMP signaling are being overlooked. We would hope that the development of this methodology will provide a stimulus for considering a broader range of signaling options for cAMP.

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