Fluorescence-Based Adenylyl Cyclase Assay Adaptable to High Throughput Screening

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Abstract: The second messenger cAMP has been implicated in numerous cellular processes such as glycogen metabolism, muscle contraction, learning and memory, and differentiation and development. Genetic evidence suggests that the enzyme that produces cAMP, adenylyl cyclase (AC), may be involved in pathogenesis in many of these cellular processes. In addition, these data suggest that membrane-bound ACs may be valuable targets for therapeutics to treat pathogenesis of these processes. The development of a robust real-time adenylyl cyclase assay that can be scalable to high-throughput screening could help in the development of novel therapeutics. Here we report a novel fluorescence-based cyclase assay using Bodipy FL GTPγS (BGTPγS). The fluorescence of the Bodipy™ moiety of BGTPγS was dramatically enhanced by incubation with the minimal catalytic core of wild-type-AC (wt-AC) and a mutant with decreased purine selectivity (mut-AC), in an AC activation-dependent manner. No increase in fluorescence was observed using Bodipy FL ATPγS (BATPγS) as substrate for either wt-AC or mut-AC. Using BGTPγS, forskolin, Gsα•GTPγS and the divalent cation Mn^{2+} potently enhanced the rate of fluorescence increase in a concentration-dependent manner. The fluorescence enhancement of the Bodipy moiety was inhibited by known inhibitors of AC such as 2'deoxy,3'AMP and 2',5'-dideoxy-3'ATP. Furthermore, the fluorescence assay is adaptable to 96-well and 384-well multiplate format and is thus applicable to high throughput screening methodologies.

Keywords: Adenylyl cyclase, fluorescence, high-throughput screening, Bodipy-FL-GTPγS.

INTRODUCTION

The hormone-regulated form of adenylyl cyclase (AC) is an integral membrane-bound enzyme localized at the plasma membrane. Hormone receptors on the cell surface communicate with AC through coupling with the heterotrimeric, guanine nucleotide binding proteins, or G proteins. The two families of Gα that regulate AC are Gsα, or stimulatory G protein (G_{olf}α and Gsα) and Giα, or inhibitory G protein (Goα, Gi_1α, Gi_2α, Gi_3α and Gzα). Gβγ also directly regulates AC activity. In addition to direct modulation of these isoforms by G proteins, other second messengers such as Ca^{2+} are potent regulators. There are nine genes that encode for at least nine forms of these enzymes (termed AC1 to AC9) [1]. The nine forms can be divided into four groups based on their regulation: AC1, AC3 and AC8 (activation by Ca^{+2}-calmodulin, inhibition by Gβγ), AC2, AC4 and AC7 (activation by Gβγ and regulation by protein kinase A), AC5 and AC6 (inhibition by Giα, inhibition by Ca^{2+}) and AC9 (regulation by calcineurin, but not regulated by the diterpene activator forskolin).

The isoform-specific regulatory properties and their differential tissue distribution engaged investigators to make AC a therapeutic target. CyclicAMP is an extremely important second messenger involved in cellular processes such as glycogen metabolism, muscle contraction, learning and memory, and differentiation and development. Moreover, many pathologic states have been described implicating defects in the signaling pathway that regulates cAMP homeostasis. Indeed, treatment of disease has classically involved the use of therapeutics that target the receptors that regulate ACs. Accordingly, β-adrenergic, adenosine, glucagon and prostanoid receptors are but a few receptor classes that function to stimulate cAMP production. Inhibition of these receptors has been used for treatment of a variety of diseases ranging from asthma, glaucoma, and cardiac failure [2, 3]. Genetic studies using transgenic and knockout mouse models also suggest that specific isoforms may be involved in cardiac diseases and potentially the treatment of disease [2-8]. Until recently, targeting effector proteins has rarely been successful. The recently reported identification of compounds that directly target adenylyl cyclase, including adenine nucleotides and nucleosides, as well as forskolin derivatives (a diterpene isolated from *Choleus forskohlii* and known activator of adenylyl cyclase) [9-11], are encouraging. The location of the nucleotide binding site, the active site, and the forskolin site have been elucidated by our work (and others) on the crystal structure of catalytic domain of adenylyl cyclase [12, 13]. These data also permit the modeling of the structure and putative mechanism of evolutionarily related adenylyl cyclases, and the closely related nucleotide lyases, guanylyl cyclases [14]. In addition, the structures have revealed the sites on adenylyl cyclases that have the potential to be pharmacologically targeted.

Part of the difficulty in the development of therapeutics that target adenylyl cyclase has been the lack of high throughput assays that directly measures cyclase activity. Common methods for detecting adenylyl cyclase activity are monitoring the conversion of isotopically-labeled ATP to cAMP, or by using various immunoassays involving RIA (radioimmunoassay) or EIA (enzyme immunoassays, eg

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HitHunter, Discover RX, Fremont CA, or cAMP-EIA, Cayman Chemicals, MI) [15]. More recently AC activity in cells has been detected by following the activation of cyclic nucleotide-gated channels (CNG channels) and Ca^{2+} mobilization as visualized by fluorescent dyes [16]. Implementation of the classic AC assays to high-throughput screening formats has been limited due to the requirements for radioisotopes, chromatography, filtration, centrifugation and/or wash steps. Here we report a novel approach to measure real-time adenylyl cyclase activity by fluorescence spectroscopy using Bodipy™-conjugated γ-thionucleotides (Invitrogen). The robust assay reflects all the regulatory properties of adenylyl cyclase including inhibition of activity by known AC inhibitors. Furthermore, the assay is adaptable to a 96-well and 384-well microplate format highlighting the feasibility of high throughput screening methodologies. The application to high throughput screening should simplify a search for cyclase ligands that may have therapeutic potential.

MATERIALS AND METHODS

The catalytic domains, C1 and C2 of wildtype and mutant (carrying point mutations Lys938Glu and Asp1028Cys and displaying increased Michaelis constant, *Km*, for Mg^{2+}•ATP and a significantly lower *Km* for Mg^{2+}•GTP) adenylyl cyclase were expressed and purified as described by Sunahara *et al.* [17, 18]. For the remainder of the manuscript the wild-type enzyme will be referred to as wt-AC whereas the mutant will be referred to as mut-AC. Bodipy FL GTPγS (BGTPγS), Bodipy FL ATPγS (BATPγS), and Bodipy FL iodoacetamide (BIA) were obtained from Invitrogen (Carlsbad, CA). All nucleotides, including 2'deoxy-3'-adenosine monophosphate (2'd-3'AMP), 2',5'-dd-3'-ATP and Mant-GTP, were obtained from Sigma (St. Louis, MO). The diterpene forskolin was obtained from Calbiochem. [^{3}H]cAMP and [^{32}P]ATP were purchased from Perkin-Elmer (Waltham, MA). The stimulatory G protein, Gsα, was purified and activated with GTPγS as described by Lee *et al.* [19]. All reagents were of analytical or molecular biology grade.

***The Detection of Spectral Changes in Bodipy FL GTPγS Fluorescence in the Presence of Adenylyl Cyclase*:** The emission spectra of four Bodipy FL compounds, Bodipy iodoacetamide (BIA), Bodipy FL ATPγS (BATPγS), Bodipy FL GTPγS (BGTPγS), and Bodipy FL thiophosphate (BSP), were measured on a Photon Technology International spectrofluorometer (Birmingham, NJ). BSP was prepared as described in Jameson *et al.* [20] Samples containing 50 nM fluorophore were prepared in 20 mM Hepes pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT (Buffer A) containing 5 mM $MnCl_2$ in a final volume of 750 μL. Fluorescence was measured using excitation at 485 nm and emission was scanned from 495 to 560 nm. Slit widths were 4 nm for excitation and 2 nm for emission.

The increase in fluorescence of BGTPγS in the presence of wt-AC or mut-AC was measured on a multiplate fluorescence detector (Victor™, Perkin-Elmer) with fixed emission wavelength of 535 nm (excitation at 485 nm). Excitation was typically measured for 100-500 ms and sampled every 30-60 s. Unless otherwise stated the final concentration of each domain is 200 nM and 10 μM for the C1 and C2 domains (or the mutant), respectively. Reactions were carried out in Buffer A in the absence or presence of 5 mM $MnCl_2$ in a volume of 100 μL (96-well plate) or 20 μL (384-well plate). In all cases, the reaction was initiated by the addition of substrate (either BATPγS or BGTPγS). The rate of change in fluorescence was determined by linear regression of the initial linear portion of curve usually within the first 300 s. Stimulation of wt-AC or mut-AC by the diterpene, forskolin (100 μM), was measured as the increase in fluorescence of the Bodipy moiety. The forskolin dose-response was measured using concentrations ranging from 100 pM to 3 μM. For G protein studies with GTPγS-bound and therefore active Gsα (Gsα-GTPγS), samples containing 500 nM C1, 2 μM C2, and 500 nM BGTPγS were mixed with varying amounts of Gsα-GTPγS (0-3 μM) in the absence or presence of 10 μM forskolin and were analyzed immediately. The Bodipy moiety was excited at 485 nm (0.2 s), and the fluorescence emission was measured at 535 nm. For inhibitor studies, the enzyme was pre-incubated in the presence of forskolin (100 μM) and inhibitor for 3 min. at 23°C. The reaction was initiated with the addition of 500 nM BGTPγS/Mn^{2+} (final).

***Adenylyl Cyclase Assays*:** Radioactive adenylyl cyclase assays were performed as described by Smigel [21]. Enzyme preparations were pre-incubated with either forskolin or GTPγS-activated Gsα for 10 min at 4 °C. The enzyme activity (from 5 nM C1 and 5 μM of either wild-type or mutant C2) was initiated by the addition of substrate ([^{32}P]ATP) and terminated (after 15 min 30°C) with a buffer containing excess cold ATP, [^{3}H]cAMP and SDS. CyclicAMP was purified using Dowex and alumina column chromatography. The conversion of [^{32}P]ATP to [^{32}P]cAMP was determined based on the recovery of the [^{3}H]cAMP standard.

***Immunoassay Detection of cGMP from BGTPγS*:** Cyclic GMP from BGTPγS was detected using an immunoassay kit (cGMP EIA from Cayman Chemicals, Ann Arbor, MI) as recommended by the manufacturer.

***Assay Setup for High-Throughput Assay*:** Three hundred and twenty compounds from a small molecule compound library (Maybridge, Cambridge, England) were prespotted (1 μL of 200 μM) on to a 384-well microtiter plate using a Beckman Biomek-FX (Center for Chemical Genomics, University of Michigan Life Sciences Institute) just prior to the assay. Enzyme preparations of wt-AC were added to the compound and allowed to incubate for 15 min at 23°C in a Buffer A. Reaction was initiated by the addition of BGTPγS in buffer so that the final Mn^{2+} concentration was 5 mM and allowed to incubate at 23°C. The reaction final volume was 20 μL. Following a 30 min incubation period, the fluorescence of the plate was monitored using a Victor fluorescence detector (Perkin Elmer).

RESULTS AND DISCUSSION

Classic methods for detecting adenylyl cyclase activity have been restricted to following the conversion of isotopically-labeled ATP to cAMP or by utilizing antibodies against cAMP coupled to various reporters [15]. While these methods have proven to be the most accurate representation of adenylyl cyclase activity, their application to high-throughput biology has been very limited. The requirements for numerous separation steps and the use and disposal of radioisotopes have deemed them cumbersome for high throughput screening assays. In addition, the cost of enzyme immuno- or radio-immunoassay is relatively high in a high-

throughput setting. As such, considerable effort has been spent on developing spectroscopic methods for measuring cAMP accumulation, most of them being cell-based. Here we describe a novel approach to achieve real-time measurements of adenylyl cyclase activation using fluorescence spectroscopy.

We take advantage of the unique spectral properties of Bodipy™-conjugated γ-thionucleotides (Invitrogen). Fig. **1A** illustrates the emission spectra of 50 nM solutions of BSP, BIA, BGTPγS, and BATPγS following excitation at 485 nm (BGTPγS structure shown in Fig. **1B**. Note that the fluorescence intensity of BSP and BIA are similar to that of BATPγS but dramatically higher than that of BGTPγS. What makes Bodipy-conjugated guanine nucleotides spectrally unique is an electron quenching effect by the guanine ring on the Bodipy moiety, resulting in fluorescence quenching. Alteration in the spatial relationship between the Bodipy moiety and the guanine ring through binding to nucleotide-binding proteins, or through cleavage of the phosphoester bond, relieves the electron quenching and results in increase quantum yield of the fluorophore. Accordingly, previous demonstrations that cleavage of the Bodipy™ moiety BGTPγS by Fhit, a diadenosine hydrolase, or alteration in the structure of BGTPγS through binding or cleavage by G proteins, results in a similar five- to seven-fold increase in fluorescence [20, 22].

In this current study we take advantage of purified forms of the minimal catalytic core of adenylyl cyclase [18]. In addition, we utilize a mutant of adenylyl cyclase (mut-AC), where the residues that dictate nucleotide specificity have been altered to recognize guanine nucleotides [17, 23]. Fig. **1C**,**D** illustrate the X-ray crystal structure of the C1 (mauve) and C2 domain (green) bound to forskolin and an ATP analogue ATPαS(RP) [14]. Panel D illustrates the highly conserved lysine (Lys_{938}) and aspartate (Asp_{1018}) residues that together coordinate the purine ring of ATP. Although these residues are not conserved in guanylyl cyclases, their positions in the cyclase fold are conserved. The substitution of

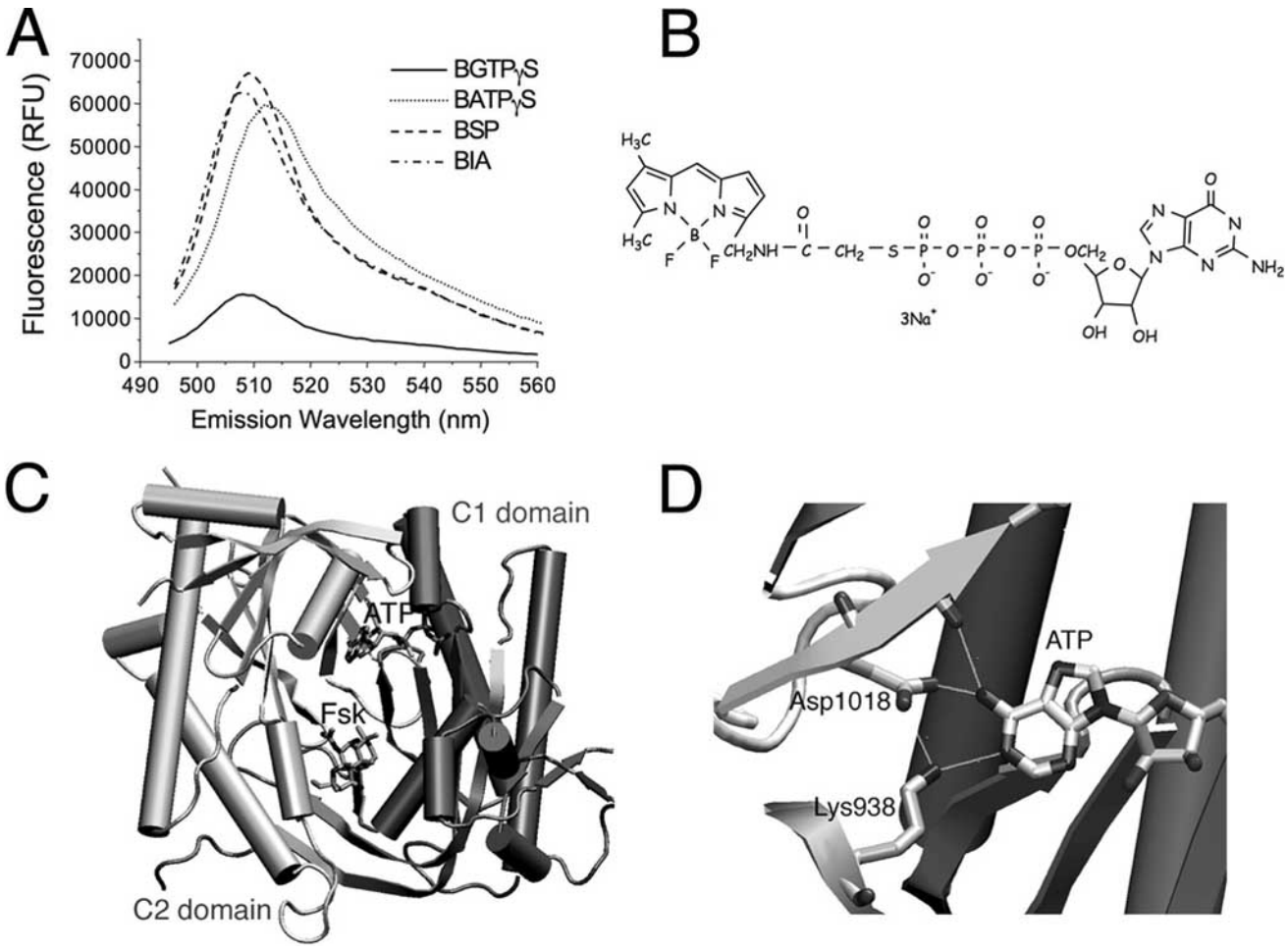

Fig. (1). A) Emission spectra of Bodipy FL iodoacetamide (BIA) Bodipy FL thiophosphate (BSP), Bodipy FL ATPγS (BATPγS) and Bodipy FL GTPγS (BGTPγS) excited at 485 nm. The fluorescence of BGTPγS in quenched in comparison to BIA, BSP and BATPγS (each at a concentration of 50 nM). **B**) Structure of BGTPγS. **C**) Structure of the catalytic domain of adenylyl cyclase. The C1 domain from AC5 (dark grey) and the C2 domain from AC2 (light grey) together form the catalytic core of AC. Bound in the catalytic core is the diterpene, forskolin (Fsk), and ATP. The coordinates for the non-hydrolyzable ATP analog, ATPαS(RP), bound to C1 and C2 were used to generate the model (PDB ID: 1CJK). **D**) Residues within the active site of adenylyl cyclase responsible for stablizing the purine ring of ATP. Highlighted are Asp_{1018} and Lys_{938} of the C2 domain and are mutated to Glu and Cys residues, respectively, in mut-AC.

Lys_{938} and Asp_{1018} with Glu and Cys, respectively, the corresponding residues in guanlylyl cyclases, converts the adenylyl cyclase to a non-selective purine cyclase (mut-AC) [17, 23]. Note that unless otherwise stated mixtures of C1•C2 or C1•mut-AC will simply be referred to as wt-AC or mut-AC, respectively.

***Forskolin Stimulates the Fluorescence Enhancement of BGTPγS by Cyclase*:** Fig. **2** illustrates that cyclase activity of both wt-AC and mut-AC increased the fluorescence of the BGTPγS in a time-dependent manner. No increases in fluorescence were observed with BATPγS with either wt-AC (Fig. **2C**) or mut-AC (not shown). In the presence of Mg^{2+}, forskolin (10 μM) dramatically enhanced the rate of fluorescence increase in the presence of the mut-AC mutant but not with wt-AC (Fig. **2A**). The increase in fluorescence was linear for at least 60 min and is consistent with enzymatic activity rather than simply binding of BGTPγS to the mut-AC. Furthermore, thermal denaturation of the enzyme during the reaction had no effect on the fluorescence, although it failed to increase with time (data not shown). Fig. **2B** illustrates that the addition of Mn^{2+} further enhanced the fluorescence increase with mut-AC; surprisingly the enhancement was observed with wt-AC as well, although to a lesser extent (Fig. **2D**). This increase in Bodipy fluorescence in the presence of wt-AC, however, is completely dependent on the presence of Mn^{2+}. The capacity of Mn^{2+} to alter nucleotide substrate specificity has been identified previously with guanylyl cyclase [24-26]. Furthermore, Mn^{2+} increased the potency of a variety of guanine nucleotide-based inhibitors of adenylyl cyclase activity [10]. The enhanced rate of fluorescence increase was sufficient to merit inclusion of Mn^{2+} in all subsequent assays.

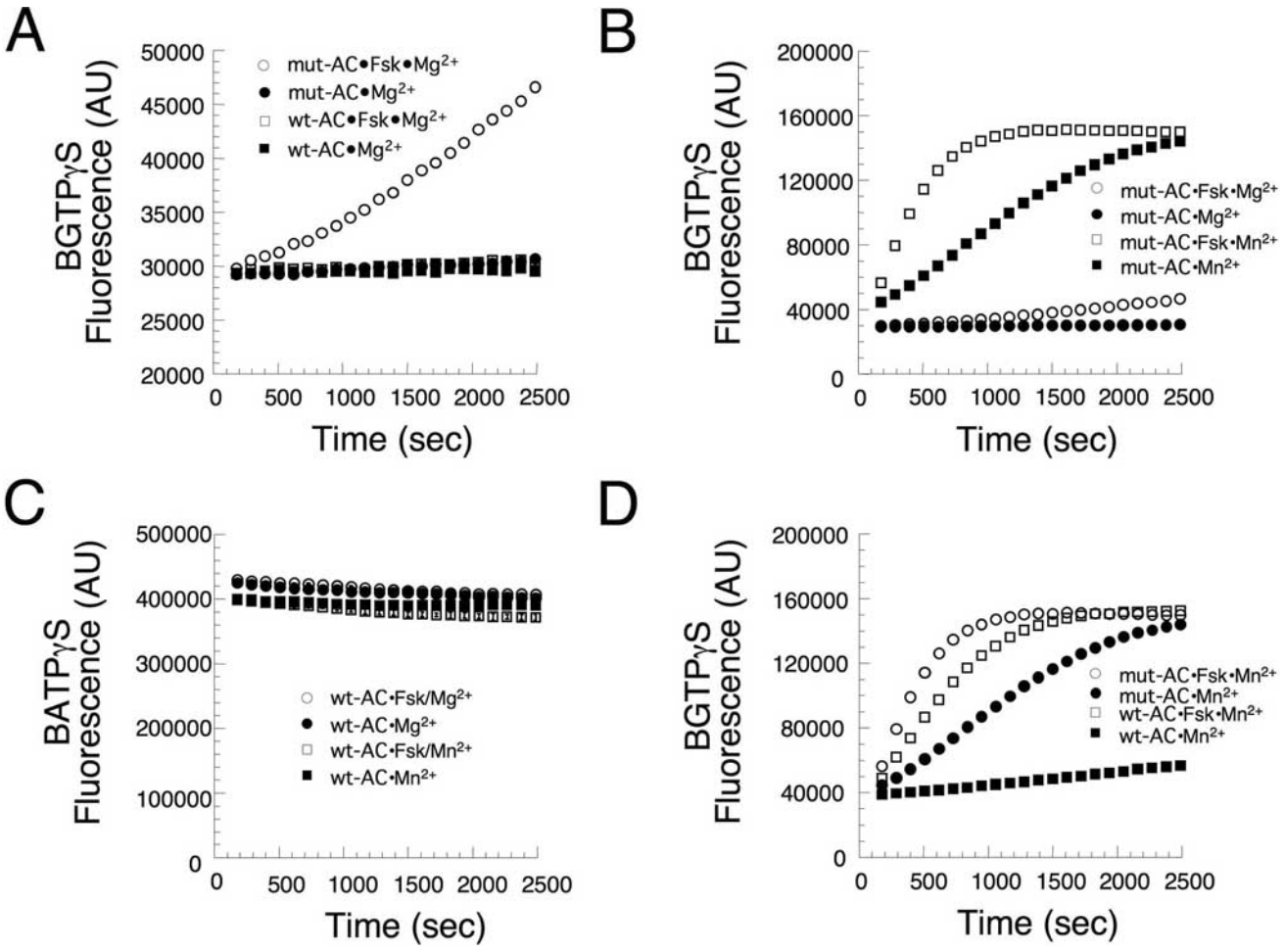

Fig. (2). Forskolin and Mn^{2+} enhance the fluorescence of the Bodipy™ moiety of BGTPγS in the presence of either wt-AC and the mut-AC mutant of adenylyl cyclase. **A**) Fluorescence enhancement of Bodipy™ occurs in a time-dependent and linear fashion in the presence of the mut-AC mutant and forskolin•Mg^{2+} (open circles). **B**) The rate of fluorescence increase is enhanced by mut-AC in the presence of the divalent cation, Mn^{2+} (squares) under basal (solid) and forskolin-stimulated (open) conditions, in comparison with Mg^{2+} (circles) under similar respective stimulation. **C**) Lack of enhancement of flurorescence of the Bodipy™ moiety of BATPγS with wt-AC under the identical conditions as in B). **D**) The presence of Mn^{2+} and forskolin (open) dramatically enhances the fluorescence of the Bodipy™ moiety of BGTPγS by wt-AC (squares), in comparison to mut-AC (circles) and under basal (solid) conditions. Note that all assays included the C1 domain (500 nM) and the C2 domain (5 μM) of either the wt-AC or the mut-AC mutant were incubated with BGTPγS (200 nM) in the presence or absence of 100 μM forskolin. Assays contained 10 mM Mg^{2+} or 5 mM Mn^{2+}. Fluorescence was excited at 485 nm and emission was collected at 525 nm every 90 s.

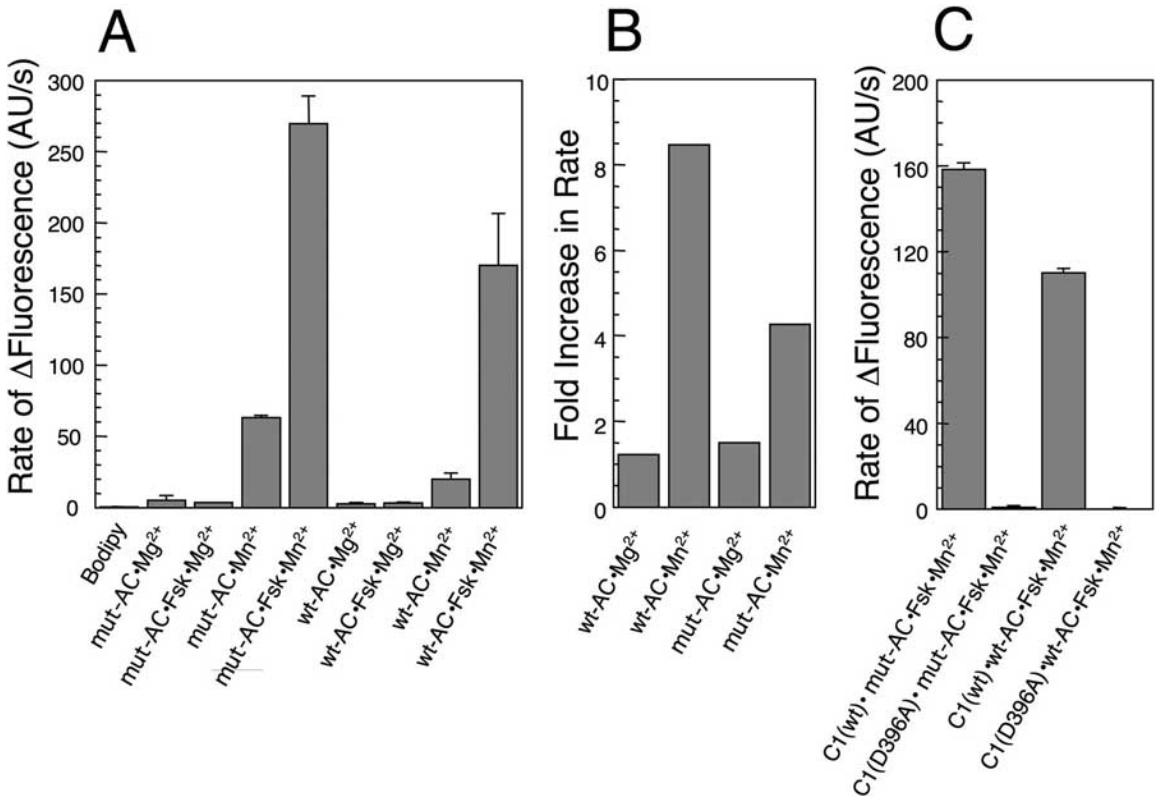

Fig. (3). Comparison of the rates of fluorescence increase using BGTPγS and various enzyme combinations under various conditions. **A**) Comparison of the rates of increase in fluorescence in Fig. **2**. **B**) A representation of the fold-stimulation by forskolin over basal conditions of wt-AC and the mut-AC in the presence of Mg^{2+} or Mn^{2+}. **C**) Comparison of the rates of fluorescence enhancement by wt-AC (C1) in comparison to the catalytically-impaired mutant (C1(Asp_{396}Ala)) reconstituted with the C2 domains of either mut-AC or wild type (C2). Reaction conditions were similar to those used in Fig. **2**.

Fig. **3** expresses the data as a fluorescence rate increase as determined by linear regression over the first 500 s after addition of BGTPγS. The rate of fluorescence enhancement varied depending on the enzyme, divalent cation, and presence of forskolin as shown in Fig. **3A**. While it is clear that forskolin-and Mn^{2+}-stimulated mut-AC displayed the fastest rate of fluorescence enhancement, Fig. **3B** clearly illustrates that the magnitude of the fold-increase in the rates of fluorescence (forskolin over basal) was greater with wt-AC.

No increases in fluorescence were observed with C1(Asp_{396}Ala) in any combination of activator or C2 domain was observed (Fig. **3C**). Asp_{396} is one of two Asp residues that are highly conserved in all cyclases (ACs and GCs) and contribute toward the coordination of the two metal sites in the active site [14]. These data support the hypothesis that the increase in fluorescence is dependent upon enzymatic cleavage of BGTPγS.

***Bodipy™ Fluorescence Enhancement by Cyclase Displays Michaelis Kinetics*:** The increase in the rate of fluorescence of BGTPγS appears to display Michaelis kinetics with an apparent maximum rate of fluorescence increase (Fmax) of 72 ± 1.2 Absorbance units (AU)/s and a *Km* 4.9 ± 0.2 μM for Mn^{2+}•BGTPγS (Fig. **4A**). Incubation with forskolin lowered the *Km* to 1.7 ± 0.2 μM while raising the Fmax to 97 ± 3). AU/s. Extrapolation of Vmax based on the calibration curve of fluorescence to the molar equivalents of BSP (not shown) suggests that the Vmax for forskolin/Mn^{2+}-stimulated mut-AC was approximately 18 ± 0.5 nmol/min/mg, substantially lower than the previously reported values for maximal velocity of wt-AC using Mg^{2+}•ATP of ~100 μmol/min/mg [18]. In addition, the *Km* value for Mn^{2+}•BGTPγS is significantly lower than the *Km* for Mg^{2+}•GTP previously reported for mut-AC [17]. Fig. **4B** illustrates how Mn^{2+} dramatically affects the activity of mut-AC in the presence or absence of forskolin and is consistent with previously described effects of Mn^{2+} on adenylyl cyclase activity [27].

As described earlier (Fig. **3C**), wt-AC in the presence of Mn^{2+} displayed a larger fold-activation in comparison to mut-AC, largely due its lower basal activity. Since one of the goals of HTS development is to ascertain conditions that display optimal signal-to-noise ratios, the use of wt-AC as an enzyme source is preferable. In order to assess the pharmacological relevance of the fluorescence assay, we tested whether known adenylyl cyclase modulators could regulate wt-AC. It is our intent that the fluorescence assay would reveal regulatory properties that are comparable to those detected by classical methods.

***Forskolin and Gsα•GTPγS Enhances Bodipy™ Fluorescence*:** Forskolin induced a concentration-dependent fluorescence enhancement of the Bodipy™ moiety using mut-AC in the presence of Mn^{2+} (EC_{50}~460 nM ± 48 nM, (Fig.

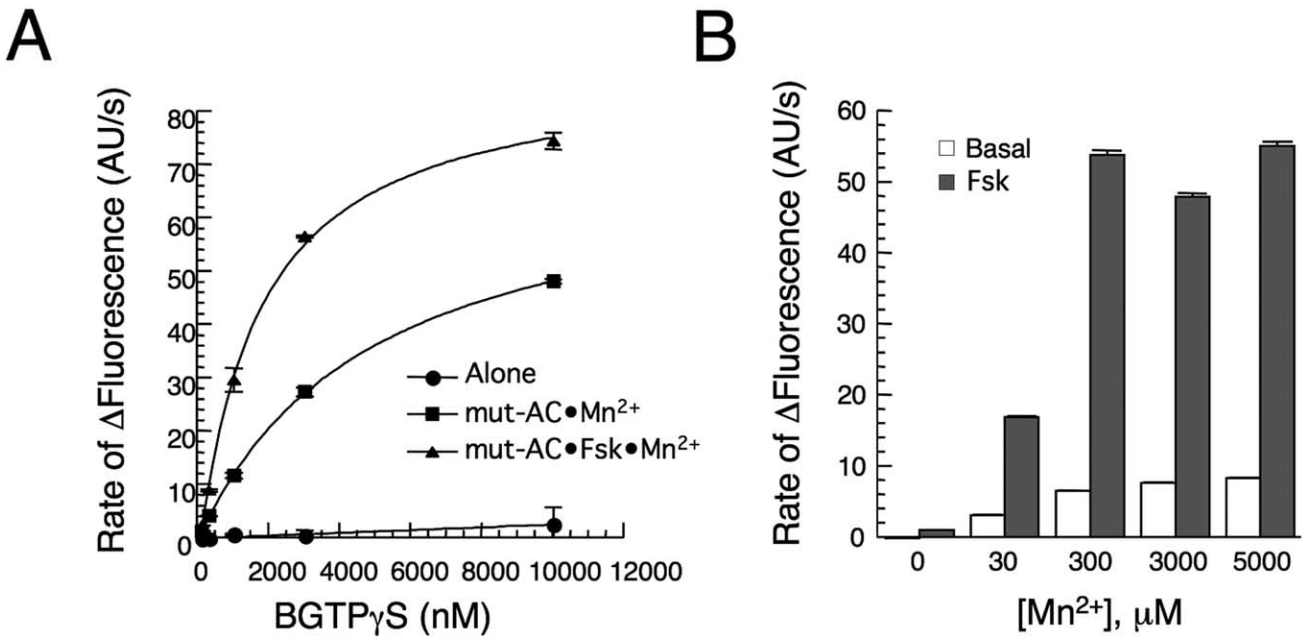

Fig. (4). The rate of change in fluorescence of Mn^{2+}•BGTPγS by forskolin-stimulated adenylyl cyclase displays Michaelis kinetics. A) The mut-AC was incubated with varying concentrations of BGTPγS (in 5 mM Mn^{2+}) in the presence (triangles) or absence (squares) of 100 μM forskolin. Also illustrated is in the basal activity of mut-AC in the presence of 10 mM $MgCl_2$. B) The effect of Mn^{+2} the rate of change in fluorescence of BGTPγS in the presence (shaded bars) or absence (open bars) of 100 μM forskolin.

5A). For comparison the EC_{50} for forskolin on wt-AC using Mg^{2+}•ATP, as measured by the classic assay that monitors the conversion of [^{32}P]ATP to [^{32}P]cAMP, was approximately 366 ± 81 nM (Fig. **5A**). Similarly, activated-Gsα (Gsα•GTPγS) enhanced the fluorescence of Bodipy™ in the presence of wt-AC and Mn^{2+} with an EC_{50} of greater than 1 μM (Fig. **5B**). Under these conditions we did not reach saturation, so a precise EC_{50} determination could not be made. It does appear that the values will be higher than values using classical activity measurements (EC_{50}~312 nM ± 68 nM, data not shown) [18].

The positively cooperative effect of forskolin on Gsα stimulation on AC activity was recapitulated in the fluorescence assay (Fig. **5B**). Forskolin (10 μM) enhanced both the apparent Vmax of AC and also decreased the EC_{50} for Gsα by greater than 30-fold, consistent with published values using the classical assays [18]. We still, however, observed a 10-fold higher EC_{50} for Gsα under these conditions. The altered EC_{50} may imply that the conformation of the enzyme that Gsα induces, in contrast to forskolin, is not optimal for BGTPγS binding and subsequent catalysis.

To demonstrate that the fluorescence enhancement following Gsα•GTPγS activation was due to cyclase activity and not GTPase activity of Gsα, we measured hydrolysis by a cGMP enzyme immunoassay (EIA, Fig. **5B** inset). Gsα•GTPγS (400 nM) stimulated the conversion of BGTPγS into cGMP and also Bodipy-pyrophosphate (B•PPi), with an activity of ~11 nmol/min/mg. Analysis of the products of BGTPγS by capillary electrophoresis confirms that B•PPi is the major fluorescent product that accumulates following activation (data not shown).

***The Fluorescence Enhancement Requires Both the C1 and C2 Domains*:** It is known that the affinities of the two domains that comprise the minimal catalytic core of AC can be enhanced by the presence of activators such as forskolin. Fig. **6** demonstrates that in the presence of forskolin, the C1 domain (C1) has an appreciable affinity for the wt-C2 domain (C2, apparent Kd~1.6 ± 0.1 μM) when assayed by fluorescence spectroscopy. Similar values were obtained using classical adenylyl cyclase assays while monitoring the conversion [^{32}P]ATP to [^{32}P]cAMP. In addition, the reciprocal experiment (i.e. varying the concentration of the C2 domain, C2), also in the presence of forskolin/Mn^{2+}, indicated an apparent Kd ~3.3 ± 0.6 μM (inset). These data support the notion that in order to obtain maximal fluorescence enhancement under forskolin stimulation at 100 nM of the C1 domain, a concentration of the C2 domain of at least 10 μM is required (100-fold excess).

***Inhibition of Fluorescence Enhancement by Adenylyl Cyclase Inhibitors*:** Forskolin-stimulated enhancement of Bodipy™ fluorescence by wt-AC using BGTPγS as a substrate was potently inhibited by the known AC inhibitor 2',5'-dd-3'-ATP (Fig. **7A**), *Ki*~ 200 ± 61 nM). Although the inhibition of fluorescence was concentration-dependent and appeared to occur through a single site, the apparent *Ki* was approximately 10-fold higher than reported for wt-AC using Mn^{2+}-ATP as a substrate [10]. Other inhibitors were also tested against forskolin-stimulated wt-AC using Mn^{2+}-BGTPγS as a substrate (Fig. **7B**). Like 2',5'-dd-3'-ATP, all compounds tested inhibited activity in a concentration-dependent manner but had higher *Ki* values than previously reported [10]. A possible explanation for this discrepancy may be due to the relatively low *Km* that we observe for Mn^{2+}•BGTPγS. The poor capacity of the P-site inhibitor 2'd-

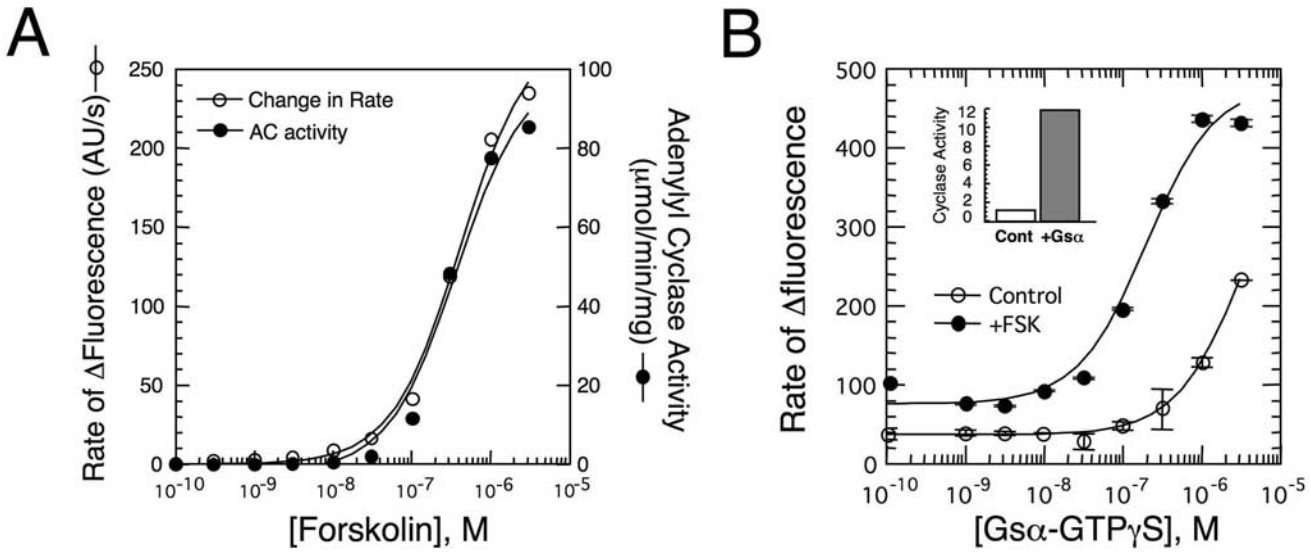

Fig. (5). The rate of change of fluorescence enhancement by the stimulatory G protein, Gsα, and forskolin correlates with adenylyl cyclase activity. **A**) Concentration-dependence of forskolin on the rate of fluorescence enhancement of BGTPγS with wt-AC in the presence of Mn^{2+} (open circles) in comparison to measurements of adenylyl cyclase activity using Mg^{2+}•[^{32}P]ATP (closed circles). **B**) Concentration-dependence of GTPγS-activated Gsα (Gsα•GTPγS) on the rate of fluorescence enhancement of wt-AC in the presence of Mn^{2+} using BGTPγS as a substrate in the absence (open circles) or presence of 10 μM forskolin (closed circles). *Inset*, bar graph representing the conversion of BGTPγS to cGMP in the absence (open bars) or presence of 400 nM Gsα•GTPγS (shaded bars) by wt-AC, as measured by EIA.

3'AMP to inhibit the fluorescence increase (IC_{50}~50 μM) is likely to be related to the poor Vmax of the enzyme using BGTPγS as a substrate (~18 nmol/min/mg). P-site inhibitor binding to the active site has previously been reported to be positively cooperative with pyrophosphate (PPi), one of the products of purine cyclases [28, 29]. The potency of P-site inhibitors is therefore dramatically influenced by the catalytic activity of the enzyme. The poor catalytic activity of AC using BGTPγS may not allow accumulation of Bodipy™-thio-PPi at high enough concentrations to display cooperative binding of 2'd-3'AMP. Alternatively, Bodipy™-thio-PPi itself may be incapable of inducing a positive allosteric effect on 2'd-3'AMP binding.

***The Application of Fluorescence Cyclase Assay to High Throughput Screening (HTS)*:** Taken together these data suggest that the fluorescence assay may be suitable as a method to screen both cyclase activators and inhibitors in a high-throughput format. All fluorescence assays described thus far have utilized the 96-well microtitre plate. We have also adapted the assay to a 384-well microtiter plate format. In doing so we have decreased the reaction volume from 100 μL to 20 μL, thus conserving reagents. This screen may be used to identify compounds that either stimulate cyclase activity (through increasing the basal fluorescence) or inhibit forskolin-stimulated cyclase activity (decreasing forskolin-stimulated fluorescence). We observe that in response to forskolin stimulation, the 384-well microplate fluorescence cyclase assay produces a significantly robust signal over basal activity and has an observed Z' score of 0.61 (not shown). Such a score would deem this assay as sufficient for HTS applications. As an example of the application of the fluorescence assay, Fig. **8A**,**B** illustrate a screen for activators of adenylyl cyclase. A single plate representing 320 compounds from a commercial compound library (diversity set based on chemical space, Maybridge, Cornwall, UK) was

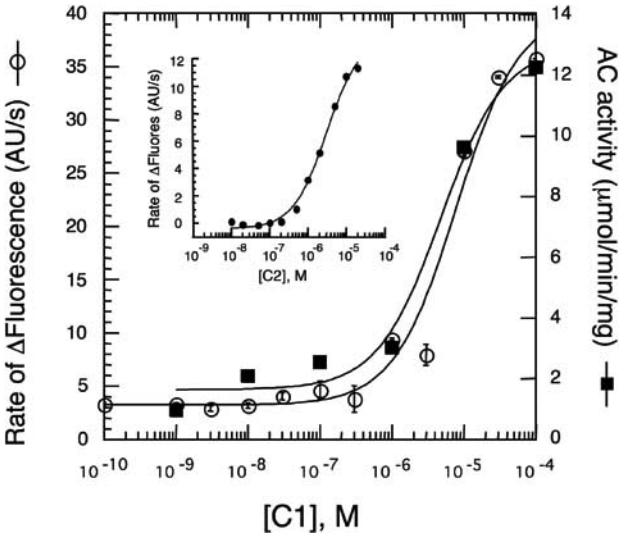

Fig. (6). Measurement of the forskolin-dependent interaction between the C1 and C2 domains by fluorescence spectroscopy. Varying concentrations of the C1 domain (C1) with a fixed concentration of the C2 domain (100 nM wild-type-C2) domain were incubated with 100 μM forskolin in the presence of 500 nM BGTPγS and 5 mM Mn^{2+}. Both the rate of change in fluorescence (using Mn^{2+}•BGTPγS, open circles) or the adenylyl cyclase activity (using Mg^{2+}•[^{32}P]ATP, closed squares) are compared. *Inset*, the rate of change in fluorescence of BGTPγS at varying concentrations of the C2 domain (wild-type-C2) with a fixed concentration of the C1 domain (100 nM C1) domain were incubated with 100 μM forskolin in the presence of 500 nM BGTPγS and 5 mM Mn^{2+}.

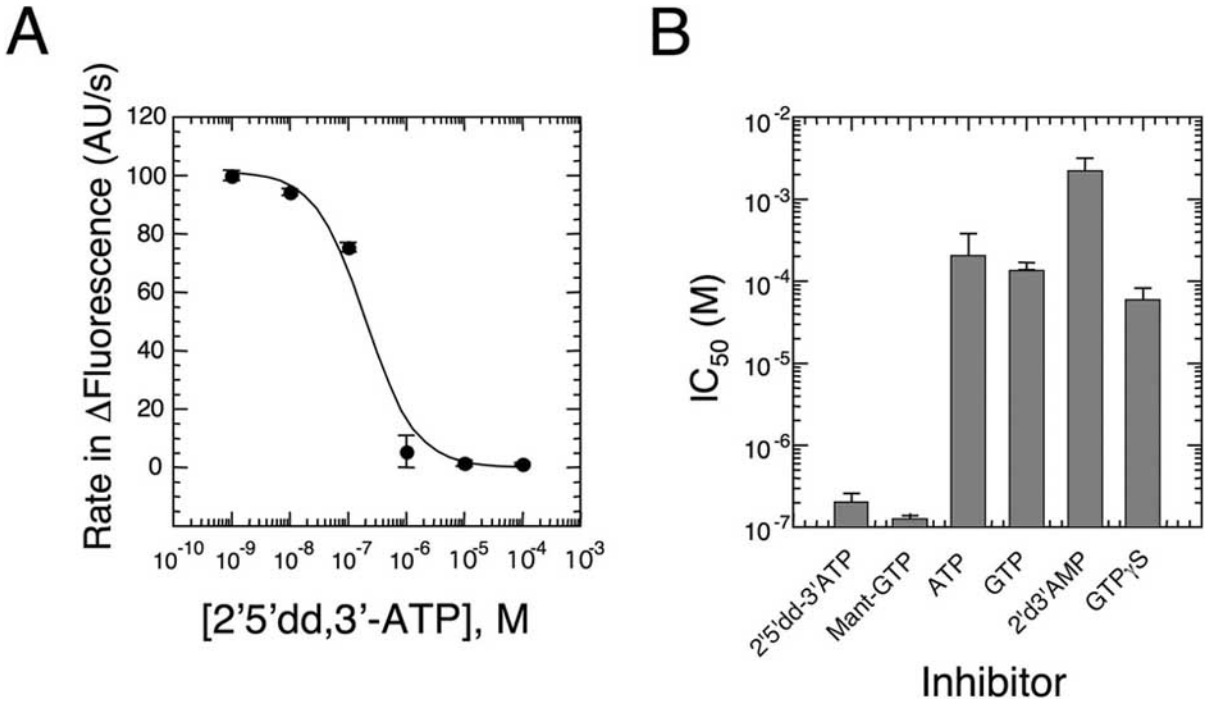

Fig. (7). Inhibition of forskolin-stimulated fluorescence enhancement of BGTPγS by various adenylyl inhibitors. **A**) Varying concentrations of adenylyl cyclase inhibitor 2'5'-dd-3'-ATP were incubated with wt-AC in the presence of 500 nM BGTPγS/Mn^{2+} and 100 μM forskolin. Data are represented as a percent of maximal rate of change of fluorescence. **B**) IC_{50}values (inhibitory concentrations at which 50% of the activity was inhibited) of various adenylyl cyclase inhibitors and substrates on forskolin-stimulated wt-AC. Values were determined from inhibition curves under conditions similar used in A).

screened for fluorescence enhancers in comparison to forskolin. Controls samples included BGTPγS and wt-AC in the absence (well #1A, 2A, 1B, 2B, M23, M24, N23 & N24), or presence of 10 μM forskolin (C1, C2, D1, D2, O23, O24, P23 & P24). Substrate, compound library and enzyme were included in rows A to P, columns 3 to 22. Fig. **8A**,**8B** illustrate the robust responses from sub-maximal concentrations of the positive control forskolin. Potential positive hits that represent adenylyl cyclase activators are identified in wells H10, H18 and K12.

Also illustrated is an example of an inhibitor screen in the 384-well microplate format (Fig. **8C**). Using forskolin-stimulated wt-AC, the same 320 compounds as above were tested for their ability to inhibit cyclase activity. Of the 320 compounds tested, none were found to significantly inhibit forskolin-stimulated activity.

In summary, we have identified a powerful fluorescence-based cyclase assay that describes the activity of adenylyl cyclase in response to stimulators, Gsα•GTPγS and forskolin as well as inhibitors such as 2'5'-dd-3'ATP. The assay entails measuring the increase in fluorescence that occurs during the conversion (and fluorescence unquenching) of BGTPγS to Bodipy-FL-γPPi, with cGMP as a biproduct. The enhanced fluorescence is a result of catalysis, rather than unquenching as a result of binding to AC: demonstrated by the resistance to thermal denaturation and the fact that the accumulation of cGMP (the other product) was assessed by enzyme immunoassay. It is important to emphasize that this fluorescence increase does not occur with BATPγS hydrolysis, as its fluorescence is not normally quenched. Although BGTPγS is not an optimal substrate, it does appear to reflect the sensitivity of AC to various modulators in a qualitatively similar manner as classical AC assays. The fluorescence assay, however, is a real-time measurement of AC activity that is adaptable to a 96-or 384-well microplate HTS format. Conversion of the assay to the 384-well format reduced reagent use by five-fold, although the sensitivity should be sufficient to reduce the assay down to a 1524-well microplate format.

Clearly, genetic studies by the Ishikawa and Storm laboratories using the mouse model have strongly suggested that pharmacological targeting of AC isoforms may be useful for the treatment and/or prevention of various ailments: cardiac hypertrophy (AC5 and AC6), memory (AC1 and AC8 [30]), anosmia (AC3 [31]) and perhaps male fertility (AC3 [32]). This concept was solidified by recent work by Iwatsubo *et al.* [33] where they reported that a small molecule inhibitor of AC5 (R,4R-3-(6-aminopurin-9-yl)-cyclopentanecarboxylic acid hydroxyamide) protected cardiac myocytes against hyper β-adrenergic receptor-stimulated apoptosis [34]. The application of HTS, using methods such as the fluorescence assay described here, might aid in the identification of modulators of AC activity. Novel small molecule or fragments derived from these screens could thus provide the foundation for the development of more potent and isoform-selective therapeutics.

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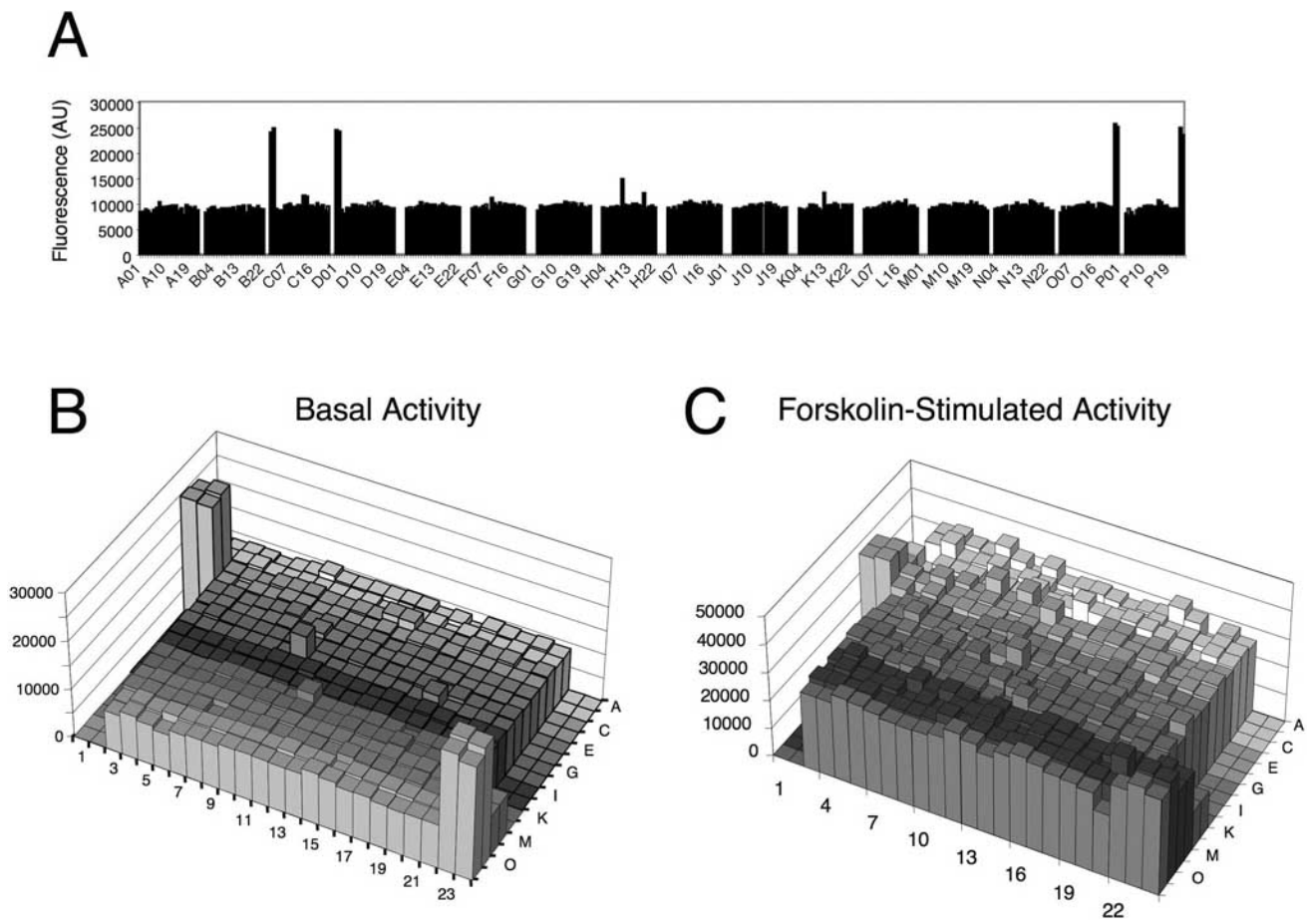

Fig. (8). Fluorescence detection of adenylyl cyclase activity (wt-AC) using 384-well microplate HTS format. **A**) and **B**) a 384-well plate was prespotted with a subset of a 320 compound diversity set library dissolved in DMSO (Maybridge). Data are represented as a bar graph from data across the entire plate (A) or as a three-dimensional illustration of the fluorescence values of the plate itself. **C**) An example of a screen of the same compounds for their capacity to inhibit forskolin-stimulated (30 μM) wt-AC activity. The assay was initiated by the addition of BGTPγS/Mn^{2+} and allowed to incubate for 30 min at 25 °C. 30 μM forskolin was used as a positive control in the upper panel (wells C1,C2, D1,D2, O23,O24, P23 and P24).

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ABBREVIATIONS

AC	=	Adenylyl cyclase
ATP	=	Adenosine triphosphate
AMP	=	Adenosine monophosphate
ADP	=	Adenosine monophosphate
AC	=	Adenylyl cyclase
2'd-3'AMP	=	2'deoxy-3'-adenosine monophosphate
2',5'-dd-3'-ATP	=	2'-5'-dideoxy-3'adenosine triphosphate
ATPαS(RP)	=	Rp enantiomer of adenosine-5'-[γ-thio] triphosphate
BATPγS	=	Bodipy FL adenosine-5'-[γ-thio]tri-phosphate
BGTPγS	=	Bodipy FL guanosine-5'-[γ-thio]tri-phosphate
BIA	=	Bodipy iodoacetamide
BSP	=	Bodipy FL thiophosphate
cAMP	=	Cyclic adenosine monophosphate
cGMP	=	Cyclic guanosine monophosphate
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
EIA	=	Enzyme immunoassay
Fhit	=	Diadenosine hydrolase
GC	=	Guanylyl cyclase
Gsα	=	Alpha subunit of the stimulator G protein Gs
GTP	=	Guanosine triphosphate

GTPγS	=	Guanosine-5'-[γ-thio]triphosphate
Ki	=	Inhibitory constant
Km	=	Michaelis constant
Mant-GTP	=	2'/3'-O-(N-methylanthraniloyl)guanosine triphosphate
RIA	=	Radioimmunoassay
Vmax	=	Maximal velocity

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