Activation and Inhibition of Adenylyl Cyclase Isoforms by Forskolin Analogs

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ABSTRACT

Adenylyl cyclase (AC) isoforms 1 to 9 are differentially expressed in tissues and constitute an interesting drug target. ACs 1 to 8 are activated by the diterpene, forskolin (FS). It is unfortunate that there is a paucity of AC isoform-selective activators. To develop such compounds, an understanding of the structure/activity relationships of diterpenes is necessary. Therefore, we examined the effects of FS and nine FS analogs on ACs 1, 2, and 5 expressed in Sf9, Spodoptera frugiperda insect cells. Diterpenes showed the highest potencies at AC1 and the lowest potencies at AC2. We identified full agonists, partial agonists, antagonists, and inverse agonists, i.e., diterpenes that reduced basal AC activity. Each AC isoform exhibited a distinct pharmacological profile. AC2 showed the highest basal activity of all AC isoforms and highest sensitivity to inverse agonistic effects of 1-deoxy-forskolin, 7-deacetyl-1,9-dideoxy-forskolin, and, particularly, BODIPY-forskolin. In contrast, BODIPY-forskolin acted as partial agonist at the other ACs. 1-Deoxy-forskolin analogs were devoid of agonistic activity at ACs but antagonized the effects of FS in a mixed competitive/noncompetitive manner. At purified catalytic AC subunits, BODIPY-forskolin acted as weak partial agonist/strong partial antagonist. Molecular modeling revealed that the BODIPY group rotates promiscuously outside of the FS-binding site. Collectively, ACs are not uniformly activated and inhibited by FS and FS analogs, demonstrating the feasibility to design isoform-selective FS analogs. The two- and multiple-state models, originally developed to conceptualize ligand effects at G-protein-coupled receptors, can be applied to ACs to explain certain experimental data.

Numerous receptors for hormones and neurotransmitters couple to the G-protein $G_\alpha$ to stimulate adenylyl cyclase (AC). AC catalyzes the conversion of ATP into the second messenger cAMP, which regulates numerous body functions including memory and learning, heart contractility, respiration, and lipolysis (Sunahara et al., 1996; Tang and Hurley, 1998; Hanoune and Defer, 2001). Traditionally, this signal transduction cascade has been targeted pharmacologically by receptor agonists or antagonists and by inhibitors of phosphodiesterases, catalyzing cAMP degradation (Iwatsubo et al., 2006).

AC is an interesting pharmacological target for several reasons. First, in many tissues, several receptors couple to $G_\alpha$ and hence AC (Birnbaumer et al., 1990). Thus, AC integrates the input from several receptors and is localized at a central position in the signaling cascade. Second, a therapeutic problem with receptor agonists is desensitization, resulting in a loss of drug efficacy during long-term treatment (Penn et al., 2000). Third, mammalian cells express nine AC isoforms with distinct regulatory properties and tissue distribution (Hanoune and Defer, 2001). Fourth, the genes for several AC isoforms were successfully knocked out in mice, and the resulting distinct phenotypes point to unique physiological functions of each AC isoform (Hanoune and Defer, 2001).

There are two principal approaches to manipulate ACs pharmacologically. First, the catalytic site can be targeted by nucleotides that inhibit AC either competitively or noncompetitively (Dessauer et al., 1999; Gille et al., 2004; Iwatsubo et al., 2006; Mou et al., 2006). Recombinant AC1 (predominantly expressed in the brain) and AC5 (the major AC isoform in the heart) are inhibited more potently by certain competitive inhibitors than AC2 (also predominantly ex-
pressed in brain) (Gille et al., 2004), and selective AC5 inhibitors are of potential value in the treatment of heart failure (Rottainder et al., 2007).

Second, the diterpene, forskolin (FS), isolated from the roots of the Indian plant Coleus forskohlii, is a very effective activator of ACs 1 to 8 but not of AC9 (Seamon and Daly, 1986; Tang and Hurley, 1998). FS binds to a defined hydrophobic pocket close to the catalytic site of AC (Tesmer et al., 1997; Tang and Hurley, 1998). FS is of interest for the treatment of various disorders, including heart failure, bronchial asthma, obesity, and glaucoma, but the development of FS analogs as drugs has been hampered by the unavailability of isoform-selective FS analogs (Seamon and Daly, 1986; Launera et al., 1989; Robbins et al., 1996; Onda et al., 2001). Another major problem in the field is the fact that we still do not know whether the FS-binding site in ACs is of any physiological relevance and whether there is an endogenous physiological ligand for this site. Very recently, FS has been identified in the cyst fluid of patients with polycystic kidney disease, but it is unknown whether FS was produced endogenously or administered exogenously by FS-containing herbal medicines (Putnam et al., 2007). Finally, FS is a very hydrophilic compound, and because of limitations of test systems with respect to organic solvent compatibility, it was often difficult to generate saturated concentration/response curves for diterpenes, rendering calculation of ligand potencies and efficacies ambiguous.

The aim of the present study was to systematically characterize the effects of FS and nine FS analogs on AC. Figure 1 shows the structures of FS and the FS analogs studied. FS analogs differ from each other in the OH substitution of C1 and C9, acetyl substitution at C7 or C8, and the type of substituent at C7. We determined the catalytic activity of mammalian AC isoforms 1, 2, and 5 expressed in Spodoptera frugiperda (Sf9) insect cell membranes. As a control, we also studied the endogenous insect cell AC. Moreover, we examined the interaction of one particularly interesting FS analog, BODIPY-FS, with purified catalytic subunits of AC and conducted molecular modeling studies with this analog.

Materials and Methods

Materials. Baculoviruses encoding ACs 1, 2, and 5 were a gift from Drs. G. Gilman and R. K. Sunahara (University of Texas Southwestern Medical Center, Dallas, TX). Wild-type baculovirus was prepared using the BaculoGold transfection kit (BD PharMingen, San Diego, CA). Sf9 insect cells were from the American Type Culture Collection (Rockville, MD). Recombinant cytosolic C1a domain from canine AC5, referred to as C1, the C2a domain from rat AC2, referred to as C2, and Gα, referred to as Gα, were purified as described previously (Tesmer et al., 2002). Gα was activated with guanosine 5'-[γ-thiol]triphosphate (GTP·S) as described previously (Tesmer et al., 2002). FS was from LC Laboratories (Woburn, MA). DMB-FS was from PerkinElmer (Wellesley, MA). Neutral alumina (super I, WN-6) was from Sigma. Fetal bovine serum was from Atlas Biologicals (Pt. Collins, CO).

Cell Culture and Membrane Preparation. Cell culture and membrane preparation were performed as described previously (Seifert et al., 1998). In brief, Sf9 cells were cultured in SF 900 II medium supplemented with 5% (v/v) fetal bovine serum and 0.1 mg/ml gentamicin. High-titer baculovirus stocks were generated through two sequential amplification steps as described previously (Seifert et al., 1998). In each amplification step, the supernatant fluid was harvested and stored under light protection at 4°C. For membrane preparation, Sf9 cells (3.0 × 10⁶ cells/ml) were infected with correspondent baculovirus encoding different mammalian ACs (1:100 dilutions of high-titer virus stocks unless stated otherwise) and cultured for 48 h. For experiments shown in Table 2, we also used 1:10 and 1:300 dilutions of virus stocks. Membranes expressing each construct and membranes from uninfected Sf9 cells were prepared as described previously (Seifert et al., 1998). In brief, cells were harvested, and cell suspensions were centrifuged for 10 min at 1000g at 4°C. Pellets were resuspended in 10 ml of lysis buffer (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml benzamide, pH 7.4). Thereafter, cell lysates were lysed with 20 to 25 strokes using a Dounce homogenizer. The resultant cell fragment suspension was centrifuged for 5 min at 500g and 4°C to sediment nuclei. The cell membrane-containing supernatant suspension was transferred into 30-ml tubes and centrifuged for 20 min at 30,000g and 4°C. The supernatant fluid was discarded, and cell pellets were resuspended in buffer consisting of 75 mM Tris/HCl, 12.5 mM MgCl₂, and 1 mM EDTA, pH 7.4. Membrane aliquots of 0.5 to 1 ml were prepared and stored at −80°C, and protein concentration for each membrane preparation was determined using the BioRad DC protein assay kit (Bio-Rad, Hercules, CA).

AC Activity Assay. AC activity was determined essentially as described in the literature (Gille et al., 2004). Before experiments, membranes were sedimented by a 15-min centrifugation at 4°C and 15,000g and resuspended in 75 mM Tris/HCl, pH 7.4. Reaction mixtures (50–μl final volume) contained 30 μg of membrane protein, 40 μM ATP/Mn²⁺ plus 10 mM MnCl₂ and FS or FS analogs at various concentrations in the presence of 3% (v/v) DMSO. With the exception of some experimental conditions shown in Fig. 2, reaction mixtures also contained 10 μM GTP·S. After a 2-min preincubation at 37°C, reactions were initiated by adding 20 μl of reaction mixture containing (final) 1.0 to 1.5 μCi/tube [α-³²P]ATP, 0.1 mM cAMP, and a regenerating system consisting of 2.7 mM monoisocitrate/l-malic acid, 1.25 μg/ml phosphoenolpyruvate, 0.125 μg/ml pyruvate kinase, and 1.0 μg/ml myokinase. Reactions were conducted for 20 min at 37°C and were terminated by adding 20 μl of 2.2 N HCl. Denatured protein was precipitated by a 1-min centrifugation at 25°C and 15,000g. The supernatant fluid (65 μl) was applied onto disposable columns filled with 1.3 g of neutral alumina. [³²P]AMP was separated from [α-³²P]ATP by elution of [³²P]cAMP with 4 ml of 0.1 M ammonium acetate, pH 7.0. Recovery of [³²P]cAMP was −80% as assessed with [³²P]cAMP as standard. [³²P]cAMP was determined by liquid scintillation counting using Ecolum scintillation cocktail (Fisher Scientific Co., Pittsburgh, PA). Data were analyzed by nonlinear regression using the Prism 4.02 program (GraphPad Software, Inc., San Diego, CA). In experiments with purified AC catalytic subunits, reaction mixtures contained 3 nM C1, 15 nM C2, 51 nM GTP·S-activated Gα, and 1.0 μCi/tube [α-³²P]ATP, 0.1 mM cAMP, 100 mM KCl, and 25 mM HEPES/NaOH, pH 7.4. Reactions were conducted for 20 min at 30°C and were terminated by adding 20 μl of 2.2 N HCl (Mou et al., 2006).

Molecular Modeling. The crystal structure 1CJK of mammalian AC subunits C1 and C2 was chosen as a template for modeling the dynamics of BODIPY-FS interaction with the enzyme (Tesmer et al., 1999). The structure was edited in SYBYL (The Tripos Associates, St. Louis, MO) as described previously (Tesmer et al., 1999). The structure was edited in SYBYL (The Tripos Associates, St. Louis, MO) as described previously (Tesmer et al., 1999).
According to an assumed pH of 7.2 (aspartate and glutamate residues left as anionic and lysine and arginine residues specified as cationic). The complex structure was dynamically equilibrated in SYBYL for 40 ps using the Tripos molecular force field (Clark et al., 1989), Gasteiger-Marsili charges (Gasteiger and Marsili, 1980), a nonbonding distance cutoff of 8.0 Å, a background dielectric constant of 78.2,

Fig. 1. Structures of FS and FS analogs. A, FS; B, 1,9dd-FS; C, 1d-FS; D, 9-d-FS; E, 7DA-FS; F, 6A7DA-FS; G, 7DA1,9dd-FS; H, 7DA1d-FS; I, DMB-FS; J, BODIPY-FS.
and initial atomic velocities as assigned according to a Boltzmann distribution suitable to 298.15K. The resulting relaxed structure was then subjected to a 100-ps analysis run under the same conditions, with the final velocities of the equilibration run taken as initial velocities for the analysis run. The resulting trajectory was analyzed visually in SYBYL to qualitatively assess the conformational stability of the bound BODIPY-FS ligand.

**Statistics.** Statistical comparisons in Tables 1 and 2 were performed using the Student’s t test. Differences were considered as statistically significant with $p < 0.05$ (*) and $p < 0.01$ (**).

**Results**

**Expression and Activation of ACs 1, 2, and 5 in SF9 Insect Cells.** SF9 cells express an as-yet unidentified membrane AC (Tang et al., 1991; Seifert et al., 1998; Gille et al., 2004). Thus, when using SF9 cells as an expression system for mammalian ACs, we had to ensure that the activities in membranes from cells infected with AC-encoding baculoviruses were well above the endogenous AC activity of the insect cells. In agreement with previous data (Tang et al., 1991), infection of SF9 cells with a baculovirus not encoding a mammalian AC, in our case a wild-type virus encoding no recombinant mammalian protein at all, reduced maximal AC activities in SF9 membranes under various experimental conditions by more than 50% (Fig. 2). Thus, the AC activity in membranes from wild-type virus-infected cells constitutes the true background of endogenous enzyme activity for analysis of mammalian ACs. Infection of SF9 cells with baculoviruses encoding AC 1, 2, or 5 resulted in basal AC activities 7 to 25-fold higher than in membranes from wild-type virus-infected cells. The FS-stimulated activities were up to 12-fold higher in membranes from AC virus-infected cells than in membranes from wild-type virus-infected cells. Thus, SF9 cells are a sensitive expression system for analyzing the effects of FS analogs on mammalian ACs. It should be noted that the FS-stimulated activities were similar for ACs 1, 2, and 5, pointing to similar AC expression levels, whereas ACs 1 and 2 exhibited higher basal activities than AC5. High basal activity of AC2 had also already been noted in a previous study (Pieroni et al., 1995).

In membranes from uninfected SF9 cells, the direct G-protein activator GTP\(_\gamma S\) substantially enhanced AC activity. This stimulation is explained by an activation of the endogenous G\(_\alpha\)-like G-protein of the insect cells (Seifert et al., 1998). The addition of GTP\(_\gamma S\) to FS-containing tubes resulted in an additive stimulation of AC in membranes from uninfected SF9 cells and wild-type virus-infected cells. The stimulatory effects of GTP\(_\gamma S\) in

**TABLE 1**

Potencies and efficacies of FS and FS analogs for activation/inhibition of AC isoforms

<table>
<thead>
<tr>
<th>Diterpene</th>
<th>Control</th>
<th>AC1</th>
<th>AC2</th>
<th>AC5</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>12.0 ± 2.8*</td>
<td>846 ± 6.4</td>
<td>66.0 ± 0.7*</td>
<td>100</td>
</tr>
<tr>
<td>DMB-FS</td>
<td>3.5 ± 4.0**</td>
<td>812 ± 9.2</td>
<td>89.0 ± 1.6*</td>
<td>100</td>
</tr>
<tr>
<td>7DA-FS</td>
<td>5.3 ± 2.0</td>
<td>85.5 ± 10.8</td>
<td>83.5 ± 6.1*</td>
<td>100</td>
</tr>
<tr>
<td>9d-FS</td>
<td>92.5 ± 12.1**</td>
<td>72.0 ± 6.2**</td>
<td>83.5 ± 9.2</td>
<td>100</td>
</tr>
<tr>
<td>1d-FS</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
</tr>
<tr>
<td>19d-FS</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
</tr>
<tr>
<td>7DA1,9d-FS</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
</tr>
<tr>
<td>7DAl-FS</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
<td>Ineffective</td>
</tr>
<tr>
<td>BODIPY-FS</td>
<td>2.9 ± 0.2</td>
<td>50.6 ± 11.8**</td>
<td>62.0 ± 1.4**</td>
<td>100</td>
</tr>
</tbody>
</table>

**AC Activity (pmol/mg/min)**

**Basal**

- GTP\(_\gamma S\) 10 \(\mu M\)
- FS 300 \(\mu M\)
- GTp\(_\gamma S\) S 10 \(\mu M\) + FS 300 \(\mu M\)

**Fig. 2.** Analysis of AC activities in membranes from uninfected SF9 cells and membranes from wild-type baculovirus-, AC1-, AC2-, and AC5 virus-infected SF9 cells. AC activity was determined as described under Materials and Methods. Each assay tube contained membranes (30 \(\mu g\) protein/tube), [\(^{35}\)P]ATP (1.0–1.5 \(\mu C/tube\)), 40 \(\mu M\) unlabeled ATP, Mn\(^{2+}\) (10 \(mM\)) and 3% (v/v) DMSO (basal), GTP\(_\gamma S\) (10 \(\mu M\)), FS (300 \(\mu M\)), or GTP\(_\gamma S\) (10 \(\mu M\)) plus FS (300 \(\mu M\)). Data shown are the mean values ± S.D. of three experiments performed in duplicate with four membrane preparations. wt-virus, wild-type baculovirus.
membranes expressing ACs 1, 2, and 5 were very small. Moreover, GTP\textsubscript{S} did not enhance or only minimally enhanced the stimulatory effect of FS in membranes expressing ACs 1, 2, and 5. The small or absent stimulatory effects of GTP\textsubscript{S} in membranes expressing ACs 1, 2, and 5 are probably due to an intrinsically low expression of the G\textsubscript{w} like G-protein of the insect cells (Seifert et al., 1998) that is further decreased by the infection per se. However, to ensure optimal conditions for AC activation in all types of membranes studied, we routinely included GTP\textsubscript{S} (10 \textmu M) in all further experiments.

**Effects of FS and FS Analogs on Catalytic Activity of Membrane ACs.** Figure 3 shows representative concentration/response curves for the effects of FS and FS analogs on the activity of insect cell AC, AC1, AC2, and AC5, and Fig. 4 shows a comparison of the effects of FS and BODIPY-FS on catalytic activity. Table 1 provides a summary of the effects of diterpenes on ACs.

Diterpenes activated insect cell AC in the order of potency FS > BODIPY-FS > 6A7DA-FS > DMB-FS > 7DA-FS > 9d-FS. With the exception of AC2, all 1d-FS derivatives (1d-FS, 1,9dd-FS, 7DA1,9dd-FS, and 7DA1d-FS) exhibited neither stimulatory nor inhibitory effects on ACs. At insect cell AC, the order of efficacy of diterpenes was FS > 6A7DA-FS > DMB-FS > 7DA-FS > 9d-FS > BODIPY-FS. In general, among all ACs studied, diterpenes exhibited the highest potencies at AC1. The order of potency at AC1 was BODIPY-FS ~ FS ~ 6A7DA-FS ~ DMB-FS ~ 7DA-FS ~ 9d-FS. The order of efficacy at AC1 was FS > 7DA-FS ~ 6A7DA-FS ~ 9d-FS > DMB-FS > BODIPY-FS.

The pharmacological profile of AC2 differed considerably from the profiles of the other ACs. Specifically, with two exceptions (DMB-FS and BODIPY-FS), the potencies of diterpenes at AC2 were lower than at the other ACs. For example, FS activated AC2 12-fold less potently than AC1. The order of potency of stimulatory diterpenes at AC2 was FS > 6A7DA-FS > DMB-FS > 7DA-FS > 9d-FS, and the order of efficacy was FS > 7DA-FS > 9d-FS > 6A7DA-FS > DMB-FS. 1d-FS, 7DA1,9dd-FS, and BODIPY-FS exhibited inhibitory effects on basal activity in membranes expressing AC2. Among these compounds, BODIPY-FS was the most potent and efficacious compound.

We also addressed the question of whether the inhibitory effect of BODIPY-FS depends on the expression level and basal activity of AC2. To address this question, we infected Sf9 cells with various titers of AC2 virus (Table 2). In fact, with decreasing virus titer, basal activity in AC2-expressing membranes decreased. However, the relative inhibitory effect of BODIPY-FS did not decrease with decreasing virus titer. The effect of BODIPY-FS did not decrease with decreasing virus titer, but the catalytic activity of membranes decreased. However, the relative inhibitory effect of BODIPY-FS depended on the expression level and basal activity of AC2. Among these compounds, BODIPY-FS was the most potent and efficacious compound.

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### Table 2

<table>
<thead>
<tr>
<th>Virus Titer</th>
<th>AC Activity (Basal)</th>
<th>AC Activity (BODIPY-FS)</th>
<th>Relative Inhibitory Effect of BODIPY-FS</th>
<th>EC\textsubscript{50}</th>
<th>%</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>44.1 ± 2.6</td>
<td>27.7 ± 0.9</td>
<td>−37.2 ± 1.7</td>
<td>1.1 ± 0.5</td>
<td>40</td>
<td>0.1</td>
</tr>
<tr>
<td>1:100</td>
<td>40.1 ± 3.9</td>
<td>23.1 ± 2.1</td>
<td>−42.4 ± 2.9</td>
<td>0.6 ± 0.4</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>1:300</td>
<td>32.5 ± 3.1</td>
<td>20.7 ± 1.3</td>
<td>−36.3 ± 1.9</td>
<td>0.5 ± 0.2</td>
<td>2.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

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We also addressed the question of whether the inhibitory effect of BODIPY-FS depends on the expression level and basal activity of AC2. To address this question, we infected Sf9 cells with various titers of AC2 virus (Table 2). In fact, with decreasing virus titer, basal activity in AC2-expressing membranes decreased. However, the relative inhibitory effect of BODIPY-FS did not decrease with decreasing virus titer.

### Fig. 3

Effects of FS and FS analogs on the catalytic activity of membrane ACs. AC activity was determined as described under Materials and Methods. Each assay tube contained a specific membrane (30 \mu g protein/tube), [\alpha-\textsuperscript{32}P]ATP (1.0–1.5 \mu Ci/tube), 40 \mu M unlabeled ATP, Mn\textsuperscript{2+} (10 mM) and GTP\textsubscript{S} (10 \mu M), 3% (v/v) DMSO, and increasing concentrations (10 nM–30 \mu M) of BODIPY-FS. Data were analyzed by nonlinear regression and best fitted to sigmoidal concentration/response curves. Data shown are mean values ± S.D. of three independent experiments in duplicate.
titer. In addition, the potency of BODIPY-FS remained unchanged over the range of virus titers studied. We tried to express AC2 even at lower levels than those shown in Table 2, but those efforts failed. It is, unfortunately, a well known weakness of the Sf9 insect cell expression system that it does not allow for reliable control recombinant protein expression (Gille and Seifert, 2003).

At AC5, diterpenes stimulated catalysis in the order of potency BODIPY-FS > H11022 6A7DA-FS > DMB-FS > 7DA-FS, and the order of efficacy was FS > 6A7DA-FS > DMB-FS > 9d-FS > TDA-FS > BODIPY-FS.

Inhibition of the Stimulatory Effects of FS on AC Catalysis by Various FS Analogs. Figure 5 shows concentration/response curves for FS at insect cell AC and ACs 1, 2, and 5 in the absence and presence of 1,9dd-FS, 1d-FS, and 7DA1,9dd-FS in a fixed concentration (100 nM). The 1d-FS derivatives shifted the concentration/response curves to the right, but they also reduced the maximum stimulatory effect.

Fig. 4. Effects of FS and BODIPY-FS on the catalytic activity of membranous ACs. AC activity was determined as described under Materials and Methods. Each assay tube contained a specific membrane (30 μg protein/tube), [α-32P]ATP (1.0–1.5 μCi/tube), 40 μM unlabeled ATP, Mn2+ (10 mM) and GTPyS (10 μM), 3% (v/v) DMSO, and increasing concentrations (10 nM–300 μM) of FS or BODIPY-FS as indicated on the abscissa. Shown are saturation curves of the relative stimulatory effects of FS (■) and the relative stimulatory or inhibitory effects of BODIPY-FS (□) on the catalytic activity of insect cell (A), AC1 (B), AC2 (C), and AC5 (D). Data were analyzed by nonlinear regression and best fitted to sigmoidal concentration/response curves. The AC activities stimulated by FS plus GTPyS in Sf9 membranes expressing ACs 1, 2, and 5 and uninfected membranes were 143 ± 0.9, 155 ± 4.1, 169 ± 5.7, and 42.1 ± 2.2 pmol/mg/min, respectively. Basal AC activities were 39.9 ± 1.7, 43.1 ± 3.5, 10.4 ± 0.4, and 2.1 ± 0.3 pmol/mg/min, respectively. Data shown are mean values ± S.D. of two independent experiments of one membrane preparation performed in duplicate. Similar results were obtained with four different membrane preparations.

Fig. 5. Inhibition of the stimulatory effects of FS on AC catalysis by various FS analogs. AC activity was determined as described under Materials and Methods. Each assay tube contained a specific membrane (30 μg protein/tube), [α-32P]ATP (1.0–1.5 μCi/tube), 40 μM unlabeled ATP, Mn2+ (10 mM) and GTPyS (10 μM), 3% (v/v) DMSO, and increasing concentrations (300 nM–300 μM) of FS in the presence of a single fixed concentration (100 μM each) of various other diterpenes. A, insect cell AC; B, AC1; C, AC2; D, AC5. ■, FS alone; ▲, FS + 1,9dd-FS (100 μM); ●, FS + 1d-FS (100 μM); ○, FS + 7DA1,9dd-FS (100 μM). Relative stimulatory effects (efficacies) were determined by dividing the fitted maximal stimulation obtained for each experimental condition by the maximum stimulation obtained for each individual membranous ACs with 300 μM FS and GTPyS (10 μM). The AC activities stimulated by FS plus GTPyS in Sf9 membranes expressing ACs 1, 2, and 5 and uninfected membranes were 138 ± 0.5, 155 ± 7.9, 171 ± 1.8, and 44.8 ± 1.1 pmol/min, respectively. Basal AC activities were 39.0 ± 3.1, 41.2 ± 3.4, 10.5 ± 0.3, and 2.3 ± 0.3 pmol/min, respectively. Data were analyzed by nonlinear regression and best fitted to sigmoidal concentration/response curves. Data shown are mean values ± S.D. of two independent experiments of two membrane preparations performed in duplicate.
of FS by 40 to 70%, i.e., 1d-FS analogs displayed mixed competitive/noncompetitive antagonism.

**Interaction of C1/C2 with FS and BODIPY-FS.** BODIPY-FS exhibits very different pharmacological properties, depending on the specific AC isoform studied (Fig. 4; Table 1). Because purified AC catalytic subunits C1/C2 have been successfully used as a model system for examining ligand/enzyme interactions by catalysis studies and molecular modeling (Mou et al., 2006), we assessed the interaction of C1/C2 with FS and BODIPY-FS. As a control, we also studied the free dye, BODIPY. Basal catalysis with C1/C2 was very low, but FS effectively stimulated the enzyme with an EC50 of 9.1 ± 2.3 μM (Fig. 6A). BODIPY-FS acted as a very weak partial agonist (efficacy > 5% of that of FS) (Fig. 6A), but due to the very low basal activity in the system and the large stimulatory effect of FS, the small stimulatory effect of BODIPY-FS could also be clearly distinguished (Fig. 6B). BODIPY-FS activated catalysis with a potency of 2.5 ± 0.3 μM, i.e., BODIPY-FS was more potent than FS. The free BODIPY dye was ineffective, indicating that the link between the FS- and BODIPY moieties was important for the pharmacological activities of BODIPY-FS. As was predicted from the weak partial agonism of BODIPY-FS, the FS analog acted as a strong partial agonist (IC50, 0.8 ± 0.3 μM) to inhibit FS-stimulated catalysis (Fig. 6C). The IC50 of BODIPY-FS for partial antagonism (Fig. 6C) fits well to its EC50 for partial agonism (Fig. 6B). The BODIPY dye itself did not inhibit FS-stimulated catalysis (Fig. 6C).

**Molecular Modeling of the Interaction of C1/C2 with BODIPY-FS.** Our 100-ps dynamic simulation of BODIPY-FS bound to C1/C2 showed that the FS moiety of this ligand remained very firmly bound to the native FS-binding site, in that FS atom positions in the final conformer resolved in the simulation vary by less than 0.5 Å root-mean-squared deviation relative to those present in the original crystal structure (Fig. 7). However, the BODIPY moiety was found to sample promiscuously its degrees of freedom, transiting a more than 150° arc during the course of a 60-ps time space during which time it experienced brief coupling with a broad range of receptor residues. In view of the fact that BODIPY-FS is a similarly potent ligand as, or even more potent ligand than, FS (Figs. 4 and 5; Table 1), our AC activity results are compatible with the assumption that the interaction of the FS moiety with the receptor is intact and that promiscuous sampling of the BODIPY moiety with various receptor residues contributes positively to ligand potency.

**Discussion**

Crystallographic studies showed that diterpenes bind to a hydrophobic cleft formed by the C1 and C2 catalytic subunits of AC opposite to the catalytic site (Tesmer et al., 1997; Tang and Hurley, 1998). In addition to hydrophobic interactions, diterpenes form hydrogen bonds with ACs, namely between the C7-OH group and the backbone oxygen of Val506 (AC5 numbering), the C7-acetyl group and Ser942 (AC2 numbering), as well as the C11-OH group and Ser508 (AC5 numbering). The amino acids interacting with FS are highly conserved among the FS-sensitive AC isoforms 1 to 8 (Tang and Hurley, 1998). Thus, it was not unexpected that in all ACs studied, deletion of the C7-acetyl group resulted in a decrease in potency and, to a lesser extent, in a decrease of efficacy of diterpenes (Table 1). The hydrogen bond between the C7-OH group and Val506 is crucial for AC activation because its deletion, alone or in combination with other structural modifications, resulted in ineffective FS analogs devoid of stimulatory effects.

The reduced potency and efficacy of DMB-FS relative to FS at all ACs (Table 1) suggests that the DMB substituent, in contrast to the BODIPY substituent (Fig. 7), exhibits unfavorable interactions with the enzyme. DMB-FS was introduced as water-soluble FS analog to facilitate experiments with intact cells (Laurenza et al., 1987), but given its pharmacological properties, DMB-FS is not a true substitute for FS.

BODIPY-FS was introduced as a fluorescent probe for localization of ACs in intact cells (Liu et al., 1998; Takahashi et al., 2002), but its functional interaction with AC has not yet been examined. In BODIPY-FS, the small C7-acetyl group is substituted by the large BODIPY group connected to C7 of the diterpene ring through a long linker (Fig. 1, A and J). The affinity of BODIPY-FS for various ACs including C1/C2 is similar to, or even higher than, that of FS (Table 1; Fig. 6). Molecular modeling revealed that the BODIPY group faces...
outside of the FS site and promiscuously interacts with several amino acids surrounding this area (Fig. 7). These interactions probably contribute to the high ligand affinity for ACs. Given the differential effects of BODIPY-FS on various ACs, it is also possible that these promiscuous interactions are different for various ACs. The fortuitous identification of BODIPY-FS as a potent FS analog opens novel opportunities for ligand design, be it fluorescent or nonfluorescent ligands. It is unusual that a fluorescent compound is a lead structure for ligand design, be it fluorescent or nonfluorescent ligands. BODIPY-FS as a potent FS analog opens novel opportunities for ligand design.

Considering the highly conserved diterpene-binding site in ACs (Tang and Hurley, 1998), it was unexpected that various ACs responded differently to the prototypical diterpene, FS, i.e., FS activated AC1 up to 12-fold more potently than ACs 2 and 5 (Table 1). These differences were unmasked because the use of a sufficiently high concentration of organic solvent [3% (v/v) DMSO], allowing effective solubilization of diterpenes. In addition, we used Mn$^{2+}$ and GTP$\gamma$S to fully activate AC (Sunahara et al., 1997; Gille et al., 2004). Together, these experimental conditions facilitated generation of saturated concentration/response curves for several diterpenes (Figs. 3–6). A previous study did not reveal differences in FS potency between AC2 and AC5 (Onda et al., 2001). It is noteworthy that Onda et al. (2001) did not obtain saturated concentration/response curves. This could be due to the use of Mg$^{2+}$ in their study and/or insufficient diterpene solubilization because the solvent conditions were not provided.

Our data suggest that amino acids other than those immediately in contact with the diterpene have an impact on diterpene affinity. In fact, there are significant structural differences between ACs in the transmembrane domains (Sunahara et al., 1996; Tang and Hurley, 1998) that may influence diterpene potency indirectly by modulating the relative mobility of the cytosolic catalytic sites. An approach to further address this issue is the construction and expression of chimeric ACs in which the transmembrane domains are exchanged.

In agreement with previous data (Laurenza et al., 1989), 1d-FS derivatives did not activate AC (Table 1). Based on $[^{3}H]$FS-binding studies, it was assumed that 1d-FS derivatives do not bind to AC (Seamon et al., 1984). However, the inhibitory effects of 1d-FS and 7DA1,9dd-FS on basal AC2 activity (Fig. 3C; Table 1) indicate that this is not true. These findings prompted us to explore the hypothesis that 1d-FS derivatives inhibit the stimulatory effects of FS on catalysis. In fact, 1d-FS, 1,9dd-FS, and 7DA1,9dd-FS inhibited FS-stimulated catalysis at all four ACs studied in a mixed competitive/noncompetitive manner (Fig. 5). These data clearly show that in contrast to previously held view (Laurenza et al., 1989), 1d-FS derivatives bind to ACs. An explanation for this apparent discrepancy is that previous $[^{3}H]$FS-binding studies with cell membranes actually did not monitor AC but rather more abundant FS-binding proteins such as ion channels and glucose transporters (Laurenza et al., 1989). This explanation is supported by the high-affinity $[^{3}H]$FS binding and the low-affinity FS-activation of AC (Table 1; Fig. 6) (Seamon et al., 1984). It is intriguing that C. forskohlii extracts that are popular over-the-counter drugs for weight reduction do not only contain FS but also 1d-FS derivatives (Ding and Staudinger, 2005). Thus, antagonism between FS and 1d-FS derivatives with respect to AC activation may actually annihilate the desired weight-reducing effect (Fig. 5) but not the undesired effects of diterpenes on hepatic enzyme induction, potentially resulting in drug interactions (Ding and Staudinger, 2005). This may also explain the paucity of controlled clinical studies demonstrating efficacy of C. forskohlii extracts in obesity.

Crystallographic studies identified a single FS-binding site in AC (Tesmer et al., 1997; Tang and Hurley, 1998). The mixed competitive/noncompetitive interaction between FS and 1d-FS derivatives is explained by the fact that those ligands are very lipophilic, impairing free ligand exchange at the FS site. Difficulties to obtain equilibrium conditions at the FS-binding site were also observed for purified catalytic AC subunits (Dessauer et al., 1997). Thus, the mixed competitive/noncompetitive interaction between FS and 1d-FS derivatives may reflect hemiequilibrium conditions (Kenakin et al., 2006) rather than the existence of a second FS-binding site.

AC2 exhibited distinct pharmacological properties. First, and in agreement with Pieroni et al. (1995), AC2 displayed...
the highest basal activity (Fig. 2). Second, except for DMB-FS and BODIPY-FS, AC2 exhibited the lowest affinity for diterpenes (Table 1). Third, and most remarkably, several 1d-FS derivatives and BODIPY-FS exhibited prominent inhibitory effects on AC2 that were not observed with another AC isoform. Thus, by analogy to the two-state model of receptor activation (Seifert and Wenzel-Seifert, 2002), AC2 could be considered as a constitutively active “FS receptor,” with the FS site isomerizing between an inactive (R) state and an active (R*) state. FS stabilizes the R* state, increasing catalysis, whereas 1d-FS derivatives and BODIPY-FS stabilize the R state and decrease the high basal catalysis rate of AC2. These findings also raise the question whether the elusive endogenous ligand for the FS site (Laurenza et al., 1989; Putnam et al., 2007) is an inverse agonist.

Although the two-state model can explain the inhibitory effects of certain diterpenes on basal AC2 activity, this model is insufficient at explaining all pharmacological effects of diterpenes. In particular, AC1 exhibited only a slightly lower basal catalysis rate than AC2, but at this AC, the most effective AC2 inverse agonist, BODIPY-FS, was actually a rather strong partial agonist and not an inverse agonist (Fig. 5; Table 1). At C1/C2, BODIPY-FS behaved as a strong partial agonist (Fig. 6). Thus, BODIPY-FS is reminiscent of a protein receptor ligand (Kenakin, 2001). In addition, the two-state model predicts that receptors with high constitutive activity exhibit increased potency and efficacy of partial agonists relative to a receptor with low constitutive activity (Seifert and Wenzel-Seifert, 2002). The two-state model also predicts that with increasing receptor expression level, the abundance of the active (R*) state increases, increasing basal effector activity and the efficacy of inverse agonists. Indeed, with increasing AC2 virus titer during the infection, the corresponding basal AC activities increased (Table 2). However, there was no increase in the efficacy of BODIPY-FS as a result of increased virus titer, expression, and activity. Moreover, a comparison of the potencies and efficacies of partial FS agonists at AC2, exhibiting high basal activity, and AC5, exhibiting rather low basal activity (Fig. 2), clearly shows that this prediction of the two-state model is not fulﬁlled. Thus, a multistate model in which a given diterpene stabilizes a ligand-specific conformation in each AC isoform with distinct catalysis-activating or -inhibiting properties is more appropriate to conceptualize our experimental findings (Kenakin, 2001, 2002; Seifert and Wenzel-Seifert, 2002; Kobila, 2007).

Finally, the exceptionally high potencies of diterpenes for AC1 activation may be of clinical relevance. In particular, AC1 is the major AC isoform in the brain and plays an important role in memory and learning (Hanoune and Defer, 2001). It is intriguing that the expression of AC1 is decreased in Alzheimer’s disease (Yamamoto et al., 2000). In a mouse model, an increase in cAMP levels through phosphodiesterase inhibition improves memory (Bach et al., 1999). Thus, it is conceivable that selective AC1 activators could be valuable drugs for the treatment of Alzheimer’s disease.

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References


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