Regulation of Forskolin Interactions with Type I, II, V, and VI Adenylyl Cyclases by $G_{s\alpha}$†

Elizabeth McHugh Sutkowski,‡ Wei-Jen Tang,§ Christopher W. Broome,‡ Joan D. Robbins,‡ and Kenneth B. Seamon*,‡

Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike (HFM-505), Rockville, Maryland 20852, and Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Received March 16, 1994; Revised Manuscript Received June 16, 1994®

ABSTRACT: Several forms of adenylyl cyclase (types I, II, V, and VI) have been expressed using the recombinant baculovirus expression system in Sf9 cells. The activation of type I adenylyl cyclase by forskolin and $G_{s\alpha}$ was not greater than additive. In contrast, there was synergistic activation of type II, V, and VI adenylyl cyclases by $G_{s\alpha}$ and forskolin. $G_{s\alpha}$ potentiated the effect of forskolin on type II adenylyl cyclase to the greatest extent. Type I and II adenylyl cyclases were photolabeled specifically by an iodinated photoaffinity derivative of forskolin ([^{125}I]-6-AIPP-Fsk). Type I adenylyl cylcase was photolabeled efficiently in the absence of $G_{s\alpha}$, and the addition of $G_{s\alpha}$ only slightly increased the labeling efficiency. In contrast, type II adenylyl cyclase was not photolabeled efficiently in the absence of $G_{s\alpha}$, and the addition of $G_{s\alpha}$ greatly enhanced the labeling efficiency. Potolabeling of type V and VI adenylyl cyclases was detected only in the presence of $G_{s\alpha}$. Neither calcium/calmodulin nor G protein $\beta\gamma$ subunits modulated the photolabeling of type I or II adenylyl cyclases. Another iodinated derivative of forskolin, [^{125}I]-6-IHPP-Fsk, bound to Sf9 cell membranes expressing type I adenylyl cyclase with high affinity in a filtration binding assay, and the specific binding was not enhanced by the addition of $G_{s\alpha}$. In contrast, specific binding of [^{125}I]-6-IHPP-Fsk to membranes expressing type II adenylyl cyclase was detected only in the presence of $G_{s\alpha}$. [^{125}I]-6-IHPP-Fsk bound to membranes expressing type I adenylyl cyclase with a K_d of 8 nM and a B_{max} of 6.4 pmol/mg protein. The K_d for binding of [^{125}]-6-IHPP-Fsk to membranes expressing type II adenylyl cyclase in the presence of $G_{s\alpha}$ was 134 nM, and the B_{max} was 11.2 pmol/mg protein. Deoxy and desacetyl derivatives of forskolin displaced the binding of [^{125}I]-6-IHPP-Fsk to membranes expressing type I and II adenylyl cyclases with a similar rank order of potency. However, the affinity of these derivatives for the type I enzyme was between 6- and 20-fold higher than that for the type II enzyme.

Forskolin, a diterpene natural product, interacts with adenylyl cyclase in a variety of different tissues to cause an increase in intracellular cyclic AMP (Seamon & Daly, 1986). At concentrations of 10 nM to 0.1 μM, forskolin potentiates the hormone activation of adenylyl cyclase (Seamon & Daly, 1986). Forskolin activates adenylyl cyclase in the absence of G_s protein[1] (Seamon & Daly, 1981); however, maximal activation of adenylyl cyclase by forskolin requires the presence of a functional G_s protein (Green & Clark, 1982; Bender & Neer, 1983). High-affinity binding sites for forskolin have been detected by a binding assay using [^{3}H]forskolin in membranes from human platelets and rat brain, which have characteristics that associate them with adenylyl cyclase (Seamon et al., 1984; Nelson & Seamon, 1986). The number of [^{3}H]forskolin binding sites is increased under conditions that promote activation of adenylyl cyclase by the G_s protein (Nelson & Seamon, 1985). Therefore, it was proposed that the high-affinity binding sites for forskolin might correspond to a ternary complex of forskolin, the catalytic subunit, and the G_s protein.

Although forskolin elicits large increases in intracellular cyclic AMP in almost all mammalian tissues and cells, the potency and efficacy of forskolin with respect to increasing cyclic AMP levels vary among tissues. The ability of forskolin to activate adenylyl cylcase in cells and tissues can be markedly affected by the presence and functional state of the G_i and G_s proteins. Additive and synergistic activations of adenylyl cyclase by guanine nucleotides and forskolin, as well as hormones and forskolin, occur in some but not all preparations. Forskolin binding sites detected in liver, lung, and heart membranes have much lower affinity for forskolin than the high-affinity binding sites detected in brain and platelets (Laurenza & Seamon, 1991). The differences among various tissues with respect to increasing cyclic AMP levels, the synergistic activation of adenylyl cyclase by forskolin and guanine nucleotides, and the affinity of forskolin binding sites (brain vs peripheral tissues) suggested that there might be differences in the adenylyl cyclase catalytic subunit or the amounts of G_s protein.

Biochemical and immunological studies originally suggested adenylyl cyclase activities that were regulated differentially

† This work was supported by a National Research Council Award, Grant-in-Aid 92G-078 from the American Heart Association, Texas Affiliate, National Institutes of Health Grant GM34497, American Cancer Society Grant BE30N, The Perot Family Foundation, The Lucille P. Markey Charitable Trust, and The Raymond and Ellen Willie Chair of Molecular Neuropharmacology.

* Author to whom correspondence should be addressed. Telephone, (301) 496-3556; Fax, (301) 402-0763.

‡ Food and Drug Administration.

§ University of Texas Southwestern Medical Center.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

[1] Abbreviations: G_s protein, guanine nucleotide-binding regulatory protein that stimulates adenylyl cyclase; G_i protein, guanine nucleotide-binding regulatory protein that inhibits adenylyl cyclase; $G_{s\alpha}$, the α subunit of the G_s protein; $rG_{s\alpha\text{-}s}$, the short form of $G_{s\alpha}$ espressed in *Escherichia coli*; GTPγS, guanosine 5′-*O*-3-thiotriphosphate; GTPγS–$rG_{s\alpha\text{-}s}$, the GTPγS-activated $rG_{s\alpha\text{-}s}$; [^{125}I]-6-AIPP-Fsk, [^{125}I]-2-[3-(4-azido-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin; [^{125}I]-6-IHPP-Fsk, [^{125}I]-2-[3-(4-hydroxy-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin; Fsk, forskolin; DDFsk, 1,9-dideoxyforskolin; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

0006-2960/94/0433-12852$04.50/0

by calcium/calmodulin and $G_{s\alpha}$ subunit association (Brostrom et al., 1977; Westcott et al., 1979; Coussen et al., 1986; Rosenberg & Storm, 1987; Mollner & Pfeuffer, 1988; Pfeuffer et al., 1989). Calmodulin-sensitive adenylyl cyclase was purified, and cDNA clones that encode this protein (type I) were isolated (Krupinski et al., 1989). Subsequently, several additional mammalian adenylyl cyclases have been cloned using probes based on the type I adenylyl cyclase sequence. Cloned adenylyl cyclases (types I–VIII) have each been expressed in eukaryotic expression systems (Bakalyar & Reed, 1990; Feinstein et al., 1991; Gao & Gilman, 1991; Ishikawa et al., 1992; Katsushika et al., 1992; Krupinski et al., 1989; Tang et al., 1991; Yoshimura & Cooper, 1992; Premont et al., 1992; Glatt & Snyder, 1993; Taussig et al., 1994; Cali et al., 1994). Studies of the individual recombinant forms of adenylyl cyclase demonstrate that the enzyme subtypes differ in their sensitivity to calcium/calmodulin, activated $G_{s\alpha}$ and $G_{i\alpha}$, the G protein $\beta\gamma$ subunit complex, and calcium (Krupinski et al., 1989; Bakalyar & Reed, 1990; Tang et al., 1991; Feinstein et al., 1991; Taussig et al., 1994; Tang & Gilman, 1991; Gao & Gilman, 1991; Ishikawa et al., 1992; Yoshimura & Cooper, 1992; Katsushika et al., 1992; Cali et al., 1994). Thus, the existence of multiple adenylyl cyclase enzymes may provide an explanation for the distinct modes of regulation of cyclic AMP levels in tissues.

The availability of membranes from cells expressing the individual types of adenylyl cyclase has allowed us to compare the interaction of forskolin with specific subtypes of adenylyl cyclase. Intermediates of forskolin derivatized at the 6- and 7-positions have been synthesized with free amino groups available for coupling to activated esters of radioactive ligands (Robbins et al., 1991). These intermediates have been used to synthesize radioactive iodohydroxyphenyl and (iodoaryl)-azido derivatives of forskolin that show selectivity for adenylyl cyclase (Robbins et al., 1991, 1992; Morris et al., 1991; Laurenza et al., 1992; Appel et al., 1992). In this paper, we have characterized the interactions of forskolin and derivatives of forskolin with recombinant adenylyl cyclases expressed in Sf9 cell membranes, as well as the regulation of the interactions by G_s protein subunits and calmodulin.

MATERIALS AND METHODS

Synthesis of Forskolin Derivatives. [^{125}I]-6-AIPP-Fsk and [^{125}I]-6-IHPP-Fsk were synthesized carrier-free (2200 Ci/mmol), as described previously (Morris et al., 1991; Robbins et al., 1991). The synthesis of 6-deoxyforskolin has also been described previously (Kosley & Spahl, 1991). 7-Desacetylforskolin and 9-deoxyforskolin were generously provided by Hoechst.

Construction of Recombinant Baculoviruses and Expression of Adenylyl Cyclases in Sf9 Cells. Adenylyl cyclases (types I, II, V, and VI) have been expressed using the recombinant baculovirus expression system. B-rACI and B-rACII were constructed and expressed as described previously (Tang et al., 1991; Feinstein et al., 1991; Tang & Gilman, 1991). B-rACV and B-rACVI were also constructed and expressed as described (Taussig et al., 1994), and the recombinant virus was selected as described (Kitts et al., 1990). Cells were lysed, and membranes were washed and resuspended in 20 mM Na Hepes (pH 8.0), 2 mM DTT, 1 mM EDTA, and 200 mM sucrose plus protease inhibitors: 22 mg/L each of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, 1-chloro-3-(tosylamido)-7-amino-2-heptanone, and phenylmethanesulfonyl fluoride, plus 3.2 mg/L each of leupeptin and lima bean trypsin inhibitor as described (Tang et al., 1991). The protein concentration of the Sf9 cell membranes was determined by dye binding (Bradford, 1976), using bovine serum albumin as the standard.

Preparation of Modulators of Adenylyl Cyclase. In all experiments, $G_{s\alpha}$ refers to GTPγS–$rG_{s\alpha\text{-}s}$, the GTPγS-activated short form of $G_{s\alpha}$ expressed in *Escherichia coli* (Graziano et al., 1989; Lee et al., 1994). The short form of $G_{s\alpha}$ ($rG_{s\alpha\text{-}s}$) was purified from *E. coli* BL21DE3 cells that harbored the pQE6/$rG_{s\alpha\text{-}s}$ as described (Lee et al., 1994); the protein was activated by incubating 20 μM $rG_{s\alpha\text{-}s}$ for 1 h at 30 °C in 20 mM Na Hepes (pH 8.0), 1 mM DTT, 5 mM $MgSO_4$, and 1 mM GTPγS. Unbound GTPγS was removed by gel filtration on a Sephadex G25 column equilibrated in 20 mM Na Hepes (pH 8.0), 1 mM DTT, and 5 mM $MgCl_2$. Drosophila calmodulin was purified from *E. coli* AR68 that carries pJFM 39 as described (Maune et al., 1992). G protein $\beta\gamma$ subunits were purified from bovine brain as described (Sternweis & Pang, 1990).

Adenylyl Cyclase Assay. Adenylyl cyclase activity was measured as described (Smigel, 1986). All assays were performed for 10 min at 30 °C in a final volume of 100 μL. The concentration of $MgCl_2$ was 10 mM. Membranes, $G_{s\alpha}$, and forskolin were incubated for 5 min at 30 °C in a total volume of 50 μL prior to initiation of the assay. The forskolin-stimulated enzymatic activities of the membranes expressing adenylyl cyclases measured in Figure 1 are representative of the enzymatic activities of the respective membranes used for all of the studies described in this paper.

Photoaffinity Labeling Procedure. To study specific photolabeling, Sf9 cell membranes (50 μg) were preincubated for 30 min on ice in the presence of TEM buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM $MgCl_2$), with or without 20 μM forskolin (Fsk) or 1,9-dideoxyforskolin (DDFsk) in a total volume of 100 μL. [^{125}I]-6-AIPP-Fsk ($(1–2) \times 10^6$ cpm, 3–6 nM) was added to the membranes, and they were incubated for an additional 30 min on ice in the dark. Immediately before photolysis, 0.9 mL of TEM buffer with or without 20 μM forskolin or 1,9-dideoxyforskolin was added, and the diluted samples were quickly photolyzed (energy = 200 000 μJ) in a Stratagene UV Stratalinker. Immediately following photolysis, 1% β-mercaptoethanol was added to each tube as a scavenger for any long-lived species. The membranes were pelleted by centrifugation at 12 000 rpm in a TOMY TMS-4 rotor for 15 min at 4 °C, washed with 10 mM Tris (pH 8.0) and 1 mM EDTA, repelleted, and prepared for electrophoresis as will be described.

To study the modulation of photolabeling of adenylyl cyclases, Sf9 cell membranes (50 μg) were preincubated for 10–30 min at 30 °C in the presence of the following reagents: A, TEM buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM $MgCl_2$]; B, A plus 40–80 nM $G_{s\alpha}$; C, A plus calmodulin (200 nM) and $CaCl_2$ (50 μM); D, combination of B and C; E, A plus G protein $\beta\gamma$ subunits (20 nM) and 0.025% Lubrol-PX; F, combination of B and E; G, combination of C and E. After the pretreatment, the membranes were photolabeled as described earlier. The proteins were precipitated from the samples containing G protein $\beta\gamma$ subunits and 0.025% Lubrol by adding 1/10 vol of 0.15% deoxycholate, incubating for 10 min on ice, and adding 1/10 vol of 72% trichloroacetic acid as described (Peterson, 1983). The proteins were pelleted by centrifugation at 12 000 rpm in a TOMY TMS-4 rotor for 15 min at 4 °C, washed with ice cold acetone, pelleted again for 5 min, and prepared for electrophoresis as will be described.

SDS–PAGE. Membranes from Sf9 cells were heated to 80 °C in the presence of 2% SDS and 0.2 mM DTT for 5 min

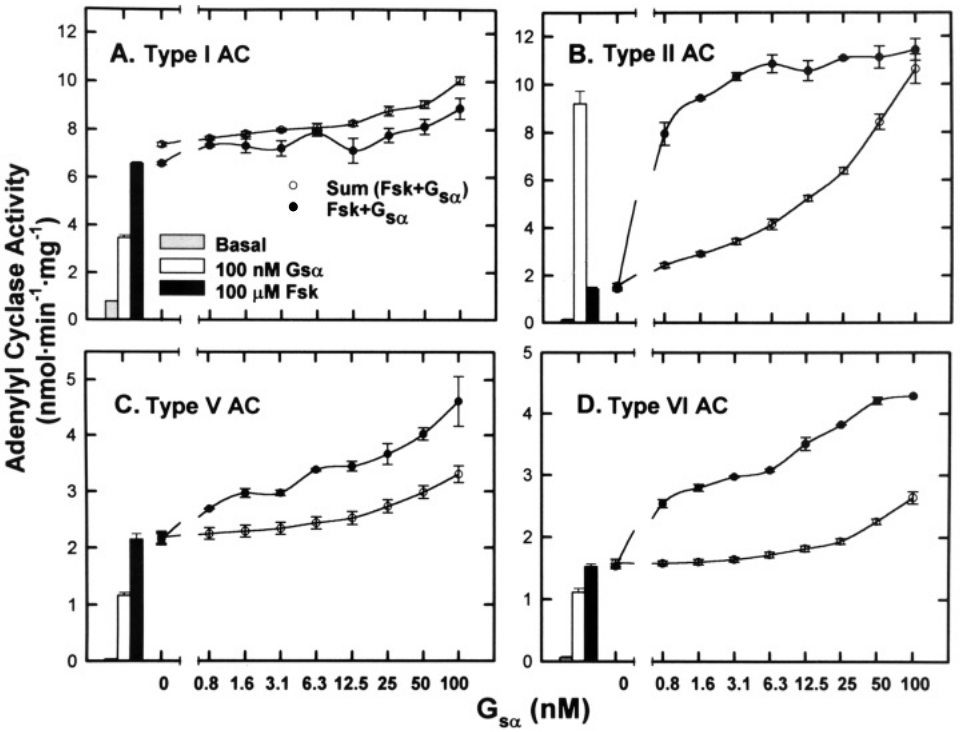

FIGURE 1: Effect of $G_{s\alpha}$ and forskolin on type I (A), II (B), V (C), and VI (D) adenylyl cyclases. Adenylyl cyclase activity was assayed in membranes (10 μg) prepared from Sf9 cells expressing type I, II, V, and VI adenylyl cyclases as described in Materials and Methods. In the bar graph portion of the figure, the membranes were assayed under basal conditions (dotted bars) or in the presence of 100 nM $G_{s\alpha}$ alone (open bars) or 100 μM forskolin alone (filled bars). In the line graph portion of the figure, the membranes were assayed in the presence of 100 μM forskolin and the indicated concentrations of $G_{s\alpha}$. The open circles are the sums of the adenylyl cyclase activities stimulated by forskolin alone and by $G_{s\alpha}$ alone. The filled circles indicate the adenylyl cyclase activity measured when both forskolin and $G_{s\alpha}$ were used in the same assay. Assays were performed in duplicate, and values are representative of the results from two separate experiments.

and then treated with 50 mM *N*-ethylmaleimide for 10 min at room temperature, prior to the addition of 4 × concentrated electrophoresis sample buffer described by Laemmli (1970). Samples were electrophoresed on 8% or 10% polyacrylamide minigels using the discontinuous buffer system described by Laemmli (1970). The ^{14}C-labeled molecular weight standards (GIBCO/BRL) were myosin (200 000), phosphorylase B (97 400), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), and β-lactoglobulin (18 400). The gels were Coomassie-stained, dried, and exposed to Kodak XAR 5 film. The incorporation of [^{125}I]-6-AIPP-Fsk was quantitated on a PhosphorImager (Molecular Dynamics).

Binding of [^{125}I]-6-IHPP-Fsk to Membranes. Binding experiments were carried out as described previously (Robbins et al., 1991; Laurenza et al., 1992). Bovine brain membranes (400 μg) were incubated for 60 min at room temperature with a constant amount (30 000 cpm, 20 pM) of [^{125}I]-6-IHPP-Fsk in the absence or presence of forskolin or forskolin analog in 12 × 75 mm glass test tubes in a total volume of 0.4 mL of 50 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$. Sf9 cell membranes were incubated under the same conditions, except that a smaller amount of membranes was added (40 μg of type I, 80 μg of type II) and the amount of [^{125}I]-6-IHPP-Fsk added was increased to 130 000 cpm (80 pM). To study the modulation of binding of [^{125}I]-6-IHPP-Fsk to Sf9 cell membranes, the membranes were preincubated for 10 min at 30 °C in the absence or presence of 40 nM $G_{s\alpha}$. The final concentration of $G_{s\alpha}$ during the incubation with forskolin or forskolin analog was 0.5–5 nM. Forskolin and forskolin analogs were tested at concentrations ranging from 0.06 nM to 10 μM. The assays were terminated by rapid filtration over Whatman GF/C filters using a Brandel cell harvester. The filters were washed three times with 4 mL of cold 50 mM Tris-HCl (pH 7.5) and counted in a γ counter. The Brandel harvester was rinsed between filter mats by the aspiration of 95% ethanol to minimize the carry-over of radioactivity. Each data point was determined in triplicate, and the average standard deviation was <10%. To determine the binding parameters, data from three separate experiments were analyzed together using the LIGAND program (MAC LIGAND V4.1), which was described previously (Munson & Rodbard, 1980).

RESULTS

Modulation of Forskolin Stimulation of Adenylyl Cyclase by $G_{s\alpha}$. Expression of recombinant adenylyl cyclases (types I, II, V, and VI) in Sf9 cells using recombinant baculovirus increases adenylyl cyclase activity in cell membranes approximately 20 times over that of control Sf9 cells (Tang et al., 1991; Feinstein et al., 1991; Taussig et al., 1993a, 1994). The distinct biochemical properties of the recombinant isoforms of adenylyl cyclase have been characterized individually. There is little effect of $G_{s\alpha}$ on the calmodulin-sensitive (type I) adenylyl cyclase in the presence of maximally effective concentrations of forskolin (Tang et al., 1991). In contrast, the calmodulin-insensitive (type II) adenylyl cyclase exhibits synergistic activation by $G_{s\alpha}$ and forskolin (Feinstein et al., 1991). Type V and VI adenylyl cyclases are insensitive to calmodulin, but are inhibited by the addition of calcium (Ishikawa et al., 1992; Yoshimura & Cooper, 1992; Katsushika et al., 1992).

The effect of the addition of exogenous $G_{s\alpha}$ on forskolin stimulation of multiple types of recombinant adenylyl cyclases (types I, II, V, and VI) expressed in Sf9 cells was compared in Figure 1. The activation of type I adenylyl cyclase by forskolin and $G_{s\alpha}$ was not greater than additive. In contrast, there was synergistic activation of type II, V, and VI adenylyl

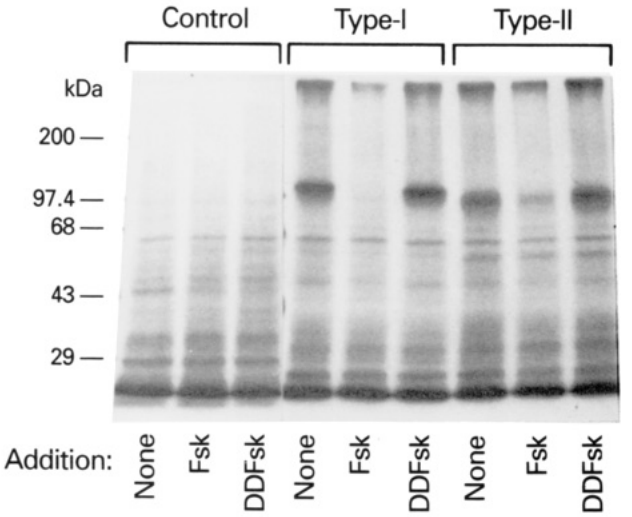

FIGURE 2: Forskolin photoaffinity labeling of control Sf9 cell membranes and Sf9 cell membranes expressing type I and II adenylyl cyclases. Membranes (50 μg) from control Sf9 cells or Sf9 cells expressing type I or II adenylyl cyclases were preincubated with either buffer with no addition (none), buffer with 20 μM forskolin (Fsk), or buffer with 20 μM 1,9-dideoxyforskolin (DDFsk) and then incubated with [^{125}I]-6-AIPP-Fsk and photolyzed as described in Materials and Methods. The samples were prepared for electrophoresis as described in Materials and Methods. Half of each control sample was run on an 8% polyacrylamide minigel, and half of each type I and type II sample was run on a 10% polyacrylamide minigel. The specific activities of the adenylyl cyclases in the membranes were in the same range as those in Figure 1.

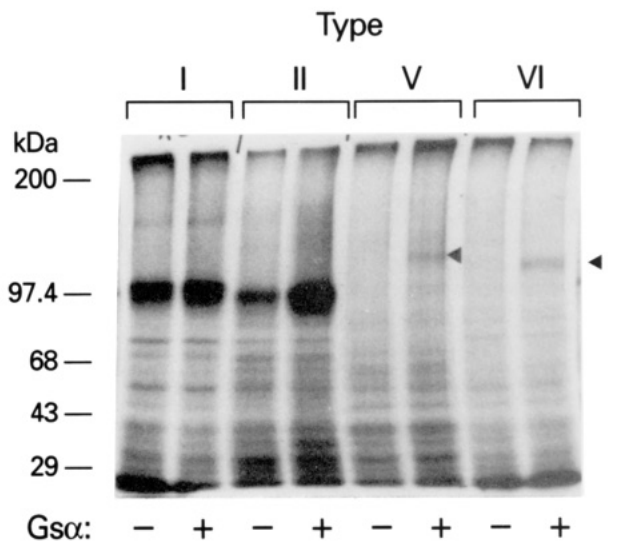

FIGURE 3: Effect of exogenous $G_{s\alpha}$ on the photoaffinity labeling of adenylyl cyclases (types I, II, V, and VI). Membranes (50 μg) from control Sf9 cells or Sf9 cells expressing type I, II, V, or VI adenylyl cyclases were preincubated for 30 min at 30 °C in the absence or presence of $G_{s\alpha}$ (80 nM) in buffer containing 10 mM $MgCl_2$. The samples were incubated with [^{125}I]-6-AIPP-Fsk and photolyzed as described in Materials and Methods. The samples were prepared for electrophoresis as described in Materials and Methods. Half of each sample was run on an 8% polyacrylamide minigel. The specific activities of the adenylyl cyclases in the membranes were in the same range as those in Figure 1.

cyclases by $G_{s\alpha}$ and forskolin, although the effect on type V adenylyl cyclase was modest. $G_{s\alpha}$ potentiated the effect of forskolin on type II adenylyl cyclase to the greatest extent.

Forskolin Photoaffinity Labeling of Adenylyl Cyclases Expressed in Sf9 Cells. A forskolin photoaffinity label, [^{125}I]-6-AIPP-Fsk, has been used to detect adenylyl cyclase in bovine brain membranes (Morris et al., 1991). The interaction of [^{125}I]-6-AIPP-Fsk with the recombinant adenylyl cyclases was studied by photolabeling membranes from Sf9 cells expressing type I, II, V, and VI adenylyl cyclases. Specific photolabeling was examined by preincubation of the membranes in buffer with no addition, 20 μM forskolin, or 20 μM 1,9-dideoxyforskolin, an inactive analog of forskolin, followed by incubation with the photolabel. After photolysis, the membrane proteins were separated by electrophoresis on SDS–polyacrylamide gels, and the labeled proteins were detected by autoradiography. [^{125}I]-6-AIPP-Fsk was incorporated into a protein with an apparent molecular weight of approximately 100 000 in membranes expressing type I and II adenylyl cyclases, and photolabeling of this protein was inhibited by forskolin but not 1,9-dideoxyforskolin (Figure 2). This result is consistent with the approximately 100 kDa protein being adenylyl cyclase. In membranes expressing type II adenylyl cyclase, there consistently was a small amount of photoincorporation into a 100 kDa protein that was not inhibited by 20 μM forskolin. Photolabeled type II adenylyl cyclase migrated slightly faster than photolabeled type I adenylyl cyclase. Similar differences in apparent molecular weight were observed with purified enzymes (Taussig et al., 1993b). In membranes expressing type V and VI adenylyl cyclases, no protein was photolabeled specifically (data not shown). In control Sf9 cell membranes, no photolabeled protein in the 100 000 molecular weight range was detected.

In a previous publication, the expression of type I adenylyl cyclase in Sf9 cell membranes was confirmed by immunoblotting experiments with subtype-specific affinity-purified antisera raised against the carboxyl-terminal residues of type I adenylyl cyclase (Tang et al., 1991). Similar experiments were performed with subtype-specific affinity-purified antisera raised against the carboxyl-terminal residues of type II adenylyl cyclase. The antiserum reacted specifically with a 100 kDa protein in Sf9 cell membranes expressing type II adenylyl cyclase (data not shown). Furthermore, the immunoreactive bands migrated to the same position as the photolabeled bands.

Modulation of Forskolin Photoaffinity Labeling of Adenylyl Cyclases by $G_{s\alpha}$. The modulation of photolabeling of adenylyl cyclases by $G_{s\alpha}$ was studied by preincubating membranes from Sf9 cells expressing the recombinant adenylyl cyclases (types I, II, V, and VI) in the absence and presence of 80 nM $G_{s\alpha}$ (Figure 3). Addition of exogenous $G_{s\alpha}$ caused only a very slight increase in photoincorporation into the type I enzyme. In contrast, exogenous $G_{s\alpha}$ greatly enhanced photoincorporation into type II adenylyl cyclase. After preincubation with $G_{s\alpha}$, proteins with apparent molecular weights of approximately 130 000 and 125 000 were photolabeled by [^{125}I]-6-AIPP-Fsk in membranes expressing type V and VI adenylyl cyclases, respectively (see arrowheads in Figure 3). Photolabeling of the indicated proteins was inhibited by forskolin but not 1,9-dideoxyforskolin (data not shown), consistent with these proteins being adenylyl cyclases. When control Sf9 cell membranes were preincubated with $G_{s\alpha}$, no difference in the labeling pattern was observed, indicating that neither the endogenous adenylyl cyclase nor $G_{s\alpha}$ itself was photolabeled (data not shown).

The addition of calcium (50 μM)/calmodulin (200 nM) alone or G protein $\beta\gamma$ subunits (20 nM) alone had no effect on the photoincorporation of [^{125}I]-6-AIPP-Fsk into type I or II adenylyl cyclases (data not shown). Also, there was no effect of calcium/calmodulin or $\beta\gamma$ subunits on the enhancement of photoincorporation into type I or II adenylyl cyclases observed in the presence of $G_{s\alpha}$.

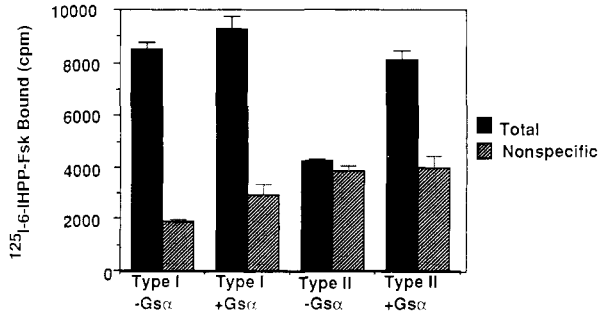

FIGURE 4: Modulation of binding of [^{125}I]-6-IHPP-Fsk to Sf9 cell membranes expressing type I and II adenylyl cyclases. Membranes from Sf9 cells expressing type I or II adenylyl cyclases were preincubated for 10 min at 30 °C in the presence or absence of $G_{s\alpha}$ (40 nM). Aliquots of the membranes (type I, 33 μg; type II, 74 μg) were then incubated for 60 min at room temperature with [^{125}I]-6-IHPP-Fsk (130 000 cpm, 80 pM) in the absence (total) or presence (nonspecific) of nonradioactive 6-IHPP-Fsk (type I, 1 μM; type II, 10 μM). The final concentration of $G_{s\alpha}$ during the incubation with forskolin or a forskolin analog as 1–3 nM. A greater amount of membranes expressing type II adenylyl cyclase was required to measure a range of total counts being bound similar to that in the membranes expressing type I adenylyl cyclase. The membranes were processed rapidly, as described in Materials and Methods, to separate bound from free ligand and counted. Each data point was determined in triplicate, and the average standard deviation was <10%. The data are representative of two different experiments. The specific activities of the adenylyl cyclases in the membranes were in the same range as those in Figure 1.

Modulation of Binding of [^{125}I]-6-IHPP-Fsk to Adenylyl Cyclases by $G_{s\alpha}$. [^{125}I]-6-IHPP-Fsk has been used as a specific high-affinity ligand to measure binding to adenylyl cyclase in bovine brain membranes (Robbins et al., 1991; Laurenza et al., 1992). The characteristics of binding of [^{125}I]-6-IHPP-Fsk and [^{3}H]forskolin to adenylyl cyclase in bovine brain membranes were shown to be identical according to a number of criteria (Laurenza et al., 1992). The binding of both ligands was modulated to the same extent by GTPγS and AlF_4^-. Total binding of [^{125}I]-6-IHPP-Fsk to type I and II adenylyl cyclases in Sf9 cell membranes was determined in the absence of nonradioactive 6-IHPP-Fsk, while nonspecific binding was determined in the presence of 10 μM nonradioactive 6-IHPP-Fsk (Figure 4). Specific binding of [^{125}I]-6-IHPP-Fsk was calculated as the difference between total binding and nonspecific binding. The total binding of [^{125}I]-6-IHPP-Fsk to membranes expressing type I adenylyl cyclase was greater than nonspecific binding in the absence of $G_{s\alpha}$; the specific binding was about 80% of the total binding. In contrast, there was very little specific binding of [^{125}I]-6-IHPP-Fsk to membranes expressing type II adenylyl cyclase in the absence of $G_{s\alpha}$. The effect of adding exogenous $G_{s\alpha}$ on the binding of [^{125}I]-6-IHPP-Fsk to type I and II adenylyl cyclases was examined in Figure 4. Exogenous $G_{s\alpha}$ caused a slight increase in both total and nonspecific binding to membranes expressing type I adenylyl cyclase. In contrast, $G_{s\alpha}$ greatly increased total binding to membranes expressing type II adenylyl cyclase, without increasing nonspecific binding.

The K_d for binding of [^{125}I]-6-IHPP-Fsk to type I adenylyl cyclase and $G_{s\alpha}$-activated type II adenylyl cyclase was determined by incubating the membranes expressing the adenylyl cyclases with a constant amount of [^{125}I]-6-IHPP-Fsk (80 pM) in the presence of increasing concentrations of nonradioactive 6-IHPP-Fsk (0.06 nM to 10 μM). To determine the binding parameters, data from three separate experiments were analyzed together as shown in Figure 5. The curves are computer-generated on the basis of the fitted parameters (type I in Figure 5A; type II + $G_{s\alpha}$ in Figure 5B).

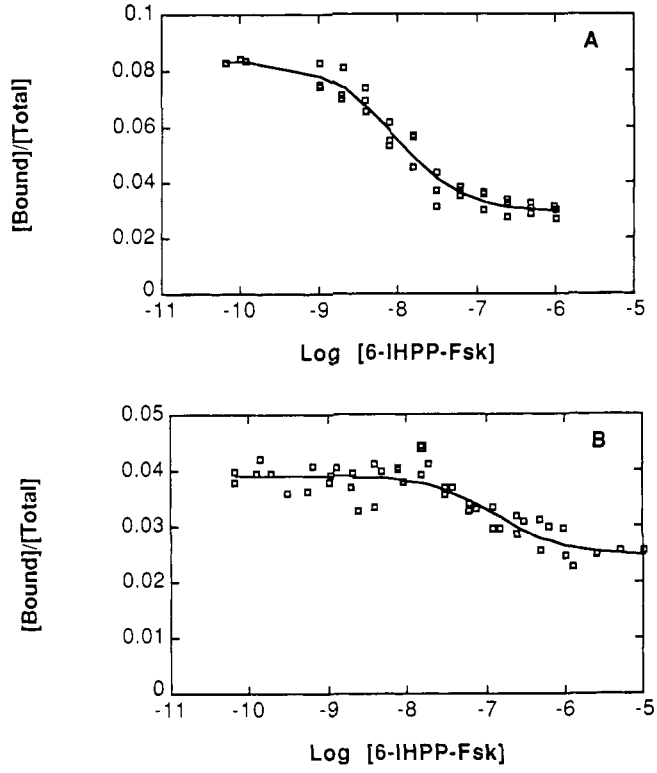

FIGURE 5: Binding of [^{125}I]-6-IHPP-Fsk to Sf9 cell membranes expressing type I and II adenylyl cyclases. Membranes from Sf9 cells expressing type II adenylyl cyclase were preincubated for 10 min at 30 °C in the presence of $G_{s\alpha}$ (40 nM). Aliquots of membranes (type I, 33 μg; type II, 74 μg) were incubated for 60 min at room temperature with [^{125}I]-6-IHPP-Fsk (130 000 cpm, 80 pM) in the presence of increasing concentrations of nonradioactive 6-IHPP-Fsk (0.06 nM to 10 μM). The final concentration of $G_{s\alpha}$ during the incubation with [^{125}I]-6-IHPP-Fsk was 0.5–5 nM. The membranes were processed rapidly, as described in Materials and Methods, to separate bound from free ligand and counted. Each data point was determined in triplicate, and the average standard deviation was <10%. To determine the binding parameters, data from three separate experiments were analyzed together using the program MAC LIGAND V4.1. For each membrane type, the data from three experiments are shown as a displacement curve (type I in A; type II + $G_{s\alpha}$ in B); the curves are computer-generated on the basis of the fitted parameters. The specific activities of the adenylyl cyclases in the membranes were in the same range as those in Figure 1.

The data were fit to a one-site model with the following parameters: membranes expressing type I adenylyl cyclase, K_d = 8 ± 1.4 nM and B_{max} = 6.4 ± 1.0 pmol/mg protein; membranes expressing type II adenylyl cyclase in the presence of exogenous $G_{s\alpha}$, K_d = 134 ± 43 nM and B_{max} = 11.2 ± 3.9 pmol/mg protein. A two-site model showed no improvement in the fit to the data.

The ability of forskolin and forskolin analogs to inhibit [^{125}I]-6-IHPP-Fsk binding to type I and $G_{s\alpha}$-activated type II adenylyl cyclases, as well as to bovine brain membranes, was determined by competition studies. For each analog, the data from two experiments were analyzed together and the K_d values were determined (Table 1). 1,9-Dideoxyforskolin was not effective, inhibiting the binding of [^{125}I]-6-IHPP-Fsk to each type of membrane by less than 20% at 10 μM (data not shown). The K_d values for forskolin and forskolin analogs were similar for bovine brain membranes and membranes expressing type I adenylyl cyclase. The K_d values determined for $G_{s\alpha}$-activated type II adenylyl cyclase ranged from 6- to 20-fold higher than those determined for type I adenylyl cyclase. The order of potency for the inhibition of binding to bovine brain membranes and membranes expressing type

Table 1: Affinities of Forskolin and Forskolin Analogs for Adenylyl Cyclase in Bovine Brain Membranes and Type I and $G_{s\alpha}$-Activated Type II Adenylyl Cyclases in Sf9 Cell Membranes

	K_d[a]		
analog	bovine brain	type I	type II + $G_{s\alpha}$
6-IHPP-Fsk	13 ± 3 nM	8.0 ± 1.4 nM	134 ± 43 nM
forskolin	34 ± 2 nM	41 ± 6 nM	226 ± 75 nM
6-deoxy-Fsk	127 ± 18 nM	223 ± 38 nM	4.5 ± 0.7 μM
7-desacetyl-Fsk	445 ± 22 nM	630 ± 63 nM	4.0 ± 1.4 μM
9-deoxy-Fsk	932 ± 65 nM	1.3 ± 0.2 μM	14 ± 1.7 μM

[a] The K_d values were analyzed for a one-site model using the MAC LIGAND V4.1 program.

I adenylyl cyclase was the same: forskolin > 6-deoxy-Fsk > 7-desacetyl-Fsk > 9-deoxy-Fsk. The order of potency for the inhibition of binding to $G_{s\alpha}$-activated type II adenylyl cyclase was similar, except that 6-deoxy-Fsk was less potent: forskolin > 6-deoxy-Fsk = 7-desacetyl-Fsk > 9-deoxy-Fsk.

DISCUSSION

Modulation of Forskolin Activation and Forskolin Photoaffinity Labeling of Adenylyl Cyclases (Types I, II, V, and VI). The expression of individual forms of adenylyl cyclase in Sf9 cells and the development of high-affinity ^{125}I-labeled derivatives of forskolin have allowed a comparison of the interactions of forskolin with different types of adenylyl cyclase and the ability of $G_{s\alpha}$ to regulate the interactions. Type I and II adenylyl cyclases were photolabeled specifically by [^{125}I]-6-AIPP-Fsk; however, type V and VI adenylyl cyclases were not photolabeled. The addition of $G_{s\alpha}$ had little effect on the photoaffinity labeling of type I adenylyl cyclase. In contrast, exogenous $G_{s\alpha}$ greatly enhanced the photoaffinity labeling of type II adenylyl cyclase, and photolabeling of type V and VI adenylyl cyclases was observed in the presence of $G_{s\alpha}$. The effects of $G_{s\alpha}$ on the ability of [^{125}I]-6-AIPP-Fsk to photolabel types I, II, V, and VI adenylyl cyclase are consistent with the effects of $G_{s\alpha}$ on the activation of these types of enzyme by forskolin (Figure 1). There is no synergistic activation of the type I enzyme by $G_{s\alpha}$ and forskolin. However, there is synergistic activation of type II, V, and VI adenylyl cyclases by forskolin and $G_{s\alpha}$.

In the absence of $G_{s\alpha}$, the amount of photolabeled adenylyl cyclase detected in membranes expressing type I adenylyl cyclase was greater than that detected in the same amount of membranes expressing type II adenylyl cyclase. This result correlates with there being greater forskolin-stimulated adenylyl cyclase activity in type I membranes than in the same amount of type II membranes (refer to Figure 1, filled bars). However, we were not able to detect any protein being photolabeled specifically in membranes expressing type V and VI adenylyl cyclases, even though these membranes contained levels of forskolin-stimulated adenylyl cyclase activity in the same range as those of membranes expressing type II adenylyl cyclase (refer again to Figure 1). Interestingly, no specific photolabeling of type III adenylyl cyclase in a crude olfactory cilia preparation[2] or of recombinant type III adenylyl cyclase was detected when the protein was expressed in 293 kidney cells[2] or Sf9 cells[3] in the presence or absence of activated $G_{s\alpha}$. These results indicate that type III, V, and VI adenylyl cyclases may have a lower affinity for the forskolin photoaffinity label, even though these enzymes are stimulated by forskolin (Bakalyar & Reed, 1990; Ishikawa et al., 1992; Katsushika et al., 1992; Yoshimura & Cooper, 1992; Premont et al., 1992).

The addition of calcium/calmodulin had no effect on the forskolin photoaffinity labeling of type I or II adenylyl cyclases. Although greater than additive activation of type I adenylyl cyclase was observed when forskolin was tested in the presence of calcium/calmodulin (Tang et al., 1991), the degree of synergism was much less than that observed between forskolin and $G_{s\alpha}$ for the activation of type II adenylyl cyclase (Tang et al., 1991; Feinstein et al., 1991; Figure 1). The lack of an effect of calcium/calmodulin on photolabeling of type II adenylyl cyclase is consistent with the type II enzyme being insensitive to calcium/calmodulin (Feinstein et al., 1991). G protein $\beta\gamma$ subunits also had no effect on the photoaffinity labeling of type I or II adenylyl cyclases, consistent with the observation that there is no synergistic effect of G protein $\beta\gamma$ subunits and forskolin on the activation of these enzymes (Tang et al., 1991; Tang & Gilman, 1991).

Characterization of Forskolin Binding Sites on Type I and II Adenylyl Cyclases. The affinities of type I and II adenylyl cyclases for forskolin and its analogs were determined quantitatively by binding studies with [^{125}I]-6-IHPP-Fsk. In the absence of $G_{s\alpha}$, there was specific binding of [^{125}I]-6-IHPP-Fsk to type I adenylyl cyclase, but little specific binding to type II adenylyl cyclase. In the presence of exogenous $G_{s\alpha}$, there was specific binding of [^{125}I]-6-IHPP-Fsk to type II adenylyl cyclase. This result is consistent with the observed synergistic activation of type II adenylyl cyclase by forskolin and $G_{s\alpha}$ (Figure 1; Feinstein et al., 1991) and with $G_{s\alpha}$-enhanced photoincorporation of [^{125}I]-6-AIPP-Fsk into type II adenylyl cyclase (Figure 3). These results are also consistent with the increase in the number of forskolin binding sites observed under conditions that promoted the activation of adenylyl cyclase by the G_s protein (Seamon & Daly, 1986; Laurenza et al., 1992; Stockmeier & Zhang, 1993). Thus, in membranes expressing type II adenylyl cyclase, the high-affinity forskolin binding site appears to be dependent upon the formation of a ternary complex of forskolin, the catalytic subunit, and the G_s protein, as previously proposed (Nelson & Seamon, 1985).

The number of forskolin binding sites (B_{max}) measured by the binding assay in Sf9 cell membranes expressing type I and II adenylyl cyclases were 6.4 ± 1.0 and 11.2 ± 3.9 pmol/mg, respectively. Interestingly, these values are close to the values calculated for the amount of enzyme present (8 pmol/mg for type I membranes and 5 pmol/mg for type II membranes), which were calculated using enzymatic activities of the membranes and turnover numbers for the purified enzymes. The turnover numbers were calculated from specific activities reported for the purified recombinant enzymes (Taussig et al., 1993b).

In the absence of $G_{s\alpha}$, [^{125}I]-6-AIPP-Fsk photolabeled type II adenylyl cyclase (Figures 2 and 3), and the majority of the photoincorporation into the type II enzyme was inhibited by forskolin. Therefore, we concluded that the majority of the photoincorporation into type II adenylyl cyclase was specific photolabeling. In contrast, there was little specific binding of [^{125}I]-6-IHPP-Fsk (less than 10% of total [^{125}I]-6-IHPP-Fsk binding) to membranes expressing type II adenylyl cyclase in the filtration binding assay in the absence of $G_{s\alpha}$ (Figure 4). Some possible explanations for the discrepancy may include differences in the radioactive ligands or differences in the level of detection between the two assays. The affinity

[2] E. McHugh Sutkowski, H. Bakalyar, R. Reed, and K. B. Seamon, unpublished observations.

[3] E. McHugh Sutkowski, W.-J. Tang, and K. B. Seamon, unpublished observations.

of [^{125}I]-6-IHPP-Fsk for type II adenylyl cyclase in the absence of $G_{s\alpha}$ may be too low to measure using a filtration binding assay. The photolabeling assay with [^{125}I]-6-AIPP-Fsk may be more sensitive since it involves covalent (irreversible) binding.

A comparison of the affinities of forskolin and its analogs for bovine brain membranes, type I adenylyl cyclase, and $G_{s\alpha}$-activated type II adenylyl cyclase showed that the rank order of potency for the inhibition of binding to both recombinant adenylyl cyclases was similar to that of crude bovine brain membranes. This similarity in the structure–activity profiles indicates that the forskolin binding sites on the two isoforms of the enzyme have some structural requirements that may be similar to those of the forskolin binding sites measured in crude bovine brain membranes. However, the affinity of forskolin and its analogs for type I adenylyl cyclase was significantly higher than that for $G_{s\alpha}$-activated type II adenylyl cyclase, indicating that there are differences in the binding sites. The affinity of forskolin analogs for adenylyl cyclase in bovine brain membranes and the type I enzyme are almost identical, suggesting that the predominant species measured by forskolin binding in bovine brain membranes represents the type I enzyme or a similar subtype.

In future experiments, binding studies with [^{125}I]-6-IHPP-Fsk will be performed with Sf9 cell membranes expressing type V and VI adenylyl cyclases to determine whether specific binding sites are measurable and whether they are modulated by $G_{s\alpha}$. The optimal conditions for forskolin binding may differ among adenylyl cyclase isoforms. Results from concentration dependence studies indicate that higher concentrations of $G_{s\alpha}$ are required to achieve maximal enhancement of photoincorporation into type V and VI adenylyl cyclases than type II adenylyl cyclase.[3] Structure–activity studies currently are being performed to determine how several forskolin derivatives compare in their ability to activate the adenylyl cyclase isoforms and whether the interactions are regulated by $G_{s\alpha}$.

Summary. Using high-affinity ^{125}I-labeled derivatives of forskolin, we have demonstrated that the interactions of forskolin with type I, II, V, and VI adenylyl cyclases are significantly different and differentially regulated by $G_{s\alpha}$. This result suggests that forskolin and the appropriate derivatives might be useful in studies on the localization and role of specific adenylyl cyclase subtypes. Future experiments will be designed to use recombinant adenylyl cyclases and the high-affinity forskolin derivatives to localize the forskolin binding site(s) on the subtypes of the enzyme.

ACKNOWLEDGMENT

We thank Ethan Lee for purified G protein $\beta\gamma$ subunits and Marian Stanzel for her superb technical support. We thank Kathy Beckingham of the Department of Biochemistry and Cell Biology, Rice University, Houston, TX, for providing *Escherichia coli* AR68 carrying pJFM 39. We thank Raymond W. Kosley, Jr., and Bettina Spahl of Hoechst-Roussel Pharmaceuticals, Somerville, NJ, for generously supplying 6-deoxy-Fsk and 9-deoxy-Fsk. We acknowledge Heather A. Bakalyar and Randall R. Reed of the Department of Molecular Biology and Genetics, the Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, for their helpful discussions and for generously providing a crude olfactory cilia preparation and 293 kidney cells expressing type III adenylyl cyclase. We thank Diane Morris for her helpful discussions and critical reading of this manuscript. W.-J. Tang thanks Alfred G. Gilman for encouragement and support.

REFERENCES

Appel, N. M., Robbins, J. D., DeSouza, E. B., & Seamon, K. B. (1992) *J. Pharmacol. Exper. Ther. 263*, 1415–1423.
Bakalyar, H. A., & Reed, R. R. (1990) *Science 250*, 1403–1406.
Bender, J. L., & Neer, E. J. (1983) *J. Biol. Chem. 258*, 2432–2439.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brostrom, C. O., Brostrom, M. A., & Wolff, D. J. (1977) *J. Biol. Chem. 252*, 5677–5685.
Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M. F., & Krupinski, J. (1994) *J. Biol. Chem. 269*, 12190–12195.
Coussen, F., Guermah, M., d'Alayer, J., Monneron, A., Haiech, J., & Cavadore, J.-C. (1986) *FEBS Lett. 206*, 213–217.
Feinstein, P. G., Schrader, K. A., Bakalyar, H. A., Tang, W.-J., Krupinski, J., Gilman, A. G., & Reed, R. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10173–10177.
Gao, B., & Gilman, A. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10178–10182.
Glatt, C. E., & Snyder, S. H. (1993) *Nature 361*, 536–538.
Graziano, M. P., Freissmuth, M., & Gilman, A. G. (1989) *J. Biol. Chem. 264*, 409–418.
Green, D. A., & Clark, R. B. (1982) *J. Cyclic Nucleotide Res. 8*, 337–346.
Ishikawa, Y. Katsushika, S., Chen, L., Halnon, N., Kawabe, J.-I., & Homcy, C. J. (1992) *J. Biol. Chem. 267*, 13553–13557.
Katsushika, S., Chen, L., Kawabe, J.-I., Nilakantan, R., Halnon, N. J., Homcy, C. J., & Ishikawa, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8774–8778.
Kitts, P., Ayres, A., & Possee, R. D. (1990) *Nucleic Acids Res. 18*, 5667–5672.
Kosley, R. W., Jr., & Spahl, B. (1991) *Abstracts of Papers*, 201st National Meeting of the American Chemical Society, Atlanta, GA, April, 1991, ORGN 25, American Chemical Society, Washington, D.C.
Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., & Gilman, A. G. (1989) *Science 244*, 1558–1564.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laurenza, A., & Seamon, K. B. (1991) *Methods Enzymol. 195*, 52–65.
Laurenza, A., Robbins, J. D., & Seamon, K. B. (1992) *Mol. Pharmacol. 41*, 360–368.
Lee, E., Linder, M. E., & Gilman, A. G. (1994) *Methods Enzymol. 237*, 146–164.
Maune, J. F., Klee, C. B., & Beckingham, K. (1992) *J. Biol. Chem. 267*, 5286–5295.
Mollner, S., & Pfeuffer, T. (1988) *Eur. J. Biochem. 171*, 265–271.
Morris, D. I., Robbins, J. D., Ruoho, A., McHugh Sutkowski, E., & Seamon, K. B. (1991) *J. Biol. Chem. 266*, 13377–13384.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Nelson, C. A., & Seamon, K. B. (1985) *FEBS Lett. 183*, 349–352.
Nelson, C. A., & Seamon, K. B. (1986) *J. Biol. Chem. 261*, 13469–13473.
Peterson, G. L. (1983) *Methods Enzymol. 91*, 95–119.
Pfeuffer, E., Mollner, S., Lancet, D., & Pfeuffer, T. (1989) *J. Biol. Chem. 264*, 18803–18807.
Premont, R. T., Chen, J., Ma, H.-W., Ponnapalli, M., & Iyengar, R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9809–9813.
Robbins, J., Laurenza, A., Kosley, R. W., O'Malley, G. O., Spahl, B., & Seamon, K. B. (1991) *J. Med. Chem. 34*, 3204–3212.
Robbins, J. D., Appel, N. M., Laurenza, A., Simpson, I. A., De Souza, E. B., & Seamon, K. B. (1992) *Brain Res. 581*, 148–152.

Rosenberg, G. R., & Storm, D. R. (1987) *J. Biol. Chem. 262*, 7623–7628.
Seamon, K. B., & Daly, J. W. (1981) *J. Biol. Chem. 256*, 9799–9801.
Seamon, K. B., & Daly, J. W. (1986) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 20*, 1–150.
Seamon, K. B., Vaillancourt, R., Edwards, M., & Daly, J. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5081–5085.
Smigel, M. D. (1986) *J. Biol. Chem. 261*, 1976–1982.
Sternweis, P. C., & Pang, I.-H. (1990) *Receptor-Effector Coupling. A Practical Approach* (Hulme, E. L., Ed.) Oxford University Press, Oxford, U.K.
Stockmeier, C. A., & Zhang, Y. (1993) *Brain Res. 610*, 156–161.
Tang, W.-J., & Gilman, A. G. (1991) *Science 254*, 1500–1503.
Tang, W.-J., Krupinski, J., & Gilman, A. G. (1991) *J. Biol. Chem. 266*, 8595–8603.
Taussig, R., Iniguez-Lluhi, J., & Gilman, A. G. (1993a) *Science 261*, 218–221.
Taussig, R., Quarmby, L. M., & Gilman, A. G. (1993b) *J. Biol. Chem. 268*, 9–12.
Taussig, R., Tang, W.-J., Hepler, J. R., & Gilman, A. G. (1994) *J. Biol. Chem. 269*, 6093–6100.
Westcott, K. R., La Porte, D. C., & Storm, D. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 204–208.
Yoshimura, M., & Copper, D. M. F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6716–6720.