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Irreversible Inhibition of Forskolin Interactions with Type I Adenylyl Cyclase by a 6-Isothiocyanate Derivative of Forskolin

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SUMMARY

Forskolin (Fsk) has been demonstrated to interact directly with the enzyme adenylyl cyclase (EC 4.6.1.1) in diverse tissues. However, the ability of Fsk to bind to and activate adenylyl cyclase varies depending on the tissue being studied. Different adenylyl cyclase subtypes have been cloned and expressed in a recombinant Sf9 expression system. This provides an opportunity to study the effects of chemically reactive derivatives of Fsk on individual adenylyl cyclase subtypes in the absence of $G_{s\alpha}$. Reaction of type I adenylyl cyclase with an isothiocyanate derivative of Fsk (6-[[*N*-(2-isothiocyanatoethyl)amino]carbonyl]forskolin) causes irreversible inhibition of Fsk binding with an IC_{50} of 300 nM and irreversible inhibition of Fsk activation with an IC_{50} of 10 μM, suggesting that there are two sites of 6-[[*N*-(2-isothiocyanatoethyl)amino]carbonyl]forskolin interaction. These studies establish the usefulness of the isothiocyanate derivative of Fsk in localizing the site(s) of Fsk interaction with type I adenylyl cyclase.

Fsk, a diterpene natural product, activates adenylyl cyclase in a variety of mammalian tissues with an EC_{50} of ~5–10 μM (1). Fsk activates adenylyl cyclase in the absence of G_s protein (the regulatory protein that stimulates adenylyl cyclase; G_i is the regulatory protein that inhibits adenylyl cyclase) (2); however, maximal activation of adenylyl cyclase by Fsk requires the presence of a functional G_s protein (3, 4). At concentrations of 0.01–0.1 μM, Fsk potentiates hormone activation of adenylyl cyclase (1).

Several mammalian adenylyl cyclases have been completely cloned, sequenced, and characterized (5–16). Studies of the individual recombinant forms of adenylyl cyclase demonstrate that all subtypes studied so far can be activated to different extents by Fsk as well as by activated $G_{s\alpha}$. Furthermore, there is synergistic activation of types II, V, and VI adenylyl cyclase by activated $G_{s\alpha}$ and Fsk; activated $G_{s\alpha}$ potentiates the effect of Fsk on type II adenylyl cyclase to the greatest extent (8, 17). The enzyme subtypes differ in their sensitivity to calcium/calmodulin, $G_{i\alpha}$, the G protein βγ subunit complex, calcium, and calcineurin (7–12, 16, 18, 19).

High affinity binding sites for Fsk (with K_d values <50 nM) have been detected in membranes from a variety of tissues and cells using [^{3}H]Fsk (20, 21) and [^{125}I]6-IHPP-Fsk (22, 23). The high affinity Fsk binding sites display structure-activity requirements consistent with the ability of Fsk to activate adenylyl cyclase (20, 24). However, it has been difficult to equate the affinity of the Fsk binding sites with the potency of Fsk as an activator of adenylyl cyclase.

The number of high affinity binding sites for Fsk is increased under conditions that promote activation of adenylyl cyclase by the G_s protein. Therefore, it was proposed that the high affinity sites may represent a complex of adenylyl cyclase and the activated G_s protein (20, 25, 26). Studies with recombinant adenylyl cyclases have demonstrated that the addition of exogenous activated $G_{s\alpha}$ is required for detection of high affinity Fsk binding sites in membranes expressing type II adenylyl cyclase (17). However, high affinity Fsk binding sites were detected in membranes expressing type I adenylyl cyclase in the absence of activators of adenylyl cyclase, and the specific binding was not enhanced by the addition of exogenous activated $G_{s\alpha}$. Therefore, because type I adenylyl cyclase is stimulated by Fsk and it binds radiolabeled derivatives of Fsk with high affinity, we chose this enzyme subtype with which to study the relationship between Fsk binding and activation in more detail.

Chemically reactive derivatives are useful reagents for determining whether ligand binding sites have reactive nucleophilic groups that may be modified irreversibly, thus

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ABBREVIATIONS: Fsk, forskolin; 6-IHPP-Fsk, 2-[3-(4-hydroxy-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)-forskolin; BrAcFsk, 7-bromoacetyl-7-desacetylforskolin; 6-AIPP-Fsk, 2-[3-(4-azido-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin; 6-NCS-Fsk, 6-[[*N*-(2-Isothiocyanatoethyl)amino]carbonyl]forskolin; 6-NCS-DDFsk, 6-[[*N*-(2-isothiocyanatoethyl)amino]carbonyl]1,9-dideoxyforskolin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEM, Tris·HCl/EDTA/$MgCl_2$; DDFsk, 1,9-dideoxyforskolin; DTT, dithiothreitol.

inhibiting the interaction of other agents. BrAcFsk irreversibly blocked high affinity Fsk binding sites in human platelet membranes and rat brain membranes without affecting Fsk stimulation of adenylyl cyclase (27). 6-NCS-Fsk, which is more selective and stable than BrAcFsk because it has the isothiocyanate group attached to Fsk through a stable carbamate linkage, irreversibly inhibited >65% of the high affinity Fsk binding sites in bovine brain membranes (22). We studied the inhibitory effects of 6-NCS-Fsk on Fsk interactions with recombinant type I adenylyl cyclase in the absence of $G_{s\alpha}$ to determine whether this chemically reactive Fsk derivative could discriminate between an Fsk binding site and an Fsk activation site on the enzyme. The results suggest that there are two sites of 6-NCS-Fsk interaction and establish this isothiocyanate derivative of Fsk as an agent that may be useful in conjunction with mass spectrometry in localizing the site(s) of Fsk interaction with type I adenylyl cyclase.

Materials and Methods

Synthesis of Fsk derivatives. [^{125}I]6-IHPP-Fsk and [^{125}I]6-AIPP-Fsk were synthesized carrier free (2200 Ci/mmol) as described previously (22, 28). The synthesis of 6-NCS-Fsk has been described previously (22). 6-NCS-DDFsk was synthesized in a similar manner except the starting material was 1,9-dideoxy-7-desacetylforskolin, which required no deprotection step.

Construction of recombinant baculoviruses and expression of adenylyl cyclases in Sf9 cells. Type I adenylyl cyclase has been expressed using the recombinant baculovirus expression system. B-rACI was constructed and expressed as described previously (7). Cells were lysed, and membranes were washed and resuspended in 20 mM NaHEPES, pH 8.0, 2 mM DTT, 1 mM EDTA, 200 mM sucrose, plus protease inhibitors (22 mg/liter each of L-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, and phenylmethylsulfonyl fluoride) and 3.2 mg/liter each of leupeptin and lima bean trypsin inhibitor as described previously (7). The protein concentration of the Sf9 cell membranes was determined by dye binding (29), using bovine serum albumin as standard.

Fsk photoaffinity labeling procedure. Photoaffinity labeling experiments were carried out as described previously (17). Sf9 cell membranes (50 μg) were preincubated for 1 hr at room temperature in the presence of TEM buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$) with or without 2 μM Fsk or Fsk analog in a total volume of 100 μl. [^{125}I]6-AIPP-Fsk (1–2 × 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 2 μM Fsk or Fsk analog was added, and the diluted samples were quickly photolyzed (energy = 200,000 μJ) in a UV Stratalinker (Stratagene, La Jolla, CA). Immediately after 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°, washed with 10 mM Tris, pH 8.0/1 mM EDTA, repelleted, and prepared for electrophoresis as described below.

To study the irreversible effects of Fsk analogs on photolabeling, Sf9 cell membranes (75 μg) were preincubated for 1 hr at room temperature in the presence of TEM buffer with or without 2 μM Fsk or Fsk analog in a total volume of 100 μl. The membranes were washed three times by resuspension with 1 ml of TEM buffer and pelleting by centrifugation at 12,000 rpm in a TOMY TMS-4 rotor for 15 min at 4°. The washed membrane pellets were resuspended in 100 μl of TEM buffer for photolysis as above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes from Sf9 cells were heated to 80° in the presence of 2% sodium dodecyl sulfate and 0.2 mM DTT for 5 min and then treated with 50 mM *N*-ethylmaleimide for 10 min at room temperature before the addition of 4× concentrated electrophoresis sample buffer described by Laemmli (30). Samples were electrophoresed on 8% or 10% polyacrylamide minigels using the discontinuous buffer system described by Laemmli (30). The ^{14}C-labeled molecular mass standards (GIBCO-BRL, Gaithersburg, MD) were myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18.4 kDa). The gels were Coomassie-stained, dried, and exposed to Kodak XAR 5 film (Eastman Kodak, Rochester, NY).

Binding of [^{125}I]6-IHPP-Fsk to Sf9 cell membranes. Binding experiments were carried out as described previously (17). Sf9 cell membranes (50 μg) were incubated for 60 min at room temperature with a constant amount of [^{125}I]6-IHPP-Fsk (30,000 cpm, ~20 pM) in the absence or presence of Fsk or Fsk analog in 12 × 75-mm glass test tubes in a total volume of 0.4 ml of 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$. Fsk and Fsk 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 aspiration of 95% ethanol to minimize carryover of radioactivity. Each data point was determined in triplicate, and the average standard deviation was <10%. To determine the binding parameters, data from at least two separate experiments were analyzed together using the LIGAND program (MacLIGAND version 4.1; ABC Software, Baltimore, MD), which was described previously (31).

To study the irreversible effects of Fsk analogs on binding of [^{125}I]6-IHPP-Fsk, Sf9 cell membranes (1.3 mg) were incubated for 60 min at room temperature with Fsk or the 6-isothiocyanate derivative of either Fsk or 1,9-dideoxyforskolin (0–10 μM) in 16 × 76-mm polycarbonate centrifuge bottles (Beckman, Palo Alto, CA) in a total volume of 0.2 ml of 25 mM Tris·HCl, pH 7.5/2.5 mM $MgCl_2$. The membranes were washed two times by resuspension in 10 ml of cold 50 mM Tris·HCl, pH 7.5, and pelleting by centrifugation at 16,000 rpm in a Sorvall SS-34 rotor for 20 min at 4°. The washed membrane pellets were resuspended in 1.8 ml of 100 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$ containing [^{125}I]6-IHPP-Fsk and 200-μl aliquots, each of which contained ~95,000 cpm [^{125}I]6-IHPP-Fsk (~60 pM), were incubated for 60 min at room temperature in the absence or presence of Fsk (10 μM) in 12 × 75-mm glass test tubes. The assays were terminated and processed as described above. Each data point was determined in triplicate, and the average standard deviation was <10%. Specific binding was calculated as the difference between total binding in the absence of Fsk and nonspecific binding in the presence of 10 μM Fsk. [^{125}I]6-IHPP-Fsk bound represents percentage of specific binding measured in control membranes (pretreated in the absence of Fsk or Fsk analog). Data from two independent experiments were averaged together and analyzed using Kaleidagraph to obtain approximate IC_{50} values.

Adenylyl cyclase assay. Adenylyl cyclase activity was measured using [α-^{32}P]ATP by a single column assay. Briefly, membranes were assayed in a 100-μl volume containing 50 mM Tris, pH 7.50, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 6 μg of bovine serum albumin, 69 μg of phosphocreatine, 13 μg of creatine phosphokinase, 1 mM cAMP and [^{3}H]cAMP (10,000 cpm/assay tube), and 0.1 mM ATP and [α-^{32}P]ATP (200,000 cpm/assay). DTT was either omitted or added fresh at a final concentration of 1 mM. The [α-^{32}P]ATP (DuPont-New England Nuclear, Boston, MA) was purified before use by anion exchange chromatography as described previously (32). The reaction mixture was incubated for 10 min at 30°, and the product was separated by $ZnCO_3$ precipitation and acid alumina chromatography as described previously (32). Each data point was determined in triplicate, and the average standard deviation was <10%.

To study the irreversible effects of isothiocyanate derivatives of Fsk on Fsk-stimulated activation of type I adenylyl cyclase, Sf9 cell

membranes (1.3 mg) were incubated for 60 min at room temperature with Fsk or the 6-isothiocyanate derivative of either Fsk or 1,9-dideoxyforskolin (0–100 μM) in 16 × 76-mm polycarbonate centrifuge bottles (Beckman) in a total volume of 2.5 ml of TEM buffer. The membranes were washed five times by resuspension in 10 ml of cold 50 mM Tris·HCl, pH 7.5, and pelleting by centrifugation at 16,000 rpm in a Sorvall SS-34 rotor for 20 min at 4°. The washed membrane pellets were resuspended in 1.0 ml of TEM buffer, and 15-μl aliquots were assayed in triplicate in the presence of DTT under either basal conditions or in the presence of 100 μM Fsk. Fsk activation was calculated as the difference between adenylyl cyclase activity measured in the presence and absence of 100 μM Fsk. Fsk activation (percent maximum) represents percentage of Fsk activation measured in control membranes (pretreated in the absence of Fsk or Fsk analog). Data from at least two independent experiments were averaged together and analyzed using Kaleidagraph to obtain approximate IC_{50} values.

Results

Inhibition of Fsk photoaffinity labeling of type I adenylyl cyclase by 6-NCS-Fsk. The [^{125}I]labeled arylazido derivative of Fsk, [^{125}I]6-AIPP-Fsk, is photoincorporated specifically into adenylyl cyclases expressed in Sf9 cells (17). The specificity of the interaction of 6-NCS-Fsk with adenylyl cyclase was examined by determining whether 6-NCS-Fsk or 6-NCS-DDFsk at a concentration of 2 μM would inhibit photoincorporation of [^{125}I]6-AIPP-Fsk into type I adenylyl cyclase (Fig. 1A, *Unwashed*). [^{125}I]6-AIPP-Fsk was incorporated into type I adenylyl cyclase (protein with apparent molecular mass of ~100,000 in *lane 1*), and photolabeling of this protein was inhibited by Fsk and 6-NCS-Fsk (*lanes 2 and 3*, respectively). In contrast, 6-NCS-DDFsk, the isothiocyanate derivative of 1,9-dideoxyforskolin, the inactive analog of Fsk, did not inhibit photolabeling of type I adenylyl cyclase (*lane 4*).

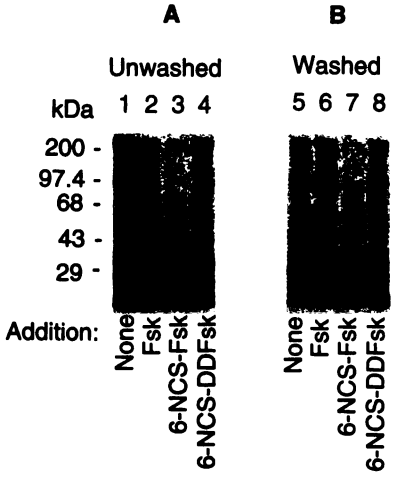

Fig. 1. A, Inhibition of Fsk photoaffinity labeling of type I adenylyl cyclase by 6-NCS-Fsk. A, Membranes (75 μg) from Sf9 cells expressing type I adenylyl cyclase were preincubated for 1 hr at room temperature with either buffer with no addition (*lane 1*), 2 μM Fsk (*lane 2*), 2 μM 6-NCS-Fsk (*lane 3*), or 2 μM 6-NCS-DDFsk (*lane 4*) and then incubated with [^{125}I]6-AIPP-Fsk and photolyzed as described in Materials and Methods. The samples were prepared for electrophoresis, and half of each sample was run on an 8% polyacrylamide minigel. B, Irreversible inhibition of Fsk photoaffinity labeling of type I adenylyl cyclase by 6-NCS-Fsk. B, Membranes (75 μg) from Sf9 cells expressing type I adenylyl cyclase were preincubated for 1 hr at room temperature with buffer with no addition (*lane 5*), 2 μM Fsk (*lane 6*), 2 μM 6-NCS-Fsk (*lane 7*), or 2 μM 6-NCS-DDFsk (*lane 8*) and washed several times to remove noncovalently bound analog. The membranes were photolyzed in the presence of [^{125}I]6-AIPP-Fsk as described in Materials and Methods. The samples were prepared for electrophoresis, and half of each sample was run on an 8% polyacrylamide minigel.

To examine whether the binding of 6-NCS-Fsk at the Fsk photoaffinity labeling site was irreversible, the Sf9 cell membranes were incubated with Fsk or Fsk analog and washed to remove noncovalently bound analog before photolabeling (Fig. 1B, *Washed*). Photolabeling of adenylyl cyclase after pretreatment with 6-NCS-Fsk was completely inhibited (*lane 7*), in contrast to pretreatment with Fsk (*lane 6*), indicating that 6-NCS-Fsk bound irreversibly to type I adenylyl cyclase at the Fsk photolabeling site. Once again, 6-NCS-DDFsk had no inhibitory effect on photolabeling of adenylyl cyclase (*lane 8*).

Inhibition of high affinity Fsk binding to type I adenylyl cyclase by 6-NCS-Fsk. The [^{125}I]labeled high affinity ligand [^{125}I]6-IHPP-Fsk bound to membranes expressing type I adenylyl cyclase with a K_d value of 8 ± 1.4 nM (17). Fsk inhibited [^{125}I]6-IHPP-Fsk binding with a K_d value of 41 ± 6 nM, whereas the inactive analog of Fsk, 1,9-dideoxyforskolin, was not effective, inhibiting the binding of [^{125}I]6-IHPP-Fsk by <20% at 10 μM (17). The ability of the isothiocyanate derivative of Fsk to inhibit high affinity Fsk binding was confirmed by studying the inhibitory effect of 6-NCS-Fsk on reversible [^{125}I]6-IHPP-Fsk binding to type I adenylyl cyclase. In this filtration binding assay, the affinity of the interaction between 6-NCS-Fsk and type I adenylyl cyclase could be determined quantitatively by competition studies (Figs. 2 and 3). The experiments in Fig. 2 did not account for the irreversible loss of binding sites, and therefore an experimentally determined apparent $K_{d(exp)}$ was calculated. 6-NCS-Fsk inhibited [^{125}I]6-IHPP-Fsk binding to type I adenylyl cyclase with a $K_{d(exp)}$ value of 12 ± 3 nM. 6-NCS-DDFsk was much less effective, inhibiting the binding of [^{125}I]6-IHPP-Fsk to the membranes by ~35% at 10 μM ($K_{d(exp)}$ = 5 ± 2 μM).

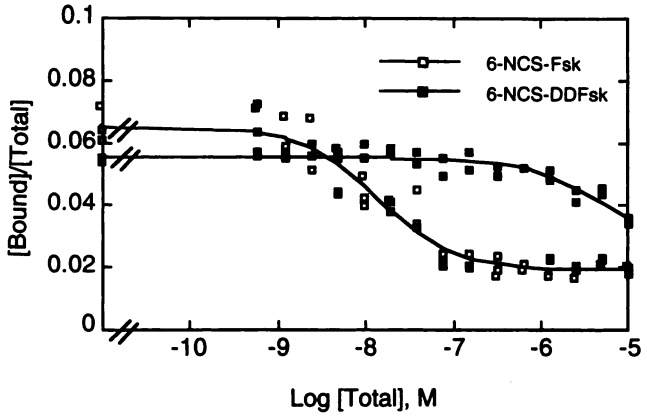

Fig. 2. 6-NCS-Fsk inhibits binding of [^{125}I]6-IHPP-Fsk to Sf9 cell membranes expressing type I adenylyl cyclases. Aliquots of Sf9 cell membranes (50 μg) were incubated for 60 min at room temperature with [^{125}I]6-IHPP-Fsk (50,000 cpm, ~30 pM) in the presence of increasing concentrations of isothiocyanate derivative (0.06 nM–10 μM). The membranes were rapidly processed as described in Materials and Methods to separate bound from free ligand and counted. Data was determined in triplicate, and the average standard deviation was <10%. To determine the binding parameters, data from at least two separate experiments were analyzed together using the program MAC LIGAND version 4.1. The data from at least two experiments are shown as a displacement curve (□, 6-NCS-Fsk; ■, 6-NCS-DDFsk); the curves are computer generated based on the fitted parameters.

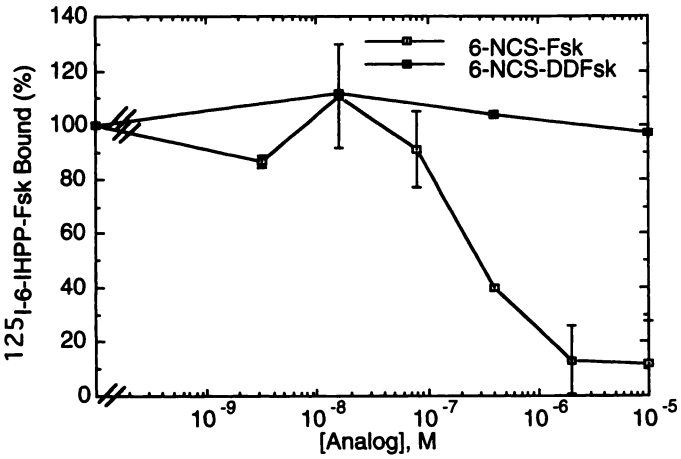

Fig. 3. The concentration dependence of irreversible inhibition of binding of [^{125}I]6-IHPP-Fsk to Sf9 cell membranes expressing type I adenylyl cyclase by 6-NCS-Fsk. Membranes from Sf9 cells expressing type I adenylyl cyclase were pretreated in the presence of increasing concentrations of isothionyl derivative (≤10 μM; □, 6-NCS-Fsk; ■, 6-NCS-DDFsk) and washed to remove noncovalently bound analog as described in Materials and Methods. Aliquots of the membranes were then incubated (in triplicate) for 60 min at room temperature with [^{125}I]6-IHPP-Fsk (95,000 cpm, ~60 pM) in the absence or presence of Fsk (10 μM). The membranes were rapidly processed as described in Materials and Methods to separate bound from free ligand and counted. The average standard deviation of the triplicates was <10%. Specific binding was calculated as the difference between total binding in the absence of Fsk and nonspecific binding in the presence of 10 μM Fsk. [^{125}I]6-IHPP-Fsk bound (%) represents percentage of specific binding measured in control membranes (pretreated in the absence of Fsk or Fsk analog). Data from two independent experiments were averaged together. *Error bars*, standard deviation. The data were analyzed using Kaleidagraph to obtain approximate IC_{50} values.

To examine whether the binding of 6-NCS-Fsk at the high affinity Fsk binding site was irreversible, the Sf9 cell membranes were incubated with increasing concentrations of isothiocyanate derivative and washed to remove noncovalently bound analog before measurement of [^{125}I]6-IHPP-Fsk binding. Pretreatment with 6-NCS-Fsk caused a concentration-dependent irreversible loss of [^{125}I]6-IHPP-Fsk binding to type I adenylyl cyclase with an IC_{50} of ~300 nM (Fig. 3). The maximum loss of binding (~90%) occurred after pretreatment with 6-NCS-Fsk at 2 μM. In contrast, pretreatment with 6-NCS-DDFsk at ≤10 μM had no irreversible effect on [^{125}I]6-IHPP-Fsk binding. The [^{125}I]6-IHPP-Fsk binding measured after the membranes were washed was the same as or slightly greater than the control sample (with no addition) when membranes pretreated with Fsk were used (data not shown). This result indicates that Fsk was removed efficiently by the washing procedure used in this experiment.

Inhibition of Fsk activation of type I adenylyl cyclase by 6-NCS-Fsk. The direct effect of the addition of Fsk and Fsk analogs on adenylyl cyclase activity in Sf9 cell membranes expressing type I adenylyl cyclase was examined in Fig. 4. Fsk stimulated adenylyl cyclase activity, causing a 2.5–5-fold increase in activation at a concentration of 100 μM (Fig. 4, *top* and *bottom*). In contrast, when 6-NCS-Fsk was added, there was no activation of type I adenylyl cyclase (Fig. 4, *top*). These experiments were performed in the absence of DTT to maintain the chemical reactivity of the isothionyl moiety of 6-NCS-Fsk toward a nucleophilic group at the Fsk site of action. It is possible that chemical modification of a nucleophilic group at the Fsk site of action prevented activation of the adenylyl cyclase by the Fsk moiety of 6-NCS-Fsk. Similar results were obtained with 6-[[*N*-(2-(bromoacetyl)-ethyl]amino]carbonyl]forskolin, which also attacks nucleophilic groups (data not shown). In Fig. 4 (*top*), the isothiocyanate derivative of the inactive Fsk analog, 6-NCS-DDFsk, did not activate the enzyme. 6-NCS-Fsk and 6-NCS-DDFsk caused slight inhibition of adenylyl cyclase activity at the highest concentrations tested (20 and 100 μM, Fig. 4, *top*).

Another chemically reactive derivative of Fsk, BrAcFsk, was shown previously to activate adenylyl cyclase in human platelets (27). However, these studies were carried out in the presence of DTT. The addition of BrAcFsk to Sf9 cell membranes expressing type I adenylyl cyclase also caused stimulation of enzyme activity when assayed in the presence of DTT (data not shown). However, when BrAcFsk was added to the Sf9 cell membranes expressing type I enzyme in the absence of DTT, no activation was observed (Fig. 4, *bottom*). We presume that BrAcFsk was acting like 6-NCS-Fsk and reacting covalently with a residue at the Fsk site of action that prevented activation of the adenylyl cyclase.

To examine whether 6-NCS-Fsk was interacting irreversibly at the Fsk activation site on adenylyl cyclase, the Sf9 cell membranes were incubated with Fsk or Fsk analog and washed to remove noncovalently bound analog, and adenylyl cyclase activity was measured in the absence and presence of 100 μM Fsk. Pretreatment with 6-NCS-Fsk caused a concentration-dependent irreversible loss of Fsk-stimulated activation of type I adenylyl cyclase with an IC_{50} of ~10 μM (Fig. 5). The maximum loss of Fsk-stimulated activation (~90%) occurred after pretreatment with 6-NCS-Fsk at 100 μM. Pretreatment with 6-NCS-DDFsk caused an irreversible loss of Fsk-stimulated activation at the highest concentration of 100 μM. However, the dose response for 6-NCS-DDFsk is shifted significantly to the right, resulting in an IC_{50} of ~70 μM. When the membranes were pretreated with 100 μM Fsk, the Fsk-stimulated activation of adenylyl cyclase measured after the membranes were washed was the same as or slightly greater than the control sample (with no addition) (see legend for Fig. 5). This result indicates that Fsk was removed efficiently by the washing procedure used in this experiment. Interestingly, Fsk (100 μM) acts to stabilize the adenylyl cyclase against a decrease in enzymatic activity that occurs during the 1-hr pretreatment at room temperature.

Discussion

A chemically reactive (isothiocyanate) derivative of Fsk (6-NCS-Fsk) was used to determine whether Fsk binding and Fsk activation occur at the same site on recombinant type I adenylyl cyclase in the absence of $G_{s\alpha}$. 6-NCS-Fsk causes specific irreversible inhibition of high affinity Fsk binding to membranes from Sf9 cells expressing type I adenylyl cyclase with an IC_{50} of ~300 nM, suggesting covalent binding of 6-NCS-Fsk to a reactive nucleophilic (amino or thiol) group at the Fsk binding site. 6-NCS-Fsk also causes a concentration-dependent loss of Fsk-stimulated activation of adenylyl cyclase. However, this irreversible inhibition of Fsk activation occurs with an IC_{50} of ~10 μM. The magnitude of the difference in the concentration ranges at which 6-NCS-Fsk causes irreversible inhibition of Fsk binding and activation (nM versus μM) suggests that there are two sites of 6-NCS-Fsk interaction.

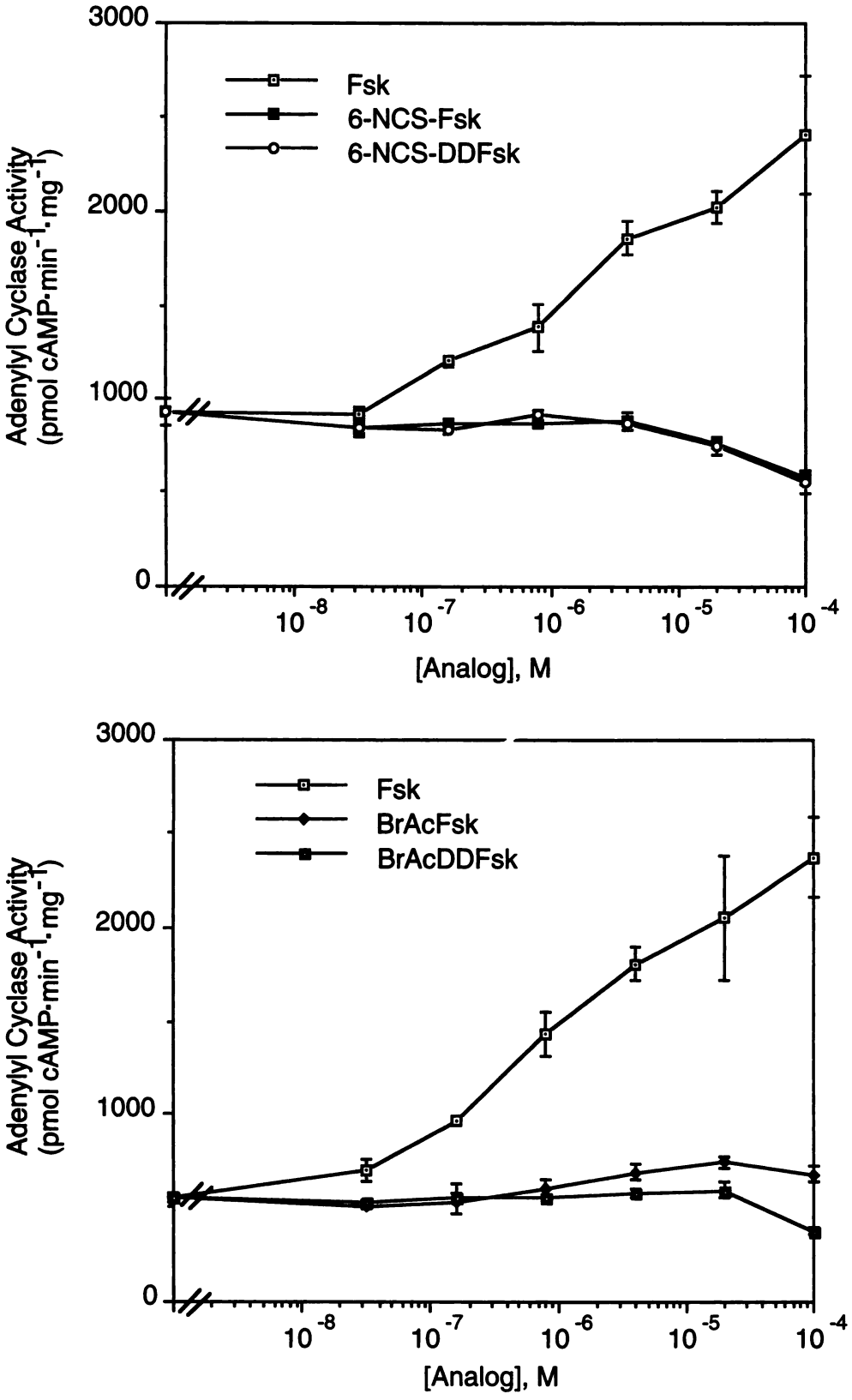

Fig. 4. Direct effect of chemical affinity derivatives of Fsk (6-NCS-Fsk and BrAcFsk) on type I adenylyl cyclase activity. Adenylyl cyclase activity was assayed in membranes (13 μg) prepared from Sf9 cells expressing type I adenylyl cyclase in the absence of DTT as described in Materials and Methods. *Top*, Membranes were assayed in the presence of increasing concentrations of Fsk (□, Fsk) or isothiocyanate derivative (■, 6-NCS-Fsk; ○, 6-NCS-DDFsk) up to a concentration of 100 μM. *Bottom*, Membranes were assayed in the presence of increasing concentrations of Fsk (□, Fsk) or bromoacetyl derivative (◆, BrAcFsk; ■, BrAcDDFsk). Each data point was performed in triplicate. *Error bars*, standard deviation. The values are representative of results from at least two separate experiments.

The 6-isothiocyanate derivative of Fsk irreversibly inhibits Fsk interactions in the same concentration ranges at which Fsk binds to and activates type I adenylyl cyclase itself in the absence of $G_{s\alpha}$. Thus, this chemically reactive derivative of Fsk should prove useful in localizing the site(s) of Fsk interaction with type I adenylyl cyclase. To examine the specificity of 6-NCS-Fsk for interacting with a nucleophilic group at the Fsk site of interaction as opposed to anywhere else on the protein, the results obtained with 6-NCS-Fsk were compared with those obtained with 6-NCS-DDFsk, the isothiocyanate derivative of

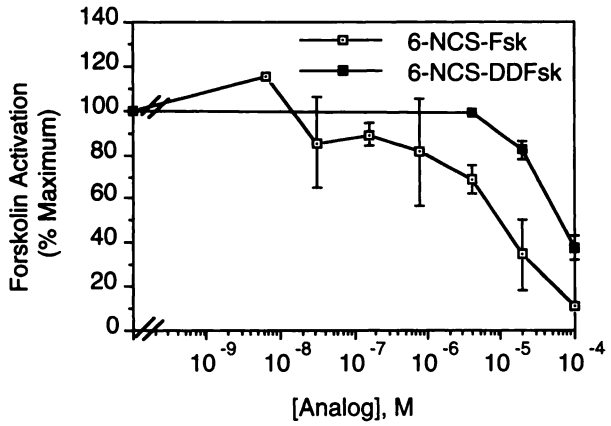

Fig. 5. Irreversible inhibition of Fsk activation of type I adenylyl cyclase by isothionate derivatives. Membranes from Sf9 cells expressing type I adenylyl cyclase were pretreated in the presence of increasing concentrations of isothionate derivative (≤100 μM; □, 6-NCS-Fsk; ■, 6-NCS-DDFsk) and washed to remove noncovalently bound analog as described in Materials and Methods. Aliquots of the membranes were assayed in the presence of DTT under either basal conditions or in the presence of 100 μM Fsk. Assays were performed in triplicate, and the average standard deviation was <10%. Fsk activation was calculated as the difference between adenylyl cyclase activity measured in the presence and absence of 100 μM Fsk. Fsk activation (% maximum) represents percentage of Fsk activation measured in control membranes (pretreated in the absence of Fsk or Fsk analog). Data from at least two independent experiments were averaged together. *Error bars*, standard deviation. The data were analyzed using Kaleidagraph to obtain approximate IC_{50} values. Listed below are the values for adenylyl cyclase activity (pmol/min) from two representative experiments. For each pretreatment condition, the concentration of Fsk or Fsk analog used in the pretreatment is listed, followed by the basal value and the value measured in the presence of 100 μM Fsk. Experiment 1: No addition (buffer alone), 7.4 ± 0.4, 21.9 ± 0.9; Fsk (100 μM), 12.0 ± 0.5, 28.4 ± 1.3; 6-NCS-Fsk (0.032 μM), 9.1 ± 0.2, 18.3 ± 2.9; 6-NCS-Fsk (0.16 μM), 9.3 ± 0.1, 21.7 ± 1.7; 6-NCS-Fsk (0.8 μM), 9.9 ± 0.4, 17.7 ± 1.3; 6-NCS-Fsk (4 μM), 9.9 ± 0.6, 19.3 ± 0.8; 6-NCS-Fsk (20 μM), 8.2 ± 0.4, 10.6 ± 1.2; and 6-NCS-Fsk (100 μM), 5.3 ± 0.1, 6.7 ± 0.3. Experiment 2: No addition (buffer alone), 5.9 ± 0.1, 23.8 ± 1.6; 6-NCS-DDFsk (4 μM), 7.2 ± 0.2, 24.9 ± 0.8; 6-NCS-DDFsk (20 μM), 6.5 ± 0.7, 21.7 ± 1.7; and 6-NCS-DDFsk (100 μM), 2.7 ± 0.2, 10.0 ± 0.1.

the inactive Fsk analog. When used at ≤10 μM, the specificity of 6-NCS-Fsk for interacting with a nucleophilic group at the high affinity Fsk binding site was easily demonstrated (Figs. 1–3). However, the specificity for the interaction of 6-NCS-Fsk at the Fsk activation site was difficult to demonstrate because higher concentrations (20 and 100 μM) were required for irreversible inhibition of Fsk activation (Fig. 5). At the higher concentrations, there probably was some modification of nucleophilic group(s) that was unrelated to the Fsk site of action and affected the enzyme activity overall.

The specificity of 6-NCS-Fsk, as well as BrAcFsk and 6-[[*N*-(2-(bromoacetyl)ethyl]amino]carbonyl]forskolin, for the adenylyl cyclase has been examined previously. The irreversible interaction of 6-NCS-Fsk at the Fsk binding site is consistent with previous studies showing that Fsk binding to rat brain and platelet adenylyl cyclase was sensitive to treatment with BrAcFsk (27). Photoincorporation of [^{125}I]6-AIPP-Fsk into adenylyl cyclase in bovine brain membranes was irreversibly inhibited by BrAcFsk but not 7-bromoacetyl-7-desacetyl-1,9-dideoxyforskolin, the chemically reactive derivative of the inactive Fsk analog, suggesting that BrAcFsk was reacting specifically with a nucleophilic group at the high affinity Fsk binding site of adenylyl cyclase (33). Although these Fsk derivatives bind also to the human erythrocyte glucose transporter (another Fsk binding protein that binds Fsk with a lower affinity), they do not bind irreversibly, presumably because there is no reactive nucleophilic group at the Fsk binding site of the transporter (22, 33). Furthermore, the irreversible inhibition of photolabeling of bovine brain adenylyl cyclase by BrAcFsk is not observed when BrAcFsk is chemically inactivated by pretreatment with β-mercaptoethanol, demonstrating that the ability of BrAcFsk to bind irreversibly requires a chemically reactive group (33). The irreversible interaction of 6-NCS-Fsk at the Fsk activation site is consistent with previous studies showing that Fsk activation of rat brain and platelet adenylyl cyclase was sensitive to treatment with the sulfhydryl reagent, *N*-ethylmaleimide (34).

High affinity Fsk binding sites display structure-activity requirements consistent with the ability of Fsk to activate adenylyl cyclase in rat brain membranes (20, 24), and Fsk interactions are alike in their sensitivity to $G_{s\alpha}$ regulation, suggesting that the two interactions occur at the same site on the protein. In type II adenylyl cyclase, Fsk activation is potentiated significantly by activated $G_{s\alpha}$, and activated $G_{s\alpha}$ is required to measure high affinity Fsk binding and detect efficient Fsk photoaffinity labeling (17). In type I adenylyl cyclase, Fsk binding and Fsk activation are insensitive to regulation by activated $G_{s\alpha}$. However, the affinity of Fsk at the high affinity binding site does not equate with the potency of Fsk as an activator of adenylyl cyclase, suggesting, alternatively, that the two interactions may occur at two different sites on the protein. In the absence of exogenous $G_{s\alpha}$, the recombinant type I adenylyl cyclase binds Fsk with high affinity; Fsk displaces [^{125}I]6-IHPP-Fsk binding with a K_d value of 41 ± 6 nM (17). Type I adenylyl cyclase is activated by Fsk with an EC_{50} of ~1 μM in the absence of exogenous $G_{s\alpha}$. The results reported here with 6-NCS-Fsk are consistent with there being two sites of Fsk interaction on type I adenylyl cyclase: a high affinity site that binds Fsk analogs and Fsk photolabel and is irreversibly alkylated by 6-NCS-Fsk with an IC_{50} of ~300 nM, and a low affinity site that stimulates enzyme activity at higher concentrations of Fsk and is irreversibly alkylated by 6-NCS-Fsk with an IC_{50} of ~10 μM. The data reported here may explain why Fsk dose-response curves for activation of adenylyl cyclase extend over many orders of magnitude.

Pretreatment with 2 μM 6-NCS-Fsk caused an irreversible loss of ~90% of the high affinity Fsk binding sites (Fig. 3), whereas 2 μM 6-NCS-DDFsk had little, if any, effect. Thus, 6-NCS-Fsk (2 μM) inhibited binding specifically at the Fsk binding site. However, after pretreatment with 2 μM 6-NCS-Fsk, ~75% of the Fsk-stimulated increase in enzyme activity remained (Fig. 5), suggesting that high affinity Fsk binding may not be required for Fsk activation of type I adenylyl cyclase. These results are consistent with the previously reported inability to detect Fsk photoaffinity labeling of other adenylyl cyclases (types III, V, and VI) that are stimulated by Fsk (17). Previous studies of the loss of [^{3}H]Fsk binding sites in human platelet membranes by BrAcFsk suggested that there may be two Fsk binding sites with high and low affinity on adenylyl cyclase (27). However, these studies were difficult to interpret because of the presence of G_s protein and the possibility of multiple types of adenylyl cyclase being present in the platelet membranes.

Because adenylyl cyclase is made up of two homologous

domains, it is tempting to speculate that high affinity Fsk binding may involve one domain and activation by Fsk may involve the other domain. In other mammalian enzymes with two homologous domains, there is precedence for only one domain being involved in catalysis (35). It has been suggested that adenylyl cyclase is regulated by an open/closed transition of the catalytic domain and that stimulators (such as Fsk) promote formation of the closed state (36, 37). This may explain why there was no activation of enzyme activity on the addition of 6-NCS-Fsk or BrAcFsk (Fig. 4). The covalent attachment of these derivatives of Fsk with a nucleophilic group at the Fsk site of action may have prevented the conformational change required for the transition between the states. It will be interesting to examine the interaction of 6-NCS-Fsk with other adenylyl cyclase subtypes.

Alternatively, high affinity Fsk binding may involve a membranous domain, and activation by Fsk may involve a soluble domain. Indeed, a soluble form of adenylyl cyclase (composed of the amino-terminal soluble portion of type I adenylyl cyclase and the carboxyl-terminal soluble portion of type II adenylyl cyclase) has been constructed that is activated by $G_{s\alpha}$ and Fsk, although it lacks the membranous domains (38). It will be interesting to study the interaction of 6-NCS-Fsk with the soluble form of the enzyme and to determine whether the soluble form retains the high affinity Fsk binding site.

The results establish the potential usefulness of the isothiocyanate derivative of Fsk in localizing the site(s) of Fsk interaction with type I adenylyl cyclase. This will involve reacting 6-NCS-Fsk with purified adenylyl cyclase, generating proteolytic fragments, and using mass spectrometry to identify the chemically modified amino acid residue.

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