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Interaction of Aminoalkylcarbamates of Forskolin with Adenylyl Cyclase: Synthesis of an Iodinated Derivative of Forskolin with High Affinity for Adenylyl Cyclase

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SUMMARY

7-(2-Aminoethyl)aminocarbonyl-7-desacetylforskolin (7-AEC-Fsk) and 6-(2-aminoethyl)aminocarbonylforskolin (6-AEC-Fsk) were synthesized and tested for their ability to activate adenylyl cyclase and inhibit the high affinity binding of [^{3}H]forskolin to bovine brain membranes. Forskolin and 7-AEC-Fsk were equipotent in activating adenylyl cyclase, with EC_{50} values of about 4 μM, whereas 6-AEC-Fsk had an EC_{50} of about 2 μM. 6-AEC-Fsk and 7-AEC-Fsk stimulated adenylyl cyclase about 7-fold over basal levels at 100 μM, whereas forskolin produced a 5-fold stimulation. Forskolin and 6-AEC-Fsk inhibited the binding of [^{3}H]forskolin to bovine brain membranes with K_d values of 41 nM and 28 nM, respectively, whereas 7-AEC-Fsk had a K_d of 83 nM. The 3-(3-iodo-4-hydroxyphenyl)propionamide derivative of 6-AEC-Fsk (6-I-HPP-Fsk) was more potent than forskolin in inhibiting [^{3}H]forskolin binding to bovine brain membranes, with a K_d of 14 nM. 6-AEC-Fsk was reacted with ^{125}I-labeled Bolton-Hunter reagent to produce 6-^{125}I-HPP-Fsk with a specific activity of 2175 Ci/mmol. 6-^{125}I-HPP-Fsk bound to bovine brain membranes with a K_d of 13 nM and a B_{max} of 3.8 pmol/mg of protein. Forskolin inhibited the binding of 6-^{125}I-HPP-Fsk to bovine brain membranes with a K_d of 31 nM, whereas 1,9-dideoxyforskolin only slightly inhibited the binding at 10 μM. The binding of 6-^{125}I-HPP-Fsk was not inhibited by agents that inhibit forskolin binding to the glucose transporter, such as D-glucose or cytochalasin B. There was no displaceable binding of 6-^{125}I-HPP-Fsk to red blood cell membranes, which contain a large concentration of the glucose transporter. Pretreatment of bovine brain membranes with an alkylating derivative of forskolin, 7-bromoacetyl-7-desacetylforskolin (BrAcFsk), led to an irreversible decrease in the binding of [^{3}H]forskolin and 6-^{125}I-HPP-Fsk. The time dependence and concentration dependence for the BrAcFsk-induced decrease in [^{3}H]forskolin binding sites were identical to those observed for the decrease in 6-^{125}I-HPP-Fsk binding sites. 6-^{125}I-HPP-Fsk binding was determined in human platelet membranes in the presence of Mg^{2+} alone and in combination with guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) or AlF_4^-. The presence of GTPγS or AlF_4^- increased the binding of 6-^{125}I-HPP-Fsk by 4.5-fold and 4-fold, respectively. An identical increase in [^{3}H]forskolin binding was observed in the presence of GTPγS or AlF_4^-.

The diterpene forskolin was first described as a unique and specific activator of mammalian and hormone-sensitive adenylyl cyclase (1, 2). Forskolin activates adenylyl cyclase rapidly and reversibly in cells, membranes, solubilized preparations, and intact animals, with an EC_{50} in the micromolar range (3). Forskolin interacts directly with the adenylyl cyclase catalytic subunit, and forskolin coupled covalently to agarose resins has been used to purify the adenylyl cyclase catalytic subunit (4–7). Although forskolin activates adenylyl cyclase with an EC_{50} in the micromolar range, high affinity binding sites for forskolin are detected in different mammalian tissues, and these sites have been associated with an activated complex of the adenylyl cyclase catalytic subunit and the guanine nucleotide regulatory protein that mediates hormonal stimulation of adenylyl cyclase, G_s (8–10).

Forskolin was first described as a specific activator of adenylyl cyclase, whose physiological effects were entirely due to increases in cAMP; however, forskolin produces effects that are unrelated to increases in cAMP (11). Forskolin inhibits the facilitated glucose transporter from a number of tissues (12–16), inhibits nicotinic acetylcholine receptor function (17–21), inhibits $GABA_A$-mediated chloride flux (22), and inhibits voltage-dependent K^+ channels (23–26). Forskolin's inhibition of glucose transport is probably mediated by the direct interaction of forskolin with the transport protein, because forskolin and forskolin derivatives can photolabel the glucose transporter (27, 28).

The structural requirements for forskolin activation of ad-

ABBREVIATIONS: GABA, γ-aminobutyric acid; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; 7-AEC-Fsk, 7-(2-aminoethyl)aminocarbonyl-7-desacetylforskolin; 6-AEC-Fsk, 6-(2-aminoethyl)aminocarbonylforskolin; 6-I-HPP-Fsk, 3-(3-iodo-4-hydroxyphenyl)propionamide derivative of 6-(2-aminoethyl)aminocarbonylforskolin; BrAcFsk, 7-bromoacetyl-7-desacetylforskolin; BrAcDDFsk, 7-bromoacetyl-7-desacetyl-1,9-dideoxyforskolin; *t*-Boc, *N-tert*-butoxycarbonyl; FMOC, *N*-(9-fluorenylmethoxycarbonyl).

enylyl cyclase were described, and derivatives of forskolin have been identified that are unable to activate adenylyl cyclase (29, 30). In particular, 1,9-dideoxyforskolin does not activate adenylyl cyclase but can reproduce cAMP-independent effects of forskolin. 1,9-Dideoxyforskolin inhibits glucose transport, nicotinic acetylcholine receptor function, $GABA_A$ receptor function, and voltage-dependent K^+ channels (11). Some information is available concerning the differences between the forskolin binding site on adenylyl cyclase and the forskolin binding site on the glucose transporter. Lipophilic analogs of forskolin are more potent at the glucose transporter than at adenylyl cyclase, whereas water-soluble derivatives of forskolin are more potent at adenylyl cyclase than at the glucose transporter (16). These studies indicate that the binding sites for forskolin on these different membrane-associated proteins are structurally different.

One of the problems in studying forskolin interactions with different proteins is the lack of specific derivatives that discriminate between the different sites of action. In addition, many of the forskolin derivatives that are used to study forskolin-binding proteins are carboxylic acid esters of forskolin derivatized at the 7-position. However, the ester linkage is subject to hydrolysis by nonspecific esterases and extremes in pH, and acyl group migration from the 7-position of forskolin to the 6-position has been observed under mild conditions (31, 32). Derivatives of forskolin with reactive groups attached to forskolin through a more stable carbamate linkage might be better suited for the preparation of forskolin analogs for biological studies. Two forskolin intermediates were developed for the synthesis of derivatives useful in biological studies, whose structures are shown in Fig. 1 (33), 7-AEC-Fsk and 6-AEC-Fsk. These were used to synthesize iodinated arylazido derivatives of forskolin that label adenylyl cyclase, the glucose transporter, and the P-glycoprotein (34, 35). In this paper, we describe the synthesis of an iodinated derivative of forskolin, 6-^{125}I-HPP-Fsk, which can be used as a specific high affinity ligand to measure adenylyl cyclase.

Experimental Procedures

Materials. Bolton-Hunter reagent, [^{3}H]cAMP, [^{3}H]forskolin, and [α-^{32}P]ATP were obtained from DuPont-New England Nuclear. Forskolin and 1,9-dideoxyforskolin were from Calbiochem. ATP, creatine phosphokinase, phosphocreatine, and other biochemicals were obtained from Sigma. BrAcDDFsk and BrAcFsk were synthesized as described (36). 6-AEC-Fsk and 7-AEC-Fsk were synthesized as described by Robbins *et al.* (33).

Synthesis of 6-^{125}I-HPP-Fsk. Bolton-Hunter reagent (1 mCi) from NEN was concentrated to dryness under N_2. A 20-μl aliquot of 6-AEC-Fsk (1 mg/ml in CH_2Cl_2) was added to the reaction vial and allowed to react at room temperature overnight. It was important to use as little volume as possible for the reaction, due to the low concentration of the ^{125}I-Bolton-Hunter reagent. The reaction was monitored by thin layer chromatography on silica plates, with ethyl acetate as developing solvent, and was visualized by autoradiography. The ^{125}I-labeled Bolton-Hunter reagent has an R_F of 0.9, and 6-^{125}I-HPP-Fsk has an R_F of 0.6. The reaction mixture was applied to a small silica column (0.9 cm × 2 cm) equilibrated with ethyl acetate. Fractions (0.3 ml) were collected and monitored by thin layer chromatography on silica plates, followed by autoradiography. The fractions corresponding to 6-^{125}I-HPP-Fsk were pooled and stored at room temperature in ethanol. The 6-^{125}I-HPP-Fsk was separated completely from 6-AEC-Fsk using these chromatographic conditions and, therefore, the labeled compounds are assumed to be carrier-free, with the specific activity of the ^{125}I-Bolton Hunter reagent (about 2175 Ci/mmol). The yields for the radioactive product range from 50 to 75% of the starting radioactivity.

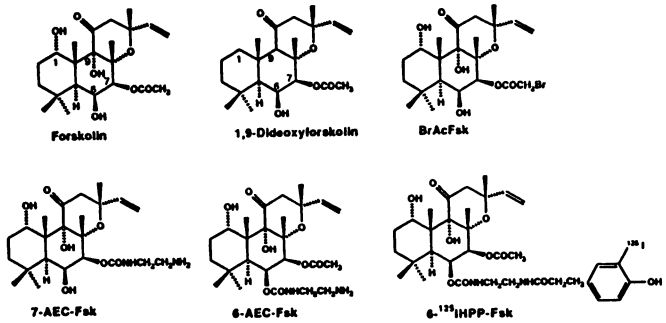

Fig. 1. Structures of forskolin and forskolin derivatives.

Preparation of membranes. Bovine brains, obtained from a local slaughterhouse, were immersed in ice-cold homogenization buffer and brought to the laboratory. The homogenization buffer contained 10 mM Tris·HCl, pH 7.4, 0.32 M sucrose, 10 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 10^{-7} M leupeptin, 10^{-6} M pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. The cortex was dissected and homogenized in a Waring blender, using a ratio of about 500 ml of buffer/brain. The homogenate was passed twice through four layers of cheesecloth and then centrifuged at 20,000 × *g* for 30 min. The pellet was resuspended to the original volume with homogenization buffer, in a Potter homogenizer, and then centrifuged at 20,000 × *g* for 40 min. The last step was repeated and the resuspended material was divided in 40-ml aliquots, frozen in liquid nitrogen, and stored at −80°.

Washed human erythrocytes and membrane ghosts were prepared by the method of Steck and Kant (37). Ghost membranes in 5 mM sodium phosphate buffer, pH 8, were isolated by centrifugation at 17,400 × *g* for 15 min, in a Sorvall SS-34 rotor, and were washed three or four times with the same buffer. Membrane ghosts were frozen at −70° until use.

Human platelet membranes were prepared as described by Siegl *et al.* (38) and stored at −70° until use.

Binding of [^{3}H]forskolin to bovine brain membranes. Briefly, the membrane suspension was prepared as described above, diluted with ice-cold 50 mM Tris·HCl buffer, pH 7.5, centrifuged at 20,000 × *g* for 10 min, and resuspended in 50 mM Tris·HCl buffer, pH 7.5, at a protein concentration of 2 mg/ml. The incubations were carried out at room temperature for 60 min, in 12- × 75-mm glass test tubes, in a total volume of 0.4 ml of 50 mM Tris·HCl buffer, pH 7.4 as described (39). The membranes (0.40 mg/tube) were incubated with 10 mM [^{3}H] forskolin in the presence of 5 mM $MgCl_2$ and 10 mM NaF. The assay was terminated by rapid filtration over Whatman GF/C filters, using a Brandel cell harvester (Gaithersburg, MD). The filters were quickly washed three times with 4 ml of ice-cold buffer and were placed in scintillation vials for determination of radioactivity. In some experiments, specific [^{3}H]forskolin binding was calculated as the difference between total binding in the absence of unlabeled forskolin and nonspecific binding in the presence of 20 μM forskolin. The nonspecific binding was about 10% of the total counts. In competition experiments the nonspecific binding and the binding parameters were analyzed using the LIGAND program (40).

Binding of 6-^{125}I-HPP-Fsk to membranes. Bovine brain membranes were prepared as described above and incubated for 60 min, at room temperature, with about 20,000 cpm of 6-^{125}I-HPP-Fsk, unless otherwise indicated. Binding equilibrium was reached by 45 min and was constant for at least 2 hr (data not shown). The assays were terminated by filtration through 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 then counted in a γ-counter. The Brandel

harvester was rinsed between filter mats by aspiration of 95% ethanol through the apparatus, in order to minimize carry-over of radioactivity and to decrease nonspecific absorption of the ligand to the filter apparatus.

Alkylation of membranes with BrAcFsk. Membranes were pretreated with BrAcFsk or other agents as described (36), with slight modifications. Briefly, suspensions of membranes were incubated at room temperature with BrAcFsk at the indicated concentrations and times. The reaction was stopped by the addition of 30 volumes of ice-cold 50 mM Tris·HCl buffer, pH 7.5, and centrifugation at 20,000 × *g* for 10 min. The pellet was resuspended in the same buffer and centrifuged as described above. This washing step was repeated five times. Membranes were then resuspended at a protein concentration of 2 mg/ml, and binding was determined as described above.

Adenylyl cyclase assay. Incubations were in a total volume of 250 μl, containing 50 mM Tris·HCl buffer, pH 7.5, 1 mM 3-isobutyl-1-methylxanthine, 5 mM $MgCl_2$, and 0.1 mM ATP. Each assay mixture contained 1 μCi of [α^{32}P]ATP and an ATP-regenerating system consisting of 5 units of creatine kinase and 2 mM creatine phosphate. Assays were initiated by the addition of 25 μl of membranes (about 100 μg of membrane protein), carried out at 30° for 10 min, and terminated by the addition of 0.5 ml of 10% trichloroacetic acid. Carrier cAMP (0.25 ml of 1 mM cAMP solution, containing about 20,000 cpm of [^{3}H]cAMP) was added, and cAMP was isolated and analyzed as described by Salomon *et al.* (41). Assays were carried out in triplicate and are reported as the mean and standard deviation.

Protein determination. The protein content was determined with the bicinchoninic acid method (42), using bovine serum albumin as standard.

Results

Activation of adenylyl cyclase. The aminoalkylcarbamates were tested for their ability to activate adenylyl cyclase in membranes from bovine brain (Fig. 2). Forskolin stimulated adenylyl cyclase in bovine brain membranes 4.5-fold over basal levels, with an EC_{50} of about 8 μM. 7-AEC-Fsk was equipotent with forskolin but was more efficacious than forskolin, producing a 6.5-fold stimulation at 100 μM. 6-AEC-Fsk was more potent than forskolin or 7-AEC-Fsk, with an EC_{50} of about 2 μM, and stimulated the enzyme activity about 7-fold at 100 μM.

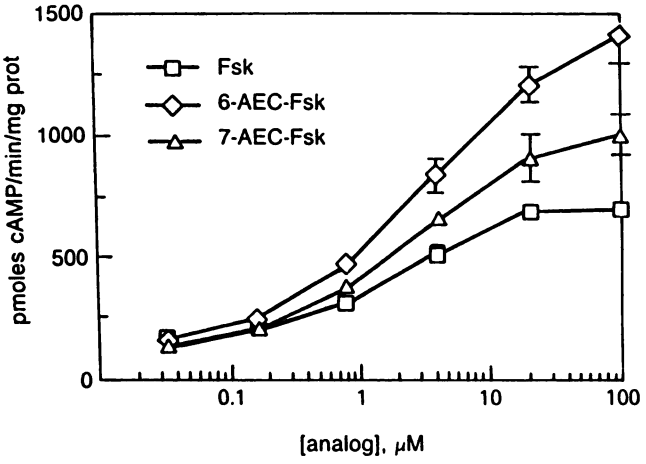

Fig. 2. Stimulation of adenylyl cyclase in bovine brain membranes by forskolin and derivatives of forskolin. Adenylyl cyclase in bovine brain membranes was assayed as described in Experimental Procedures, with the indicated concentrations of forskolin (□), 6-AEC-Fsk (◇), and 7-AEC-Fsk (△). The data are from one experiment and are representative of three different experiments. Data are expressed as the mean of triplicate determinations with the standard deviation indicated, unless the value is too small to be represented.

Inhibition of [^{3}H]forskolin binding to bovine brain membranes. The aminoalkylcarbamates of forskolin were tested for their ability to inhibit [^{3}H]forskolin binding to bovine brain membranes (Fig. 3). The K_d values for binding to bovine brain membranes were calculated using the LIGAND program and are given in Table 1. Forskolin bound to bovine brain membranes with a K_d of 41 ± 5 nM. 6-AEC-Fsk and 6-I-HPP-Fsk were more potent than forskolin, with K_d values of 28 ± 2 nM and 14 ± 2 nM, respectively. 7-AEC-Fsk was less potent than 6-AEC-Fsk, with a K_d of 83 ± 14 nM. 1,9-Dideoxyforskolin only slightly inhibited the binding at 10 mM.

Binding of 6-^{125}I-HPP-Fsk to bovine brain membranes. The assay for detecting the binding of 6-^{125}I-HPP-Fsk to bovine brain membranes was remarkably reproducible, using the procedure given in Experimental Procedures. The assays were carried out in triplicate, and the data points did not generally differ by more than about 5%. The washing of the filter apparatus with 95% ethanol was carried out to minimize carry-over of radioactivity between filter mats and was felt to be important for the consistency of the results. The K_d value of 6-^{125}I-HPP-Fsk for binding to bovine brain membranes was determined by incubating membranes with a constant amount

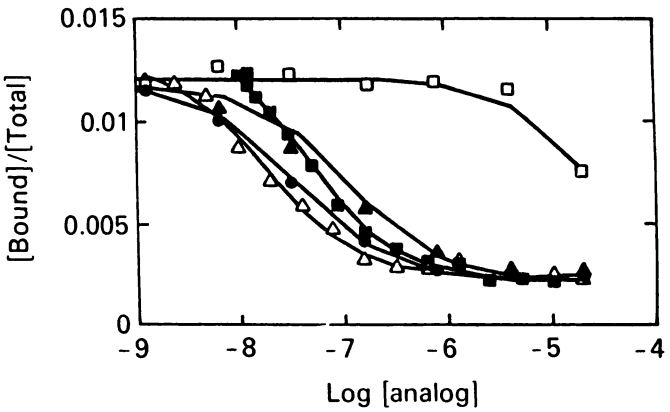

Fig. 3. Inhibition of [^{3}H]forskolin binding to bovine brain membranes by forskolin and derivatives of forskolin. Bovine brain membranes were incubated with the indicated concentrations of forskolin (■), 6-AEC-Fsk (●), 7-AEC-Fsk (▲), 6-I-HPP-Fsk (△), or 1,9-dideoxyforskolin (□) for 60 min at room temperature, in the presence of 10 nM [^{3}H]forskolin. Binding was determined as described in Experimental Procedures. Each data point was determined in triplicate, and the standard deviation for each point was <10%. The data were then analyzed using the program LIGAND, and the *line* drawn through the data points corresponds to the computer-fitted K_d, as given in Table 1.

TABLE 1

K_d values for forskolin and analogs, measured by inhibition of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk binding

The K_d values were analyzed for a one-site model using the LIGAND program. A two-site model did not lead to a better fit of the data.

Analog	K_d	
	[^{3}H]Forskolin[a]	6-^{125}I-HPP-Fsk[b]
	nM	
Forskolin	41 ± 5	31 ± 4
6-AEC	28 ± 2	26 ± 8
7-AEC	83 ± 14	95 ± 26
6-I-HPP-Fsk	14 ± 2	13 ± 3

[a] The K_d values were calculated from the data shown in Fig. 3.
[b] The K_d values were calculated from the data shown in Fig. 5.

of 6-^{125}I-HPP-Fsk, in the presence of different concentrations of nonradioactive 6-I-HPP-Fsk. The data were then analyzed using the program LIGAND. Each data point was determined in triplicate, and the standard deviation for each data point was <10%. Data from three separate experiments are shown in Fig. 4. The data from all three experiments were analyzed together, using the LIGAND program, and were fit to a one-site model with the following parameters: K_d = 16 ± 2 nM and B_{max} = 3.8 ± 0.3 pmol/mg of protein. There was no improvement in the fit by using a two-site model for the fit.

The ability of forskolin analogs to inhibit 6-^{125}I-HPP-Fsk binding to bovine brain membranes was determined by competition studies (Fig. 5), and the K_d values were determined with the LIGAND program (Table 1). The K_d values for forskolin and analogs determined from their inhibition of 6-^{125}I-HPP-Fsk binding to bovine brain membranes agreed well with the K_d values determined from their inhibition of [^{3}H]forskolin binding (Table 1). The K_d for forskolin determined from its ability to inhibit 6-^{125}I-HPP-Fsk binding was 31 ± 4 nM, whereas the K_d for [^{3}H]forskolin was 41 ± 5 nM. 1,9-Dideoxyforskolin only slightly inhibited the binding of 6-^{125}I-HPP-Fsk at 10 μM (Fig. 5). 6-AEC-Fsk had a K_d of 26 ± 8 nM and was more potent than 7-AEC-Fsk, which had a K_d of 95 ± 26 nM.

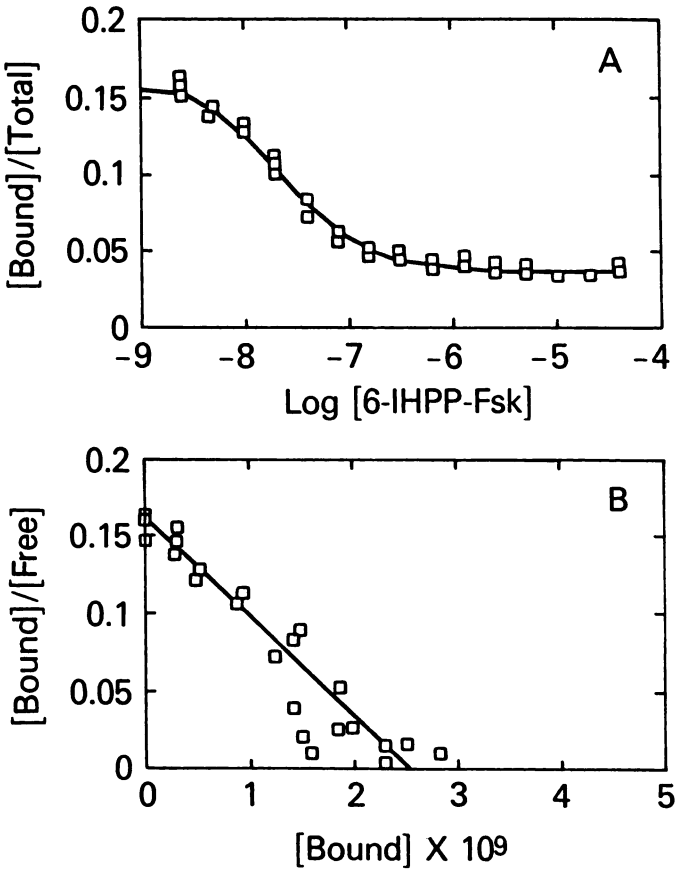

Fig. 4. Binding of 6-^{125}I-HPP-Fsk to bovine brain membranes. Bovine brain membranes were incubated with about 20,000 cpm of 6-^{125}I-HPP-Fsk in the presence of different concentrations of nonradioactive 6-I-HPP-Fsk, for 1 hr at room temperature, and then processed as described in Experimental Procedures. The data from all three experiments were analyzed together using the LIGAND program and were fit to a one-site model. The binding parameters were K_d = 16 ± 2 nM and B_{max} = 3.8 ± 0.3 pmol/mg of protein. Data from three different experiments are show as a displacement curve in A, and the Scatchard plot is shown in B; the curves are computer generated based on the fitted parameters.

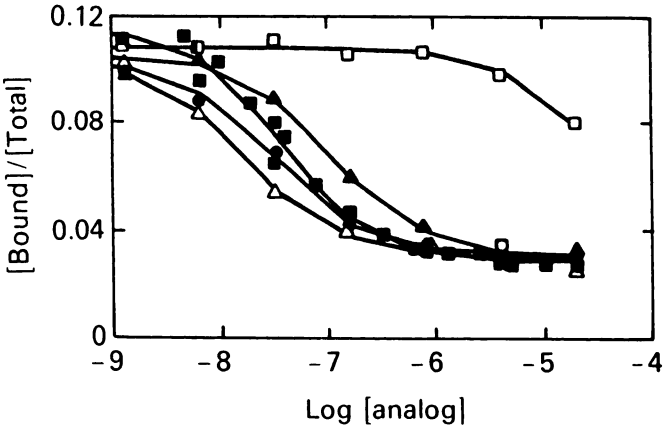

Fig. 5. Inhibition of 6-^{125}I-HPP-Fsk binding to bovine brain membranes by forskolin and forskolin derivatives. Bovine brain membranes were incubated with the indicated concentrations of forskolin (■), 6-AEC-Fsk (●), 7-AEC-Fsk (▲), 6-I-HPP-Fsk (△), or 1,9-dideoxyforskolin (□) for 60 min at room temperature, in the presence of about 20,000 cpm of 6-^{125}I-HPP-Fsk. Binding was determined as described in Experimental Procedures. Each data point was determined in triplicate, and the standard deviation for each point was <10%. The data were then analyzed using the program LIGAND, and the *line* drawn through the data points corresponds to the computer-fitted K_d as given in Table 1.

TABLE 2

Compounds that do not inhibit 6-^{125}I-HPP-Fsk binding to bovine brain membranes

The agents were assayed at the indicated concentrations, as described in Experimental Procedures, using bovine brain membranes. The experiment was repeated three times, with assays in triplicate. None of the agents inhibited the specific binding of 6-^{125}I-HPP-Fsk by >10% at the indicated concentrations.

Agent	Concentration
D-Glucose	0.5 M
3-*O*-Methyl-D-glucose	0.5 M
2-Deoxy-D-glucose	0.5 M
Phloretin	20 μM
Phloridzin	20 μM
Diethylstilbestrol	20 μM
Dexamethasone	20 μM
Cortisone	20 μM
Androsterone	20 μM
17-β-Estradiol	20 μM
Cytochalasin B	20 μM
Carbachol	20 μM
Phencyclidine	20 μM
Verapamil	20 μM
Diltiazem	20 μM
Thioridazine	20 μM
Vincristine	20 μM
Vinblastine	20 μM
Actinomycin D	20 μM

Agents that bind to the glucose transporter were tested for their ability to inhibit 6-^{125}I-HPP-Fsk binding to bovine brain membranes (Table 2). Cytochalasin B and D-glucose were ineffective at inhibiting 6-^{125}I-HPP-Fsk binding at concentrations of 20 μM and 0.5 M, respectively. Phloretin and phloridzin, at concentrations of 20 μM, had no effect on 6-^{125}I-HPP-Fsk binding. The steroids diethylstilbestrol, androsterone, and dexamethasone also had no effect on 6-^{125}I-HPP-Fsk binding to bovine brain membranes, at concentrations of 20 μM. The calcium channel blockers verapamil and nifedipine, as well as the *Vinca* alkaloids vincristine and vinblastine, had no effect on binding.

Modulation of 6-^{125}I-HPP-Fsk binding in human platelet membranes. [^{3}H]Forskolin binding to bovine brain membranes and human platelet membranes is increased when membranes are treated with agents that activate the G_s protein (8–10). 6-^{125}I-HPP-Fsk and [^{3}H]forskolin binding was determined in human platelet membranes in the presence of GTPγS or AlF_4^-, with and without $MgCl_2$ (Fig. 6). The amount of 6-^{125}I-HPP-Fsk and [^{3}H]forskolin bound to platelet membranes was increased about 4-fold in the presence of $MgCl_2$ and AlF_4^- or GTPγS (Fig. 6). The maximal increase in binding observed with either GTPγS or AlF_4^- required the presence of Mg^{2+}. A 2-fold increase in binding was observed for either [^{3}H]forskolin or 6-^{125}I-HPP-Fsk in bovine brain membranes, using the same experimental conditions (data not shown).

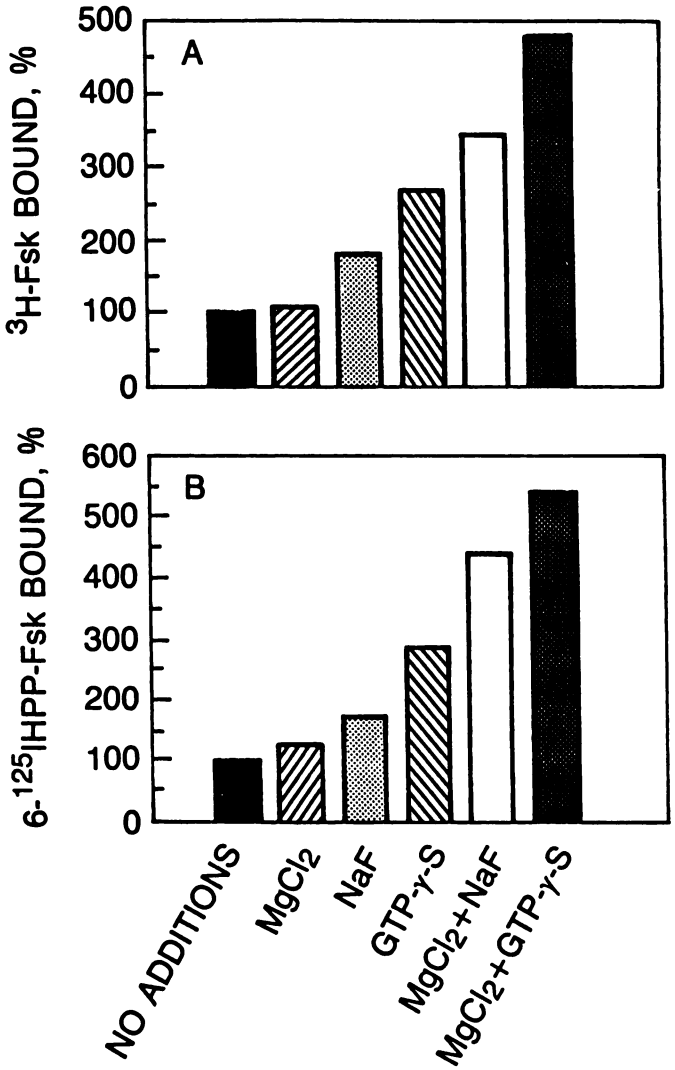

Fig. 6. Modulation of [^{3}H]forskolin (A) and 6-^{125}I-HPP (B) binding to human platelet membranes by $MgCl_2$, NaF, and GTPγS. Human platelet membranes (400 μg of protein/assay) were incubated with 10 μM [^{3}H] forskolin (A) or 20,000 cpm of 6-^{125}I-HPP-Fsk (B), in the presence of the indicated agents. All assays contained 50 mM Tris-HCl, pH 7.5 in the presence or absence of 5 mM $MgCl_2$ (*$MgCl_2$*), 10 mM NaF and 10 μM $AlCl_3$ (*NaF*) or 100 μM GTP-γ-S (*GTP-γ-S*) as indicated. After incubation at room temperature for 1 hr, the membranes were filtered as described in Experimental Procedures and the amount of specific binding was determined. The data are plotted as percentage of control binding (determined in the presence of 50 mM Tris, pH 7.5). The standard deviations from triplicate assays were <10% and are not plotted. The data are representative of two different experiments.

The binding of 6-^{125}I-HPP-Fsk to human platelet membranes was determined in the presence of 5 mM $MgCl_2$ alone and in the presence of 10 mM NaF and 10 μM $AlCl_3$, to promote the activation of G_s. The major effect of activation was to increase the B_{max} for binding. 6-^{125}I-HPP-Fsk bound to nonactivated membranes and activated membranes with a K_d of 6 ± 3 nM (two experiments). The B_{max} was 194 ± 58 fmol/mg of protein (two experiments) in the absence of activation and 660 ± 200 fmol/mg of protein (two experiments) in membranes activated with fluoride.

Effect of BrAcFsk on 6-^{125}I-HPP-Fsk binding. BrAcFsk inhibits 6-^{125}I-HPP-Fsk binding to bovine brain membranes with an IC_{50} of 0.8 μM (data not shown). BrAcFsk was tested for its ability to irreversibly block 6-^{125}I-HPP-Fsk and [^{3}H]forskolin binding. Membranes were incubated with no additions, 20 μM forskolin, 20 μM BrAcFsk, 20 μM bromoacetic acid, or 20 μM BrAcDDFsk. The membranes were then washed extensively, and the amount of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk binding was determined (Fig. 7). The amount of bound [^{3}H]forskolin or 6-^{125}I-HPP-Fsk was not affected by preincubation with forskolin, bromoacetic acid, or BrAcDDFsk. In contrast, there was only about 15% of the specific binding of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk in membranes that were pretreated with BrAcFsk.

The irreversible decrease in binding of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk produced by BrAcFsk was dose dependent, with 50% of the binding being lost at a concentration of BrAcFsk of about 4 μM (Fig. 8). The decrease in binding of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk showed the same concentration dependence on BrAcFsk.

The irreversible decrease in [^{3}H]forskolin and 6-^{125}I-HPP-Fsk binding by BrAcFsk was studied as a function of time. Membranes were treated with 20 μM BrAcFsk, in the presence and absence of 200 μM forskolin, for different times; the membranes were immediately diluted with cold buffer and washed three times with cold buffer. The irreversible decrease in the binding of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk showed the same time dependence (Fig. 9). The inclusion of 200 μM forskolin during the pretreatment could partially prevent the decrease in binding. In the presence of 200 μM forskolin, there was only about a 60% decrease in specific binding, even at 30 min (Fig. 9).

Discussion

Forskolin has been used principally as an agent to study adenylyl cyclase. In this regard, forskolin activates adenylyl cyclase *in vitro* and *in vivo* (for review, see Ref. 3), and forskolin esters have been covalently attached to solid supports and used to purify the catalytic subunit of adenylyl cyclase (4–7), thus allowing the cloning of at least one form of the bovine brain catalytic subunit (43). Photoaffinity labels derived from 7-desacetyl-7-hemisuccinylforskolin label the glucose transporter in different tissues, demonstrating a direct interaction of forskolin with the transporter (27, 28). Forskolin also interacts with a number of different membrane proteins, including the glucose transporter, the nicotinic acetylcholine receptor, and voltage-dependent K^+ channels (for review, see Ref. 11). Currently, there is great interest in defining analogs of forskolin that might have enhanced selectivity at the different sites of

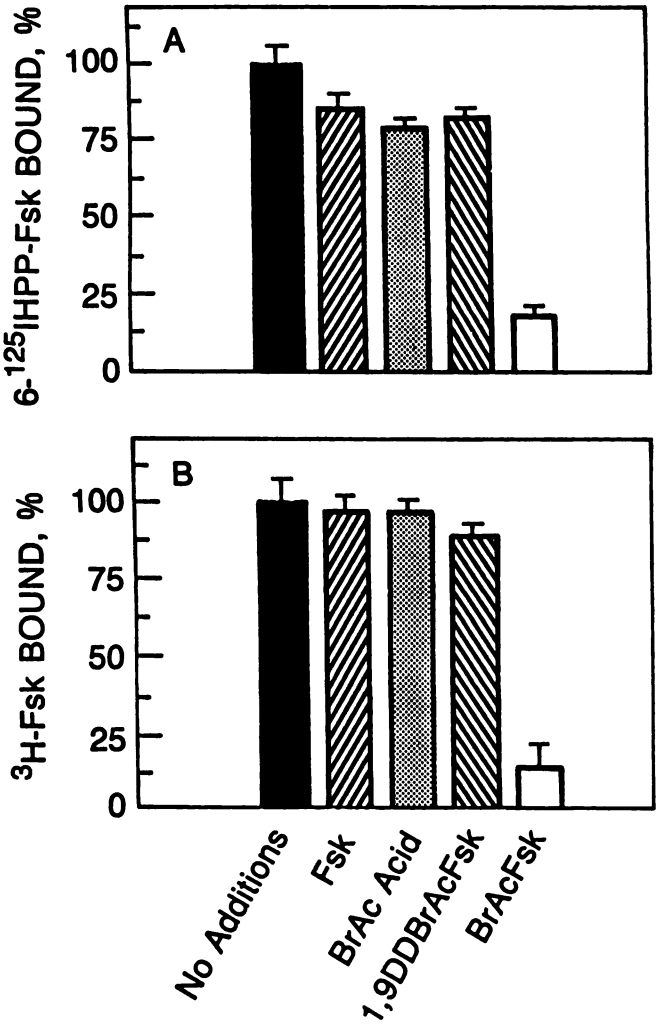

Fig. 7. Effect of pretreatment of bovine brain membranes with BrAcFsk on 6-^{125}I-HPP-Fsk (A) and [^{3}H]forskolin (B) binding. Bovine brain membranes were incubated for 20 min at 25° in the presence of 50 mM Tris·HCl, pH 7.5 (*No Additions*), or with 20 μM forskolin (*Fsk*), 20 μM bromoacetic acid (*BrAc Acid*), 20 μM BrAcDDFsk (*1,9DDBrAcFsk*), or 20 μM BrAcFsk (*BrAcFsk*). The membranes were then washed extensively and resuspended in 50 mM Tris·HCl buffer, pH 7.5, and the binding of 6-^{125}I-HPP-Fsk (A) or [^{3}H]forskolin (B) was determined as described in Experimental Procedures. The percentage of specific binding, compared with incubations with no additions, is plotted as the average of triplicate determinations, with the standard deviation indicated. The data are representative of two different experiments.

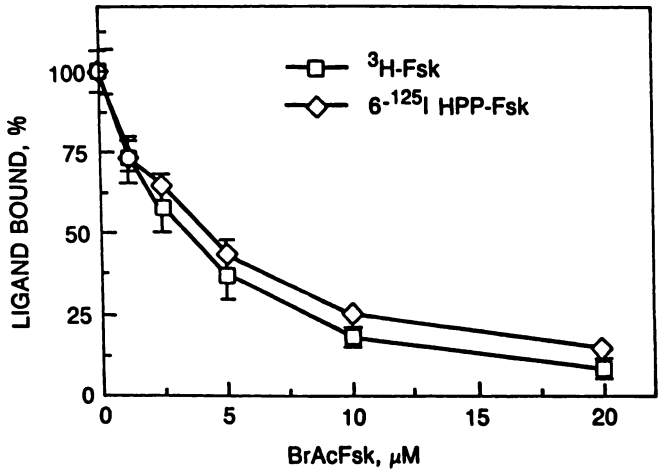

Fig. 8. Concentration dependence of the loss of [^{3}H]forskolin and 6-^{125}I-HPP-Fsk binding to bovine brain membranes by pretreatment with BrAcFsk. Bovine brain membranes were incubated with the indicated concentrations of BrAcFsk for 20 min at room temperature. Membranes were then washed extensively, and binding of [^{3}H]forskolin (□) or 6-^{125}I-HPP-Fsk (◇) was determined as described in Experimental Procedures. The percentage of control binding, determined in the absence of additions, is plotted as the average of triplicate determinations, with the standard deviation indicated. The data are representative of two different experiments.

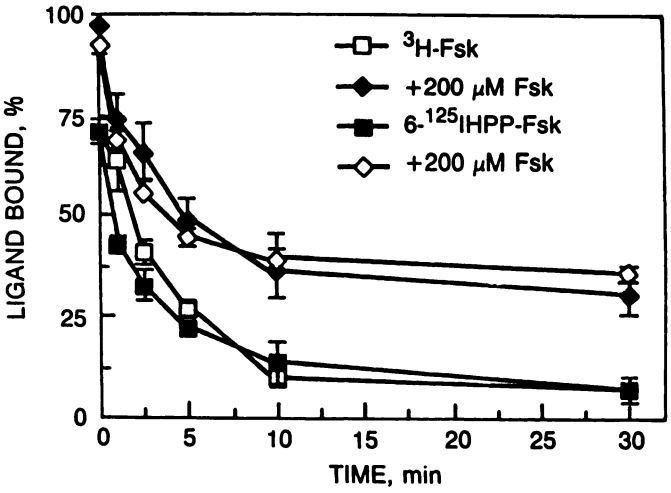

Fig. 9. Time dependence for the loss of [^{3}H]forskolin and 6-^{125}I-HPP-Fsk binding to bovine brain membranes by pretreatment with BrAcFsk. Bovine brain membranes were incubated at 25° for the indicated times, in the presence of 20 μM BrAcFsk alone (□, ■) or in the presence of 200 μM forskolin (◆, ◇). At the indicated times, the membranes were diluted with 30-fold cold buffer and washed extensively, and the specific binding of [^{3}H]forskolin (□, ◆) or 6-^{125}I-HPP-Fsk (■, ◇) was determined. The percentage of control binding, determined in the absence of additions, is plotted as the average of triplicate determinations, with the standard deviation indicated. The data are representative of two different experiments.

action of forskolin.

We have previously attempted to synthesize aminoalkylesters of forskolin by reacting 7-desacetylforskolin with protected amines such as FMOC-β-alanine or *t*-Boc-β-alanine.[1] The ester derivatives of FMOC-β-alanine and *t*-Boc-β-alanine were synthesized in good yield, using carbodiimide coupling (31). However, the aminoethyl esters were not stable after removal of the amine-protecting group. Removal of the *t*-Boc protecting group with trifluoroacetic acid resulted in degradation of forskolin, whereas removal of the FMOC group with piperidine resulted in a mixture of the 7-desacetyl derivative and the migrated 6-aminoethyl ester derivative. Therefore, we synthesized forskolin intermediates containing primary amines coupled through a stable carbamate linkage (44). The 7-(2-aminoethyl)-aminocarbonyl derivatives were synthesized because of previous studies suggesting that analogs of forskolin with lipophilic groups at the 7-position were relatively potent at inhibiting the glucose transporter (28). Other studies demonstrated that analogs of forskolin with heterocyclic amino acids esterified at the 6-position were potent at activating adenylyl cyclase (45, 46). We, therefore, synthesized the 6-(2-aminoethyl)aminocarbonyl derivative of forskolin, envisioning that derivatives of

[1] K. B. Seamon, Unpublished data.

this compound would have high affinities at adenylyl cyclase. The synthetic procedure used to synthesize the aminoethylcarbamates has also been used to prepare aminobutylcarbamates, aminohexylcarbamates, and aminoheptylcarbamates, and thus it is a general method for attaching functional groups with different chain lengths to forskolin (33).

6-AEC-Fsk and 7-AEC-Fsk were potent at activating adenylyl cyclase. 6-AEC-Fsk was slightly more potent than 7-AEC-Fsk or forskolin; however, it is difficult to define EC_{50} values of these compounds, because there was no apparent plateau in the activation at the highest concentration used (100 μM). This is frequently observed with forskolin and forskolin derivatives, and EC_{50} values for forskolin activation have been defined as that concentration of forskolin (or derivative) that produces 50% of the activation produced by 100 μM forskolin. It is interesting to note that 6-AEC-Fsk and 7-AEC-Fsk were more efficacious than forskolin in activating adenylyl cyclase, as indicated by a greater fold stimulation over basal activity. The water solubility of forskolin is limited to about 40 μM and, therefore, it is difficult to determine whether an apparent plateau in activation curves is due to saturation of the enzyme by forskolin or due to limited solubility. The increased efficacy of 6-AEC-Fsk and 7-AEC-Fsk, in comparison with forskolin, could be due to the enhanced water solubility of 6-AEC-Fsk and 7-AEC-Fsk. The nonradioactive analog of 6-^{125}I-HPP-Fsk was tested for its ability to stimulate bovine brain adenylyl cyclase. However, due to the limited solubility of the compound, it was not possible to make aqueous solutions at concentrations greater than 20 μM. 6-I-HPP-Fsk did activate adenylyl cyclase, although with an EC_{50} greater than that of forskolin. It is difficult to interpret these results, due to the limited solubility of the compound.

6-^{125}I-HPP-Fsk was synthesized carrier-free, using commercially available Bolton-Hunter reagent and 6-AEC-Fsk. The reaction is essentially complete after an overnight incubation, and the yields for the radioactive product range from 50% to 75% of the starting radioactivity. 6-^{125}I-HPP-Fsk can be separated completely from the precursor 6-AEC-Fsk, because 6-AEC-Fsk does not elute from the silica column, using ethyl acetate as solvent. 6-^{125}I-HPP-Fsk is also separated from unreacted Bolton-Hunter reagent by chromatography on silica gel in ethyl acetate.

The affinity of 6-^{125}I-HPP-Fsk for binding to bovine brain membranes determined by direct binding is 14 nM, which is essentially identical to its affinity determined by inhibition of [^{3}H]forskolin binding. Furthermore, the affinities of forskolin analogs at the 6-^{125}I-HPP-Fsk binding sites are essentially identical to their affinities at the [^{3}H]forskolin binding sites. 1,9-Dideoxyforskolin does not inhibit the binding of [^{3}H]forskolin or 6-^{125}I-HPP-Fsk binding. 6-AEC-Fsk and 6-I-HPP-Fsk are more potent than 7-AEC-Fsk and 7-I-HPP-Fsk at the [^{3}H]forskolin and 6-^{125}I-HPP-Fsk binding sites. These results suggest that the binding sites detected with 6-^{125}I-HPP-Fsk are probably the same as those detected with [^{3}H]forskolin.

The number of forskolin binding sites in different regions of rat brain has been determined and varies from about 100 fmol/mg of protein to 800 fmol/mg of protein (39). The number of [^{3}H]forskolin binding sites in the bovine brain membranes utilized in this study is 476 ± 47 fmol/mg of protein, which is well within the range reported in other studies (39). The number of 6-^{125}I-HPP-Fsk binding sites determined by analysis of six pooled experiments is 1008 ± 80 fmol/mg of protein. This number is about 2-fold higher than that observed with [^{3}H]forskolin. The difference may be due to the higher affinity of 6-^{125}I-HPP-Fsk (K_d = 16 nM), compared with the K_d of [^{3}H]forskolin (K_d = 30 nM). It is possible that there is some dissociation of [^{3}H]forskolin from the binding sites during the washing steps. Alternatively, it is now known that there are multiple adenylyl cyclases in brain, and these different adenylyl cyclases could demonstrate different selectivity for [^{3}H]forskolin and 6-^{125}I-HPP-Fsk. Studies with cloned and expressed adenylyl cyclase (47) will be informative regarding the differential interaction of forskolin with different types of adenylyl cyclase. However, it is interesting to note that the distributions of [^{3}H]forskolin and 6-^{125}I-HPP-Fsk sites in rat brain, determined using quantitative autoradiographic techniques, are essentially identical (48). There is also very good correspondence between the numbers of 6-^{125}I-HPP-Fsk and [^{3}H]forskolin binding sites in human platelet membranes. The number of 6-^{125}I-HPP-Fsk binding sites determined in platelet membranes activated with AlF_4^- is 660 fmol/mg of protein, which compares well with the number of [^{3}H]forskolin binding sites, 455 fmol/mg of protein (9).

The binding of [^{3}H]forskolin to bovine brain membranes and human platelet membranes is increased in the presence of Mg^{2+} and GTPγS or AlF_4^-, suggesting that the high affinity binding sites for forskolin represent a complex of the catalytic subunit of adenylyl cyclase and the activated G_s protein (8–10). The binding of 6-^{125}I-HPP-Fsk to human platelet membranes is also increased in the presence of Mg^{2+} and GTPγS or AlF_4^-, and the increase in the number of binding sites is identical to that observed for [^{3}H]forskolin binding. The increase in binding requires the presence of Mg^{2+}, which is required for the activation of the $G_{s\alpha}$ protein. Therefore, the number of 6-^{125}I-HPP-Fsk binding sites can be modulated by agents that activate the G_s protein.

BrAcFsk is an α-haloacetyl derivative of forskolin that activates adenylyl cyclase and inhibits the high affinity binding of forskolin to bovine brain membranes and human platelet membranes (36). Pretreatment of human platelet membranes with BrAcFsk produced an irreversible loss of 6-^{125}I-HPP-Fsk and [^{3}H]forskolin binding sites. The loss of 6-^{125}I-HPP-Fsk and [^{3}H]forskolin binding sites exhibited the same dependence on BrAcFsk concentration and the same time dependence. The irreversible loss of 6-^{125}I-HPP-Fsk binding sites and [^{3}H]forskolin binding sites could be partially prevented by including 200 μM forskolin. These results indicate that there is a nucleophilic group at the forskolin binding site of adenylyl cyclase that can be alkylated by BrAcFsk.

The high affinity binding of 6-^{125}I-HPP-Fsk is not associated with the glucose transport protein. Forskolin binding to the glucose transporter has been characterized by direct binding studies, photoaffinity labeling, and the ability of forskolin analogs to inhibit glucose transport (reviewed in Ref. 11). Forskolin binding to the glucose transporter is inhibited by cytochalasin B and by D-glucose. However, the high affinity binding of [^{3}H]forskolin to membranes is not inhibited by cytochalasin B or by D-glucose (39). In a similar manner, there is no effect of cytochalasin B or D-glucose on the binding of 6-^{125}I-HPP-Fsk to bovine brain membranes. The glucose transport inhibitors phloretin and phloridzin also do not inhibit the binding of 6-^{125}I-HPP-Fsk to bovine brain membranes (Table

2). We have attempted to determine high affinity binding of 6-^{125}I-HPP-Fsk to human red cell membranes, which contain high concentrations of the glucose transporter. There is no forskolin-displaceable binding of 6-^{125}I-HPP-Fsk to human red cell membranes, using the filtration assay that is used to measure the high affinity binding of 6-^{125}I-HPP-Fsk (data not shown). Forskolin and D-glucose are structurally similar, and it has been suggested that forskolin binds to the glucose transporter with the 6-hydroxyl group of forskolin binding at a site similar to that of the 1-hydroxyl of D-glucose (11, 16). The model, therefore, predicts that derivatives of forskolin at the 6-hydroxyl group would not be potent at the glucose transporter, as is observed for 6-alkylcarbamates of forskolin (33).

The characteristics of the binding of [^{3}H]forskolin and 6-^{125}I-HPP-Fsk are identical, according to a number of different criteria. 1) Forskolin and derivatives have the same K_d values determined by competition with each ligand. 2) Binding of both ligands is modulated by GTPγS and AlF_4^-. 3) BrAcFsk irreversibly blocks binding sites for both ligands. 4) Binding of both ligands is insensitive to agents that act at the glucose transporter or that inhibit the interaction of forskolin with the P-glycoprotein (35). Therefore, it seems reasonable to conclude that [^{3}H]forskolin and 6-^{125}I-HPP-Fsk bind to the same sites associated with adenylyl cyclase. We have not observed any differences in the characteristics of 6-^{125}I-HPP-Fsk binding or [^{3}H]forskolin binding to bovine brain membranes or human platelet membranes. However, 6-^{125}I-HPP-Fsk can be synthesized with a specific activity of 2175 Ci/mmol, which is considerably greater than that of [^{3}H]forskolin (about 40 Ci/mmol), and, therefore, 6-^{125}I-HPP-Fsk should be useful in detecting very small amounts of adenylyl cyclase using binding assays or autoradiographic analysis of tissue sections. This ligand should also be useful for studying the differences in the forskolin-binding characteristics of different forms of hormone-sensitive adenylyl cyclase that are found in peripheral tissues and other forms of adenylyl cyclase that are prepared using recombinant molecular biological techniques. It is now known that there are multiple adenylyl cyclases in brain, and these different adenylyl cyclases could demonstrate different selectivity for [^{3}H]forskolin and 6-^{125}I-HPP-Fsk.

The synthetic procedure described for the synthesis of 6-^{125}I-HPP-Fsk has been used to synthesize other functionalized derivatives of 6-AEC-Fsk and 7-AEC-Fsk. These include fluorescent derivatives of forskolin[1] and iodinated photoaffinity labels that bind with high affinity and selectivity to adenylyl cyclase, the glucose transporter, and other forskolin-binding proteins (34, 35). 6-AEC-Fsk and 7-AEC-Fsk have been coupled to cross-linked agarose and have proven useful in the affinity purification of adenylyl cyclase.[2] Furthermore, 6-^{123}I-HPP-Fsk has been synthesized and provides an iodinated derivative of forskolin that may be useful for *in vivo* studies of adenylyl cyclase.[1] It is anticipated that 6-AEC-Fsk will provide a number of interesting derivatives of forskolin with specificity for adenylyl cyclase.

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