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Differences between high-affinity forskolin binding sites in dopamine-rich and other regions of rat brain

(striatum/guanylyl imidodiphosphate/cholera toxin)

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ABSTRACT **[^{3}H]Forskolin bound to high- and low-affinity sites in the rat brain. The high-affinity site was discretely located, with highest densities in the striatum, nucleus accumbens, olfactory tubercule, substantia nigra, hippocampus, and the molecular layers of the cerebellum. This site did not correlate well with the distribution of adenylate cyclase. The high-affinity striatal binding site may be associated with a stimulatory guanine nucleotide-binding protein. Thus, the number of sites was increased by the addition of Mg^{2+} and guanylyl imidodiphosphate. Cholera toxin stereotaxically injected into one rat striatum increased the number of binding sites, and no further increase was noted following the subsequent addition of guanyl nucleotide. High-affinity forskolin binding sites in non-dopamine-rich brain areas (hippocampus and cerebellum) were modulated in a qualitatively different manner by guanyl nucleotides. In these areas the number of binding sites was significantly reduced by the addition of guanyl nucleotide. These results suggest that forskolin may have a potential role in identifying different functional/structural guanine nucleotide-binding proteins.**

The diterpene forskolin possesses an interesting spectrum of pharmacological activities. It stimulates adenylate cyclase both in peripheral tissues and in brain by a mechanism that involves a direct interaction with the catalytic subunit (C) of the enzyme adenylate cyclase (1). The presence of guanine nucleotide-binding proteins (G proteins), although not necessary for this action of forskolin, has been implicated as important for the expression of full enzymatic activity (2). This direct stimulation of adenylate cyclase usually occurs at forskolin concentrations in the micromolar range (1). At much lower concentrations, forskolin can also act synergistically with hormones that activate adenylate cyclase to greatly increase the generation of cAMP (3).

These two actions of forskolin have been explained as a consequence of the compound binding to two discrete sites: a low-affinity site associated with the catalytic subunit and a high-affinity site, the location of which is unknown. The occupancy of this high-affinity site by forskolin enhances stimulatory G protein (G_s)–C coupling (4).

Support for this model has come from studies of a lymphoma cell line. Forskolin restored the response to isoproterenol in S49 lymphoma cells defective in G_s–C coupling but not in other cell variants where either G_s was defective or receptor–G_s coupling was impaired (5).

These two separate actions of forskolin have been described in many different tissues and for a variety of hormones, suggesting that the two sites have a widespread distribution. However, when the distribution of forskolin binding sites in rat brain was examined autoradiographically by using [^{3}H]forskolin, high-affinity [^{3}H]forskolin binding sites showed a very uneven pattern of distribution (6). High densities of binding sites were observed, particularly in the limbic system and the basal ganglia. The highest density of binding was in the corpus striatum, in which adenylate cyclase is particularly sensitive to stimulation by forskolin (7).

The present paper examines more closely the high-affinity binding sites for forskolin in the rat central nervous system and the possibility that some of these sites are associated with the molecular mechanisms underlying G_s–C interactions.

MATERIALS AND METHODS

[^{3}H]Forskolin Binding. Striatal or hippocampal tissue was dissected from 10 to 20 Sprague–Dawley (200–250 g) rats and homogenized in 10 vol of 50 mM Tris·HCl (pH 7.4) by using a Brinkman Polytron at a setting of 12 for 15 sec. Debris and nuclei were removed by centrifugation at 1000 × *g* for 10 min. Membranes were sedimented from the supernatant by centrifugation at 50,000 × *g* for 60 min. The final pellet was resuspended in 50 mM Tris·HCl (pH 7.4), which usually contained 10 mM $MgCl_2$, at a protein concentration of 0.5–1.0 mg/ml. Membranes could be stored up to 4 weeks at −20°C without a loss of activity.

Membranes (30–60 μg of protein) were added together with [12-^{3}H]forskolin (0.5–20 nM; DuPont; specific activity of 20–40 Ci/mmol; 1 Ci = 37 GBq) to 50 mM Tris·HCl (pH 7.4) containing 10 mM $MgCl_2$ in a final volume of 500 μl. Specific binding was determined by the addition of 1 μM forskolin to half of the tubes. Following an incubation period of 1 hr at room temperature, free and bound ligand were separated by filtration through Whatman GF/C filters by using a cell harvester or by centrifugation in a microcentrifuge. Specific binding was expressed as fmol/mg of protein.

Autoradiography. Brains were removed and rapidly frozen at −70°C. They were mounted on blocks with plastic embedding media (Tissue-Tek; Raymond A. Lamb, London) and were allowed to equilibrate to cryostat temperature (−23°C). Sections (10 μm) were cut on a cryostat microtome and thaw-mounted on chrome alum/gelatin-coated microscope slides, which were stored at −20°C until further use (1–3 days). Subsequently the slides were equilibrated to room temperature (23°C) and incubated for 20 min with 20 nM [^{3}H]forskolin in 50 mM Tris·HCl (pH 7.4), with additions of 10 mM $MgCl_2$ and 10 μM guanylyl imidodiphosphate (p[NH]ppG) as indicated in the text. Nonspecific binding to the slides was defined by the addition of 20 μM forskolin to half of the slides. After incubation, slices were dipped once in ice-cold buffer, washed three times for 1 min in ice-cold

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Abbreviations: G protein, guanine nucleotide-binding-protein; C, catalytic subunit of adenylate cyclase; p[NH]ppG, guanylyl imidodiphosphate; G_s, stimulatory G protein coupled to adenylate cyclase.

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buffer, and finally dipped in ice-cold deionized water. They were rapidly dried under a stream of cold air, mounted on acid-free card, and apposed to ^{3}H-sensitive film for 6–9 weeks. The films (Amersham Hyperfilm) were developed and analyzed densitometrically using a Quantimet image analyzer (Cambridge Instruments, Cambridge, U.K.) and calibrated against ^{3}H microscales (Amersham).

Cholera Toxin Treatment. Male rats (250 g) were anesthetized by i.p. injection of Equithesin [4.25% (wt/vol) chloral hydrate/2.1% $MgSO_4$/39.6% propane-1,2-diol/10% absolute alcohol/16.2% sodium pentobarbitone (containing 10% vol/vol alcohol and 20% vol/vol propylene glycol)] at 3 ml/kg of body weight. They were mounted in a stereotaxic frame, and 4 μl of sterile saline with or without 4 μg of cholera toxin (Sigma) was injected at a rate of 1 μl/min into the right striata. Penetration was allowed to proceed for 5 min before the cannula was removed and the wound was closed. The stereotaxic coordinates used were according to the atlas of Paxinos and Watson (8) (3 mm lateral, 1 mm anterior, 5 mm ventral) using bregma and brain surface as the zero coordinates. After an 18-hr recovery period, the rats were tested for circling behavior in a perspex chamber (diameter = 50 cm). The animals were allowed to habituate to the apparatus for 5 min, and then the activity was recorded for a further 5 min. This measurement was repeated 6 hr later. Any animal not exhibiting turning was discarded. The remaining animals were sacrificed, and their brains were removed. Both striata were dissected, and membranes were prepared from pooled right and left striata. In some animals the whole brain was frozen for autoradiography.

Adenylate Cyclase Measurement. Striata were homogenized in 50 vol of ice-cold 10 mM Tris/2 mM EDTA, pH 7.4, and centrifuged at 35,000 × *g* for 10 min. The pellet was washed once by homogenization and centrifugation at 35,000 × *g* for 10 min. The final pellet was resuspended at a concentration of 16 mg of wet weight per ml. The assay consisted of 40 μg of membrane protein, 4 mM $MgSO_4$, 1 mM isobutylmethylxanthine, 5 mM phosphocreatine, 50 units of creatine phosphokinase per ml, 1 mM ATP containing 2–3 × 10^6 dpm of [α-^{32}P]ATP (10–50 Ci/mmol; Amersham), 80 mM Tris (pH 7.4), and 0.4 mm EDTA in a total volume of 200 μl. After a 5-min incubation period at 37°C, the reaction was terminated by the addition of 100 μl of 50 mM Tris containing 45 mM ATP, 2% $NaDodSO_4$, and [^{3}H]cAMP (30–50 Ci/mmol, ≈20,000 dpm; Amersham). Any cAMP formed during the incubation was separated by sequential chromatography over Dowex, followed by alumina columns (9). The samples were assayed by liquid scintillation counting and corrected for recovery of added [^{3}H]-cAMP. The results were expressed as pmol of cAMP formed per min per mg of protein. Protein was measured by the method of Bradford (10).

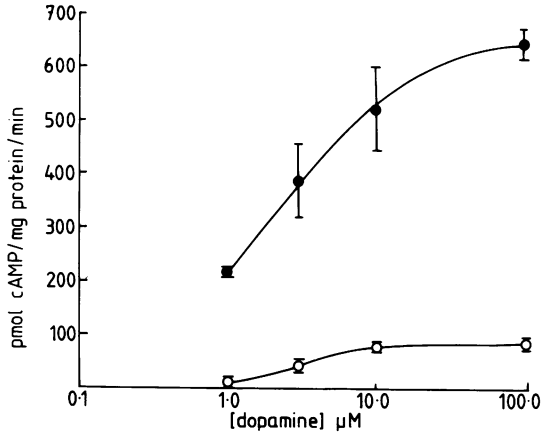

FIG. 1. The effect of forskolin (10 nM) on dopamine-stimulated adenylate cyclase from rat striatal membranes. Striatal membranes were incubated with either dopamine (○) or dopamine + 10 nM forskolin (●) for 5 min at 37°C in the presence of 1 mM ATP containing [^{32}P]ATP. Sulpiride (1 μM) was present throughout the incubation. At the end of the incubation, the amount of [^{32}P]cAMP formed was estimated, and the results were expressed as pmol of cAMP formed per min per mg of protein. Results are given as the mean ± SEM of six observations. Basal adenylate cyclase activity was 72.5 ± 3.4, and, in the presence of forskolin, the adenylate cyclase activity increased to 177 ± 9.3 pmol per min per mg of protein. All values were corrected for these basal levels.

RESULTS

[^{3}H]Forskolin bound to rat striatal membranes in a specific, saturable fashion. Analysis of the binding data revealed two binding sites: a high-affinity site (K_d = 9.3 ± 1.2 nM) with low capacity (B_{max} = 888 ± 144 fmol/mg) and a low-affinity site (K_d = 1.8 ± 0.2 μM) with high capacity (B_{max} = 280 ± 86 pmol/mg of protein).

A concentration of forskolin (10 nM) similar to the K_d for the high-affinity binding site was tested for its ability to enhance the stimulatory effects of dopamine on adenylate cyclase activity in striatal membranes. Dopamine is a major transmitter in the striatum, and its interaction with D1

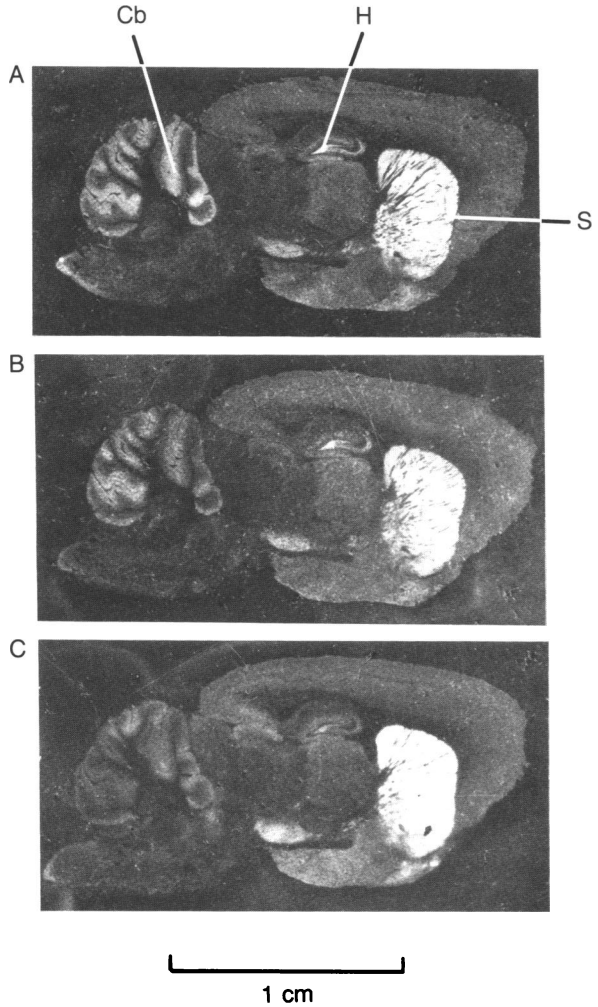

FIG. 2. Autoradiograph of [^{3}H]forskolin binding to sagittal sections of rat brain. Sections were incubated for 20 min at 21°C with 20 nM [^{3}H]forskolin containing either no additions (*A*), 10 mM $MgCl_2$ (*B*), or 10 mM $MgCl_2$ and 10 μM p[NH]ppG (*C*). Cb, cerebellum; H, hippocampus; S, striatum.

Table 1. Effect of 10 mM Mg^{2+} and 10 μM p[NH]ppG on [^{3}H]forskolin binding to rat striatum

Additions	Membranes: K_d, nM	Membranes: B_{max}, fmol/mg of protein	Membranes: nH	[^{3}H]Forskolin in tissue sections, fmol/mg of wet weight
None	11.3 (2.4)	560 (38)	1.06	28.7 (3.8)
$MgCl_2$	12.2 (2.7)	895 (147)	1.20	43.6 (4.3)*
$MgCl_2$ and p[NH]ppG	9.3 (2.0)	1856 (335)†	1.23	62.3 (5.2)†

Membranes were incubated with various concentrations (0.2–50 nM) of [^{3}H]forskolin and, where indicated, Mg^{2+} or Mg^{2+} and p[NH]ppG for 1 hr at room temperature. These data were subjected to Scatchard analysis (one site) by computer analysis; each curve was derived from at least eight observations (in duplicate) from four experiments. nH, Hill coefficient. For autoradiographic experiments, the results were taken from measurements of 18–26 sections from one rat brain. *P* values were determined by comparison with the control of the relevant group using computer-assisted analysis of variance followed by Neuman–Keuls multiple range test (RS/1 package BBN Software Products, Cambridge, MA). The SEM is given in parentheses.
*$P < 0.05$.
†$P < 0.01$.

dopamine receptors leads to stimulation of cAMP formation (11). Dopamine doubled the basal enzyme activity in rat striatum, with an EC_{50} of 3.2 μM (Fig. 1), a result that is in keeping with previous reports from many laboratories (12). In the presence of forskolin, the maximum adenylate cyclase response to dopamine increased from 88 pmol of cAMP formed per min per mg of protein to 604 pmol of cAMP formed per min per mg of protein, with little change in the EC_{50} (1.2 μM). Similar results were obtained in the presence and absence of sulpiride, a D2 dopamine receptor antagonist, indicating that forskolin altered dopamine actions at D1 receptors.

These experiments are consistent with suggestions that the high-affinity binding sites for forskolin in rat striatum are involved in synergistic adenylate cyclase responses in situations where G_s–C coupling has been promoted. Neither cAMP nor dopamine (up to 1 mM) had any affinity for the high-affinity [^{3}H]forskolin binding site.

Autoradiographs of sagittal sections of rat brain demonstrated the discrete localization of [^{3}H]forskolin binding sites, with striatum, substantia nigra, nucleus accumbens, hippocampus, and the molecular layer of the cerebellum showing particularly heavy densities of specific binding (Fig. 2*A*). This pattern of localization is similar to that reported by Gehlert *et al.* (6). If the forskolin binding sites in all regions of brain are associated with a G_s–C interaction, then the sites

Table 2. Effect of 10 mM Mg^{2+} and 10 μM p[NH]ppG on [^{3}H]forskolin binding to rat hippocampus

Additions	Membranes: K_d, nM	Membranes: B_{max}, fmol/mg of protein	Membranes: nH	[^{3}H]Forskolin in tissue sections, fmol/mg of wet weight
None	9.3 (2.3)	123 (7)	1.20	26.7 (3.5)
$MgCl_2$	17.4 (6.3)	176 (8)*	1.40	18.3 (1.6)*
$MgCl_2$ and p[NH]ppG	9.7 (2.8)	112 (20)	1.32	15.8 (0.6)*

Experimental details are as described in Table 1 except that for the membranes each value represents the mean of two experiments. The SEM is given in parentheses.
*$P < 0.01$ from the control of the relevant group using computer-assisted analysis of variance followed by Neuman–Keuls multiple range test.

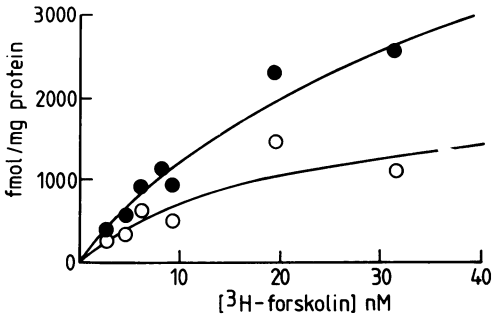

FIG. 3. The effect of cholera toxin on [^{3}H]forskolin binding in rat striatal membranes. Rats were injected with 4 μg of cholera toxin into the right striata stereotaxically and were allowed to recover for 24 hr. The animals were sacrificed, and membranes were prepared from toxin-treated (●) or control (uninjected) striata (○). The membranes were incubated with increasing concentrations of [^{3}H]-forskolin, and the results were analyzed by computer-assisted curve fitting to a one-component binding site. The results represent the mean of six observations from two experiments.

should all be Mg^{2+}-sensitive and modulated by p[NH]ppG. The effect of Mg^{2+} (10 mM) was indeed to increase the labeling of high-affinity [^{3}H]forskolin binding sites in striatum (Fig. 2*B*). The binding sites in striatum were also increased by the further addition of p[NH]ppG, a stable GTP analogue (Fig. 2*C* and Table 1). Interestingly, however, the high-affinity [^{3}H]forskolin binding sites in the hippocampus exhibited a *reduced* density of binding in the presence of the guanine nucleotide (Table 2 and Fig. 2) compared with that in the presence of Mg^{2+}, and this was also the case in the cerebellum. This effect was consistent and reproducible, occurring in all sections examined and from more than one brain sample.

The effects of Mg^{2+} and p[NH]ppG upon [^{3}H]forskolin binding to striatal and hippocampal membranes were also examined by using *in vitro* preparations (Tables 1 and 2). Mg^{2+} increased the number of binding sites in striatal membranes without significantly altering their affinity for [^{3}H]forskolin (Table 1). Addition of p[NH]ppG further significantly increased the B_{max}, again without significant changes in the K_d. These effects were not seen, however, in hippocampal membranes *in vitro* (Table 2). Here addition of p[NH]ppG and Mg^{2+} significantly reduced the B_{max} for

Table 3. Effect of 10 μM p[NH]ppG on [^{3}H]forskolin binding to rat striatum from control and cholera toxin-treated rats

Additions	B_{max} in membranes, fmol/mg of protein: Control	B_{max} in membranes, fmol/mg of protein: + Toxin	[^{3}H]Forskolin in tissue sections, fmol/mg of wet weight: Control	[^{3}H]Forskolin in tissue sections, fmol/mg of wet weight: + Toxin
$MgCl_2$	482 (29)	1056 (149)*	20.0 (2.3)	41.75 (4.6)*
$MgCl_2$ and p[NH]ppG	1103 (83)*	1036 (49)*	55.6 (6.7)*	85.0 (10.2)*

Membranes were prepared from rats injected with 4 μg of cholera toxin into the right striata 24 hr before they were sacrificed. Both right and left striata were removed, and the left striata acted as a control. Incubations were for 1 hr at room temperature with 0.2–50 nM [^{3}H]forskolin. These data were derived from computer-assisted fitting to a one site model; each curve contained not less than eight points (measured in duplicate) from three experiments. Tissue sections were incubated with 20 nM [^{3}H]forskolin. Autoradiographs were measured against ^{3}H standards using a Quantimet image analyzer and expressed as fmol of [^{3}H]forskolin per mg wet tissue weight (n = 20 sections from one brain). The SEM is given in parentheses.
*$P < 0.01$ compared with control values using computer-assisted analysis of variance followed by Neuman–Keuls multiple range test.

[^{3}H]forskolin compared with that in the presence of Mg^{2+} alone. Thus, the results obtained with membrane preparations mimicked those obtained by autoradiography.

The dependence of the binding sites in dopamine-rich areas of brain upon Mg^{2+} and their modulation by p[NH]ppG are in keeping with a possible association with a G_s–C mechanism. Another criterion for the involvement of a G protein is that cholera and pertussis toxins have characteristic effects on the functions of known G proteins, and these can be used in either intact cells or cell membranes (13). We examined the effect of cholera toxin treatment on the high-affinity [^{3}H]forskolin binding sites in striatum. Cholera toxin was injected stereotaxically into the right striatum, and the left striatum from the same animal was used as a control. The activity of the toxin was verified in two ways. The animals were tested for behavioral asymmetry due to increased cAMP in only one striatum, which resulted in contralateral turning behavior. Successfully treated animals showed a pronounced circling, which increased significantly with time (control, 0 turns per 5 min; toxin-treated, 14 ± 3 turns per 5 min 18 hr after treatment and 31 ± 7 turns per 5 min 24 hr after treatment). Of 15 animals tested, 2 did not exhibit turning behavior and were rejected. None of the saline-injected controls exhibited any turning behavior. Membranes from control and toxin-treated striata were also examined for dopamine D1 receptor binding by using the antagonist ligand [^{3}H]SCH23390 (14). Dopamine displaced specific [^{3}H]SCH23390 binding with an IC_{50} of 9.4 μM; in the presence of 20 μM p[NH]ppG, the curve was displaced and the affinity of dopamine for the site decreased (IC_{50} = 26.6 μM). In the toxin-treated membranes, however, the effect of p[NH]ppG was abolished (IC_{50} = 20 μM, n = 2). Both of these results confirmed that the cholera toxin treatment was fully effective.

Striatal membranes from the right striata of saline-injected and toxin-treated rats were used for [^{3}H]forskolin binding. Cholera toxin treatment caused an increase in the number of high-affinity [^{3}H]forskolin binding sites (Fig. 3). Further-

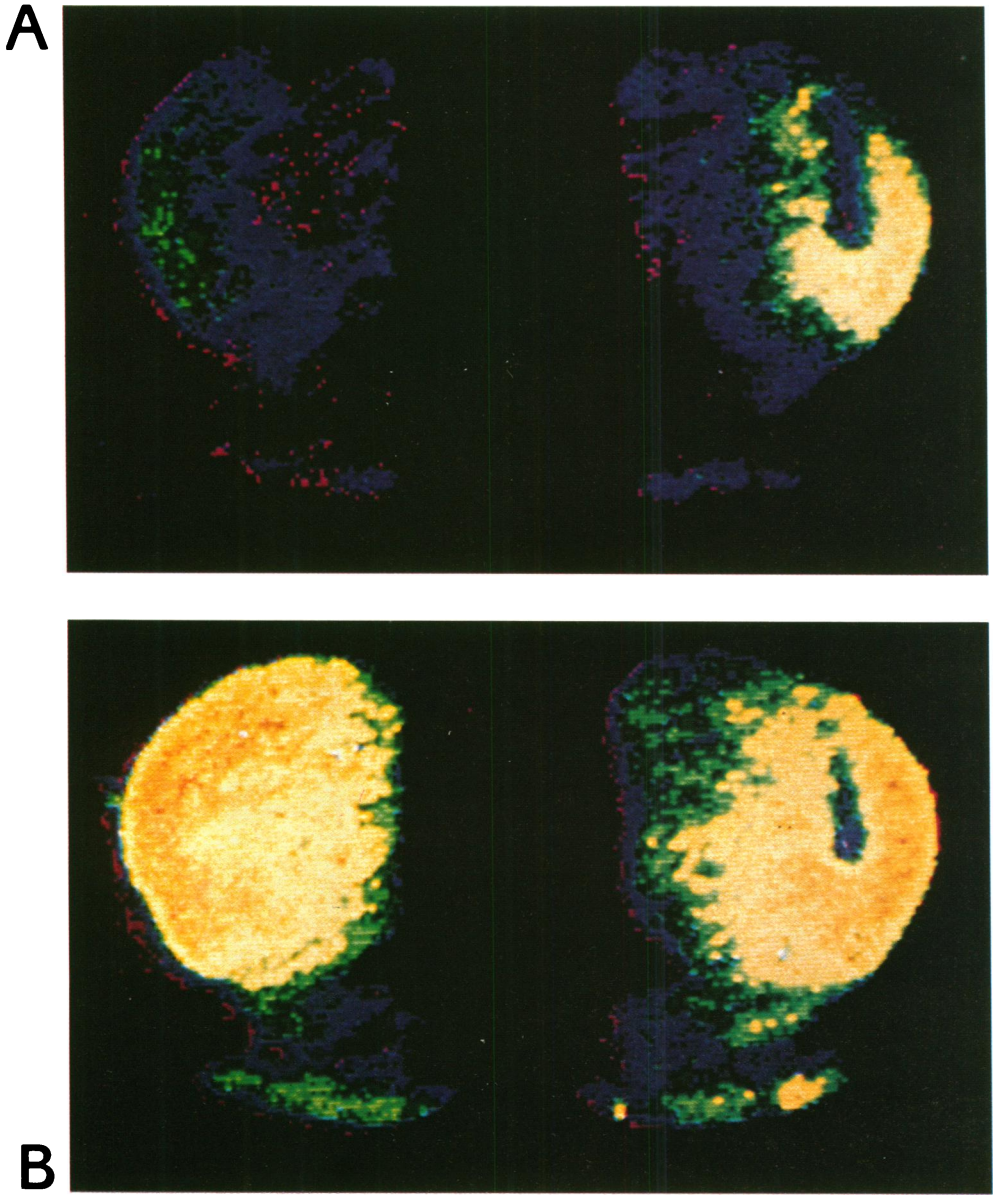

FIG. 4. The effect of cholera toxin pretreatment on [^{3}H]forskolin binding to coronal sections of rat brain. Autoradiographs were prepared from rats injected with 4 μg cholera toxin in the right striata stereotaxically and were allowed to recover for 24 hr. The animals were sacrificed, and slices were incubated with 20 nM [^{3}H]forskolin containing either 10 mM $MgCl_2$ (*A*) or 10 mM $MgCl_2$ and 10 μM p[NH]ppG (*B*) for 20 min at 21°C. Color pictures were produced using an image analyzer. Binding-site density is color-coded with high-to-low densities represented in the order of orange, yellow, green, and blue.

more, the increase in the number of binding sites normally observed upon the addition of p[NH]ppG to striatal membranes *in vitro* was no longer seen after treatment with cholera toxin (Table 3). In none of these conditions was there any significant change in the affinity constant for [^{3}H]forskolin binding. These *in vitro* biochemical results were similar to those obtained in autoradiographic experiments (Fig. 4). Coronal sections of rat brain sectioned at the level of the striatum show the effect of cholera treatment on [^{3}H]forskolin binding after unilateral toxin injections. The density of binding was greatly increased around the site of the injection, but the ability of p[NH]ppG to further increase the binding was attenuated.

DISCUSSION

Examination of the forskolin sites in rat brain striatal membranes by direct ligand-binding methods showed the presence of two binding components with K_d values of 11 nM and 1.8 μM. The present results are similar to those obtained by Seamon *et al.* (16), who reported sites with K_d values of 18 nM and 1 μM for [^{3}H]forskolin using whole rat forebrain membranes.

The high-affinity binding sites for forskolin are generally assumed to be associated with the ability of low concentrations of forskolin to synergize with a variety of hormones that stimulate adenylate cyclase. The present results demonstrate that in rat striatum forskolin will synergize in the production of cAMP with dopamine in membrane preparations. The synergistic interactions between dopamine and forskolin in membranes resulted in a large increase in the maximum response but only modest changes in the potency of dopamine. This situation may involve an enhanced G_s–C coupling; thus, these observations are consistent with the high-affinity binding site for forskolin being associated with a G_s protein.

Other conditions that promote G_s–C coupling are high Mg^{2+} and guanyl nucleotides (17). Both of these were capable of increasing [^{3}H]forskolin binding to striatal membranes; the changes observed were increases in the number of high-affinity binding sites rather than changes in the K_d.

A third criterion for the involvement of G proteins is sensitivity to cholera toxin. Cholera toxin catalyzes the NAD-dependent ribosylation of the α subunit of G_s, which promotes the release of GTP from G_s, inhibits GTP hydrolysis, and promotes the dissociation of the α subunit from the complex. In striatal membranes from cholera toxin-treated animals, [^{3}H]forskolin binding was considerably increased and could no longer be further enhanced by the addition of p[NH]ppG *in vitro*. These results are consistent with the high-affinity [^{3}H]forskolin binding site being associated with a G_s binding protein.

The results obtained using striatal membrane preparations were confirmed by autoradiography of brain sections, using as far as possible the same conditions employed in the membrane binding experiments. However, differences do occur due to the sampling procedures employed by the two methods. Membrane preparations are from grossly dissected tissue from a large number of animals, whereas autoradiographic measurements are made on more discrete areas from four animals. The autoradiographs of [^{3}H]forskolin binding also revealed other interesting features of the mechanism of action of forskolin. Both the adenylate cyclase catalytic subunit and G_s are widely distributed entities, and the actions of forskolin on the two proposed sites occur in a wide variety of tissues and with a range of stimulatory transmitters (3). The highly discrete localization of [^{3}H]forskolin binding sites in certain brain regions seems to be at odds with this concept. Furthermore, the binding sites in different brain regions responded differently to the addition of the guanine nucleotide p[NH]ppG together with Mg^{2+}. Increases were noted in the number of binding sites within dopamine-rich areas of brain (striatum, substantia nigra, nucleus accumbens, and olfactory tubercule), whereas decreases in the number of binding sites were observed in the hippocampus and cerebellum. Gehlert (18), in a recent autoradiography study, also noted that there were differences between the behavior of the binding sites in the hippocampus and striatum, although no change in the number of forskolin binding sites in the hippocampus on addition of p[NH]ppG was observed, rather than the decrease seen in this study.

Thus, it may be that forskolin offers an insight into the existence of different G_s protein subspecies in different areas of the central nervous system, suggesting that G_s–C interactions within different brain regions are not all mediated by identical molecular mechanisms. Dopamine-rich areas of brain appear to possess a particular form of G_s–C coupling, which is uniquely sensitive to forskolin. Interestingly, a recent paper mapping brain G proteins by *in situ* hybridization showed a surprisingly low density of G_s sites in rat striata (19), which may suggest the existence of other G_s type proteins whose mRNA does not hybridize with existing oligonucleotide probes.

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