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Acute *In Vivo* Amphetamine Produces a Homologous Desensitization of Dopamine Receptor-Coupled Adenylate Cyclase Activities and Decreases Agonist Binding to the D1 Site

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SUMMARY

We have previously reported that, 30 min after a single injection of 7.5 mg/kg *d*-amphetamine sulfate, there was a significant 25% decrease in the apparent V_{max} for stimulation of adenylate cyclase activity by the D1 receptor-selective partial agonist SKF 38393 in rat striatal membranes, as compared with saline-injected controls. This desensitization was seen in the striatal but not the mesolimbic forebrain. In the present study this desensitization was further characterized by using various ligands that interact with the three components of the D1 receptor-coupled adenylate cyclase complex to determine the site of modification that resulted in the desensitization. The desensitization was not associated with a change in the stimulation of adenylate cyclase at the level of the catalytic subunit or the guanyl nucleotide-regulatory protein N_s. Receptor number, as assessed by the binding of the D1 selective antagonist [^{3}H]SCH 23390, was unaltered in the desensitized state. In contrast, the number of high affinity binding sites, as measured with the agonist [^{3}H]dopamine, was decreased 30% by acute amphetamine exposure. This suggests that the amphetamine-induced desensitization may be the result of an uncoupling of the receptor from N_s. In order to further assess the effects of amphetamine on receptor/N_s coupling, we measured the ability of the guanyl nucleotide guanosine-5′-(β,γ-imido)triphosphate [Gpp(NH)p] to decrease high affinity [^{3}H]dopamine binding to striatal membranes. The inclusion of 100 μM Gpp(NH)p in the assay decreased the number of receptors in the high affinity state by 40% and 52% in membranes from saline- and amphetamine-pretreated rats, respectively. These results imply that amphetamine treatment does not modify the ability of Gpp(NH)p to decrease high affinity agonist binding. It is possible that amphetamine treatment decreases the number of receptors that can couple to N_s but the remaining receptors can still form a high affinity complex and are sensitive to the effects of Gpp(NH)p. We also report that maximal D2 dopamine receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity was decreased in striatal membranes prepared from amphetamine-treated rats as compared with saline-injected controls, implying that the D2 pathway was desensitized by amphetamine treatment. Conversely, acute amphetamine injection did not alter the ability of either the adenosine A_2 receptor to stimulate or the muscarinic cholinergic receptor to inhibit adenylate cyclase activity in the rat striatum. These results suggest that acute amphetamine treatment produces a dopamine receptor-specific or homologous desensitization.

Because of the postulated role of the neurotransmitter DA in a variety of pathological states, including schizophrenia (1, 2), regulation of dopaminergic neurotransmission is an area of intensive research. Abuse of the central nervous system stimulant AMPH is associated with toxic reactions that are clinically indistinguishable from paranoid schizophrenia (3). In rats, AMPH produces a dose-dependent series of behaviors. An IP injection of a low dose of AMPH, 1.0 mg/kg, results in enhanced locomotor and exploratory behavior; injection of higher doses (2.5–7.5 mg/kg) produces a stereotyped response consisting of repetitive head and limb movements and intense sniffing and/or licking with head oriented toward the cage floor. It is well established that these behaviors are produced by the release of DA from presynaptic nerve terminals in the striatal and mesolimbic forebrain regions (4). How this released DA generates the characteristic behavioral effects of AMPH is not known. The DA that is released in the striatum can interact with D1 and D2 DA receptors (5, 6). Both subtypes of DA receptors are believed to play a role in the behavioral response to AMPH (7–10). The D1 receptor is coupled to the stimulation of adenylate cyclase (EC 4.6.1.1) through N_s (11); the D2 receptor in the striatum inhibits adenylate cyclase through N_i (12, 13). There-

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ABBREVIATIONS: DA, dopamine (3-hydroxytyramine); AMPH, amphetamine; N_s, stimulatory guanyl nucleotide-regulatory protein; N_i, inhibitory guanyl nucleotide-regulatory protein; IP, intraperitoneal; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Gpp(NH)p guanosine-5′-(β,γ-imido)triphosphate; IBMX, isobutylmethylxanthine; BSA, bovine serum albumin; ACh, acetylcholine; PIA, N^6-1-phenylisopropyladenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; SCH 23390, (*R*)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-(1*H*)-3-benzazepine-7-ol; SKF 38393, (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride.

fore, it is of interest to determine the regulation of D1 and D2 DA receptor-coupled adenylate cyclase activities in response to increased synaptic DA concentrations produced by AMPH pretreatment.

Our laboratory has recently described a desensitization of D1 receptor-coupled adenylate cyclase activity in the rat striatum 30 min after a single IP injection of 2.5 or 7.5 mg/kg AMPH (14). There was a 25% decrease in the V_{max} for stimulation of adenylate cyclase activity by SKF 38393 in rat striatal crude membranes as compared with saline-injected controls. Twenty-four hours after AMPH treatment, there was no significant difference in stimulation by SKF 38393 between the saline and AMPH groups.[1] This desensitization was not seen in the mesolimbic forebrain region (14). Barnett and Kuczenski (5) have also reported a reversible desensitization of DA-stimulated adenylate cyclase activity after acute AMPH in the rat striatum.

The goal of the present study was to further characterize this desensitization. First, the effects of AMPH on the components of the D1 receptor-coupled adenylate cyclase were examined to determine the site that contributed to the desensitization. We found that the desensitization was accompanied by a decrease in high affinity agonist binding, suggesting that the site of modification is the receptor. Secondly, we assessed the specificity of the desensitization by examining the effects of *in vivo* AMPH on DA D2, adenosine A_2, and muscarinic cholinergic receptor coupling to adenylate cyclase. Our results demonstrated that *in vivo* AMPH pretreatment produced a desensitization that was specific for D1 and D2 receptor-coupled adenylate cyclase activities, implying that the desensitization is homologous.

Materials and Methods

Female Holtzman rats, 150–250 (Harlan Spraque Dawley, Inc., Madison, WI) were used in all experiments. Animals were injected IP with an equal volume of either 7.5 mg/kg AMPH or 0.9% saline and sacrificed 30 min later by decapitation. Control rats were paired with AMPH-treated rats in the same experiment on each day. The animals were rated behaviorally 1 min before sacrifice and were described by one of the following three categories: still, asleep or awake but stationary; moderate activity, intermittent or continuous sniffing, rearing, grooming, or ambulation; intense stereotypy, continuous sniffing, and/or licking with head oriented toward the cage floor with no ambulation. The striata were dissected on ice and homogenized in the appropriate buffer using a glass/Teflon homogenizer. Crude membrane fractions were prepared for one of the following assays.

Adenylate cyclase assay. Striatal tissue was homogenized in 9 volumes of ice-cold 10 mM Tris maleate buffer containing 1.2 mM EGTA and 1 mM $MgSO_4$, pH 7.5. Membranes were prepared by centrifugation for 20 min at 27,000 × *g*. This procedure was repeated once and the final resuspension was to a protein concentration of 5 mg/ml. Protein concentrations in this and all other assays were determined by the method of Lowry *et al.* (16). Adenylate cyclase activity was measured according to the procedure of Krishna *et al.* (17) as modified by Roberts-Lewis *et al.* (14). The activity was measured in the presence and absence of GTP, Gpp(NH)p, $MnCl_2$, NaF, and calmodulin. The reaction was conducted at 37° for 8 min and stopped by heating for 1 min at 95°. All enzyme activities were adjusted for the recovery of [^{3}H]cAMP, which was consistently between 80 and 95%. Dopamine stimulation required the presence of GTP. For each of the effectors, stimulation of enzyme activity is expressed as pmol of cAMP/min/mg of protein produced.

To measure adenosine receptor-mediated stimulation of adenylate cyclase activity, papaverine at a concentration of 0.1 mM replaced IBMX as the phosphodiesterase inhibitor, adenosine deaminase was included in the assay to decrease the concentration of endogenous adenosine (18), and the incubation was for 5 min at 37°. Stimulation was measured in the presence and absence of 1 μM GTP and various concentrations (100 nM–30 μM) of the adenosine analogue PIA. PIA stimulation is expressed as pmol of cAMP/min/mg of protein produced above that seen in the presence of 1 μM GTP alone.

Inhibitory adenylate cyclase assay. DA-mediated inhibition of forskolin-stimulated adenylate cyclase activity was measured according to the procedure of Onali *et al.* (12) with modifications. Striatal tissue was homogenized in 10 volumes of HDE buffer, which contained 10 mM HEPES/NaOH, pH 7.4, 1 mM dithiothreitol, and 1 mM EGTA with 10% (w/w) sucrose added. A crude membrane fraction was prepared by centrifugation at 27,000 × *g* for 20 min. This step was repeated and the homogenate was resuspended in the above buffer minus sucrose with the pH adjusted to 8.0. After a 30-min incubation on ice, the centrifugation step was repeated and the final resuspension was to a protein concentration of 2 mg/ml in HDE buffer, pH 7.4. BSA was added to a final concentration of 1 mg/ml. SCH 23390, at a concentration of 100 nM, was included in the assay to prevent activation of the D1 receptor. Each assay tube contained 80 mM Tris·HCl, pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, 1 mM cAMP, 5 mM phosphoenolpyruvate, 1.33 mM dithiothreitol, 0.5 mM IBMX, 0.33 mM EGTA, 66 μg of BSA, 40 μg of pyruvate kinase, 0.5 mM ATP, and 1 μCi/tube [α-^{32}P]ATP in a final assay volume of 100 μl. The reaction was initiated by the addition of 10–15 μg of crude membrane protein and proceeded at 25° for 20 min. The reaction was stopped, and the levels of [^{32}P]cAMP were quantitated by the procedure described above for the stimulatory assay. Activity was measured in the presence and absence of 50 μM GTP, 1 μM forskolin, and various concentration of DA (30 nM–500 μM). The ability of ACh (1–300 μM) to inhibit adenylate cyclase activity was measured in the presence of 100 μM GTP and 0.3 μM forskolin; 10 μM eserine was included to inhibit acetylcholinesterase. The inhibition of activity produced by ACh and DA are expressed as per cent decrease from the activity measured in the presence of their respective forskolin plus GTP concentrations.

[^{3}H]SCH 23390 binding assay. Binding of the D1-selective antagonist [^{3}H]SCH 23390 to striatal membranes was performed according to the procedure of Schulz *et al.* (19). Saturation binding data were determined experimentally by incubation with various concentrations of [^{3}H]SCH 23390 (0.03–10 nM). Nonspecific binding was measured at each concentration of [^{3}H]SCH 23390 by the inclusion of 10 μM (+)-butaclamol. The incubation was at 25° for 60 min; bound ligand was separated from free using Whatman GF/C filters (Maidstone, England) under vacuum filtration. Radioactivity remaining on the filters was quantitated using liquid scintillation counting.

[^{3}H]Spiroperidol binding assay. The procedure of Creese *et al.* (20) was used to determine the binding of [^{3}H]spiroperidol, with the following modifications. Crude membranes were prepared by centrifugation at 48,000 × *g* for 15 min. This procedure was repeated twice and the final resuspension was to a protein concentration of 0.14 mg/ml in an ion mix as previously described (20). The concentrations of [^{3}H]spiroperidol used for saturation analysis ranged from 0.3 to 30 nM. Nonspecific binding was determined at each concentration of [^{3}H]spiroperidol by the inclusion of 1 μM (+)-butaclamol. The incubation was for 35 min at 37°. The reaction was stopped, and radioactivity remaining on the filters was quantitated as described above.

[^{3}H]DA binding assay. The procedure of Leff and Creese (21) was followed with slight modifications. Membranes prepared from a 48,000 × *g* centrifugation were preincubated in 50 volumes of 50 mM Tris·HCl, containing 2 mM $MgCl_2$, for 30 min at 37°. After two washing steps, the final resuspension was to 2.0 mg/ml protein in a 20 mM MOPS/19 mM Tris buffer containing 1 mM EDTA, 5 mM $MgSO_4$, 10 μM pargyline, and 0.1% ascorbic acid at an assay pH of 7.2 at 22°. Membranes were incubated with increasing concentrations of [^{3}H]DA

[1] P. H. Roseboom, *et al.*, unpublished observation.

(0.2–40 nM) for 60 min at 22°. Nonspecific binding was determined at each concentration of [^{3}H]DA by the inclusion of 10 μM (*cis*)-flupenthixol, and 20 nM spiroperidol was included in all tubes to prevent labeling of the D2 site. Bound was separated from free according to the procedures described above.

Data analysis. The apparent EC_{50} and V_{max} values for stimulation and inhibition of adenylate cyclase activity were determined by fitting the dose-response curves using a nonlinear regression least squares curve fitting program (GraphPad; ISI) to a sigmoidal function $Y=P_{min}+(Pmax-P_{min})\cdot[D^n/(D^n+EC_{50}^n)]$ where EC_{50} is the concentration of the agonist required to produce half maximal response, n is the Hill coefficient or slope factor, D is the drug concentration, and P_{min} and P_{max} represent the minimum and maximum enzyme activities, respectively. Statistical comparisons of the data were made using Student's t tests.

K_D and B_{max} values for [^{3}H]SCH 23390 and [^{3}H]spiroperidol binding were determined from Scatchard analysis. The data fit to a single site and linear regression was used to calculate the actual values. Binding data obtained with the agonist [^{3}H]DA were analyzed using the nonlinear least squares curve-fitting program SCAFIT (22), which provides estimates of the affinity and number of receptors in each affinity state. R_S and K_S are defined here as the B_{max} and K_D for those receptors that bind ligand with high affinity in membranes prepared from control animals; R_A and K_A are the same values for tissue from AMPH-treated animals. Deviation of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance (23). In order to determine whether a significant difference in the high affinity binding parameters existed between saline and AMPH, the data were first analyzed simultaneously with the K_D and B_{max} both constrained to be estimated as a common value between treatment groups. The data were then analyzed with either the K_D or B_{max} allowed to vary between saline and AMPH, and in the fourth analysis both parameters could assume different values. The improvement in fit produced by the various methods was assessed using the variance ratio (F statistic) derived from the residual variance (the ratio of the sum of squares of the residuals divided by the degrees of freedom) (122). This same procedure was used to analyze the effects of Gpp(NH)p on [^{3}H] DA binding to membranes from saline- and AMPH-pretreated animals.

Materials. [α-^{32}P]ATP (specific activity, 38 Ci/mmol), [^{3}H]SCH 23390 (specific activity, 75–85 Ci/mmol), and [^{3}H]cAMP were purchased from Amersham Corp. (Arlington Heights, IL); [^{3}H]spiroperidol (specific activity, 27 Ci/mmol) and [^{3}H]DA (specific activity 42 Ci/mmol) were obtained from New England Nuclear Research Products (Boston, MA); forskolin was obtained from Calbiochem-Behring Corp. (La Jolla, CA); cAMP, phosphoenolpyruvate, ATP, PIA, papaverine, DA, ACh, eserine, and $MnCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO); pyruvate kinase, adenosine deaminase, GTP, and Gpp(NH)p were obtained from Boehringer Mannheim (Indianapolis, IN). NaF was purchased from Mallinckrodt (St. Louis, MO); IBMX was obtained from Aldrich Chemical Co. (Milwaukee, WI); calmodulin was purified from bovine testes by the method of Dedman *et al.* (24); *d*-amphetamine sulfate was purchased from the University of Michigan Laboratory of Animal Medicine; spiroperidol was a generous gift of Janssen Pharmaceutica N. V. (Beerse, Belgium); (+)-butaclamol was donated by Ayerst Laboratories (New York, NY). (*cis*)-Flupenthixol was provided by H. Lundbeck and Co. A/S (Copenhagen, Denmark), and SCH 23390 maleate was graciously donated by the Schering Corp. (Bloomfield, NJ).

Results

Effects of AMPH on D1 receptor-mediated stimulation of adenylate cyclase activity and D1 receptor binding. We have previously shown that *in vivo* administration of AMPH produces a dose- and time-dependent desensitization of stimulation of adenylate cyclase activity by SKF 38393 in rat striatal membranes as compared with saline-injected controls (14). This is demonstrated in Fig. 1, showing that *in vivo* administration of 7.5 mg/kg AMPH 30 min before decapitation resulted in a significant 21% decrease in the V_{max} for *in vitro* stimulation of adenylate cyclase activity by SKF 38393 as compared with saline treatment (see the legend to Fig. 1 for the enzyme kinetic constants). This desensitization was observable only at doses (2.5 and 7.5 mg/kg) and times after AMPH treatment (30 and 60 min) when the rats were engaged in stereotyped behavior.

To specify the site of modification that resulted in the acute desensitization, ligands that interact with D1 receptor-stimulated adenylate cyclase at each of the three components were used. Administration of AMPH produced no significant change in the ability of either GTP or Gpp(NH)p to stimulate adenylate cyclase activity through N_s (Table 1). Additionally, the activity in the presence of 10 mM NaF, which also activates adenylate cyclase at the level of N_s, was not modified by pretreatment with AMPH. The activity was, in units of pmol/min/mg of protein, ± standard error, 345 ± 45 for the saline group and 332 ± 57 for the AMPH group (n = 3). Stimulation of the catalytic subunit by 5 mM $MnCl_2$ was not significantly different, with activity at 314 ± 12 pmol/min/mg of protein for the saline group and 325 ± 16 for the AMPH group, n = 3. Calmodulin-stimulated adenylate cyclase activity was also unaltered. The V_{max} values were, in units of pmol/min/mg of protein, ± standard error (n = 4), 249 ± 16 and 231 ± 16 for saline and AMPH, respectively; EC_{50} values for calmodulin were, in units of μM (standard error range), 0.35 (0.21–0.57) and 0.20 (0.17–0.23) for saline and AMPH, respectively.

The effect of AMPH on the D1 receptor number was determined by measuring the binding of the selective antagonist [^{3}H]SCH 23390 to crude membranes prepared from rat striatal tissue. This binding was found to saturate and display a single affinity state as described previously (19, 26). Scatchard analysis of saturation binding data revealed no difference in the total number of sites, B_{max}, or in the equilibrium dissociation con-

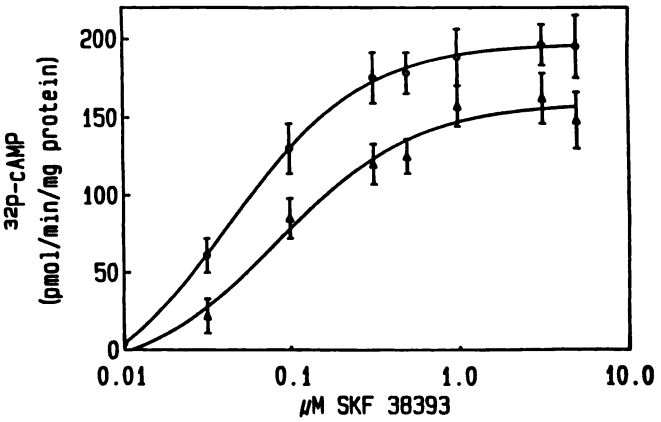

Fig. 1. Stimulation of adenylate cyclase activity by SKF 38393 in striatal membranes prepared from rats receiving a 30-min treatment with either 7.5 mg/kg AMPH (△) or saline (○). Each *point* represents the mean enzyme activity (pmol/min/mg of protein) ± standard error for five to seven animals. The kinetic constants were determined by the method of Wilkinson (25). The V_{max} values were, in units of pmol/min/mg of protein, ± standard error, 199 ± 17 for saline and 157 ± 14 for AMPH and represent a statistically significant decrease as determined by Student's t test, $p < 0.05$; the EC_{50} value was significantly increased, $p < 0.005$, with values of 0.05 ± 0.004 μM and 0.08 ± 0.009 μM for saline and AMPH, respectively.

TABLE 1

Apparent kinetic constants for stimulation of adenylate cyclase activity by GTP and Gpp(NH)p

Rats were injected IP with 7.5 mg/kg AMPH or an equivalent volume of saline and killed 30 min after treatment. Striatal membranes were prepared as described in Materials and Methods. Kinetic constants for stimulation of adenylate cyclase activity above basal were determined by fitting the individual dose-response curves to a sigmoidal curve using a nonlinear least squares curve fitting program (GraphPad, ISI) and represent the mean ± standard error for the given number of rats (*n*). The EC_{50} values were averaged as the logarithm base 10; therefore, the standard error (numbers in parentheses) is expressed as a range. Basal enzyme activities in the GTP experiment were, in units of pmol/min/mg of protein, 123 ± 9 for the saline group and 135 ± 12 for the AMPH group. In the GppNHp experiment the values were 119 ± 9 for the saline group and 147 ± 15 for the AMPH group.

Agonist	*n*	Apparent kinetic constants	
		EC_{50}	V_{MAX}
		μM	*pmol/min/mg of protein*
GTP			
Control	5	0.27 (0.19–0.37)	220 ± 32
AMPH	5	0.25 (0.21–0.29)	199 ± 23
GppNHp			
Control	4	0.42 (0.27–0.66)	417 ± 85
AMPH	3	0.51 (0.30–0.88)	397 ± 95

TABLE 2

K_D and B_{max} values for [^{3}H]SCH 23390 and [^{3}H]spiroperidol binding in striatal membranes from rats that received 7.5 mg/kg AMPH or saline (control) 30 min before decapitation

Linear regression was performed on Scatchard transformations of the saturation curve data to determine the equilibrium binding parameters. B_{max} values are presented as mean ± standard error obtained in the indicated number of experiments (*n*). The K_D values were averaged as the logarithm base 10; therefore, the standard error (numbers in parentheses) is expressed as a range. The experimental procedures are detailed in the Materials and Methods section.

Ligand	*n*	K_D	B_{max}
		nM	*fmol/mg of protein*
[^{3}H]SCH 23390			
Saline	5	0.15 (0.15–0.16)	959 ± 46
AMPH	5	0.16 (0.15–0.17)	932 ± 73
[^{3}H]spiroperidol			
Saline	5	0.13 (0.096–0.16)	463 ± 22
AMPH	5	0.18 (0.15–0.21)	463 ± 34

stant, K_D, for the binding of [^{3}H]SCH 23390 in membranes prepared from either saline- or AMPH-treated rats (Table 2).

In several models of desensitization, agonist binding is modified in the absence of changes in antagonist binding. In the present study, we measured the binding of the agonist [^{3}H]DA to striatal membranes over a concentration range of 0.20 to 40 nM (Fig. 2A). The specific binding ranged from 70 to 30% of total binding. Nonlinear regression analysis using the program SCAFIT (22) revealed that the experimental data best fit a two-site model. This can be more readily seen in the curvilinear nature of the Scatchard plots (Fig. 2C). Fig. 2B shows saturation data from 0.20 to 8 nM [^{3}H]DA to highlight binding to the high affinity population. AMPH produced a significant decrease in binding at almost every concentration of [^{3}H]DA below 8 nM. This was not the result of different concentrations of endogenous DA being carried over into the binding assay. DA concentrations that would be present in the binding assay

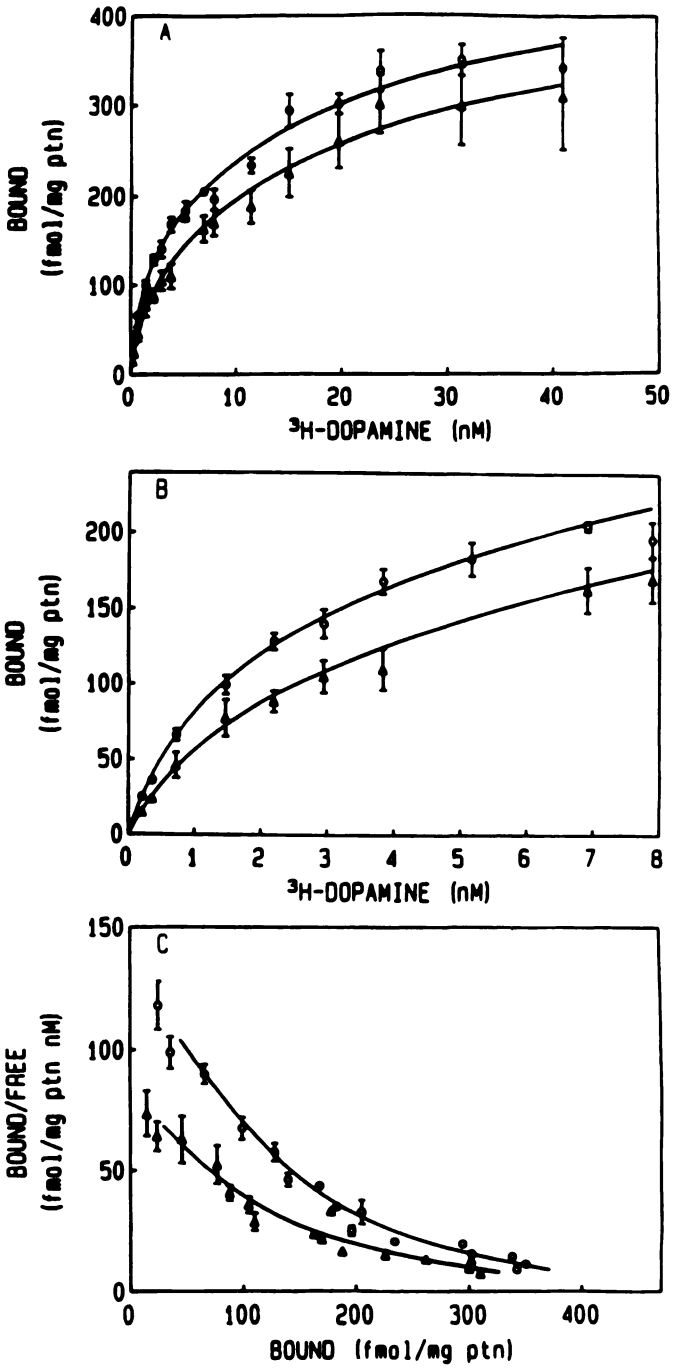

Fig. 2. Saturation curves and Scatchard plots for [^{3}H]DA binding to striatal membranes from rats that received 7.5 mg/kg AMPH (Δ) or saline (O) 30 min before decapitation. The data *points* represent the average ± standard error of five determinations. A shows the saturation data obtained over a concentration range of 0.2 to 40 nM [^{3}H]DA; B depicts the same data as A from 0.2 to 8 nM to allow better visualization of binding at the lower concentrations. Nonspecific binding was determined in the presence of 10 μM (*cis*)-flupenthixol, and 20 nM spiroperidol was included to prevent labeling of the D2 site. The full saturation curves best fit a two-site model and the *lines* are the theoretical "best fit" curves as determined by the nonlinear least squares curve-fitting computer program SCAFIT. C, Scatchard analysis of the [^{3}H]DA binding to both high and the low affinity components. [^{3}H]DA concentration ranged from 0.2 to 40 nM. The *lines* represent Scatchard transformations of the theoretical curve derived from a two-site fit of the saturation analysis using SCAFIT. The decrease in high affinity agonist binding produced by AMPH treatment could be explained entirely by a lowering of the B_{max} value.

TABLE 3

[^{3}H]DA binding data: effects of constraining equilibrium binding parameters on goodness of fit

Results of SCAFIT analysis of [^{3}H]DA saturation data from striatal membranes taken from rats that were treated for 30 min with a single injection of either saline or 7.5 mg/kg AMPH. The combined saturation curves from five saline and five AMPH rats were fit simultaneously to a two-site model with various constraints placed on the analyses. See Materials and Methods for details. The improvement in fit produced by relaxing the constraints was assessed using the variance ratio (*F* statistic) derived from the residual variance (the ratio of the sum of squares of the residuals divided by the degrees of freedom).

Constraints	Sum of squares	Degrees of freedom Degree of fit	*F* Statistic	
			Compared with fit 1	Compared with fit 4
Fit 1 $R_S = R_A$ $K_S = K_A$	93,445	149		
Fit 2 $K_s = K_A$	58,710	148	86.7[a]	1.26
Fit 3 $R_S = R_A$	63,506	148	69.8[a]	13.4[a]
Fit 4	58,213	147		

[a] Statistically significant improvement in fit, $p < 0.01$.

were measured with high performance liquid chromatography coupled to electrochemical detection and were not different between the two groups. The values were determined in two saline- and AMPH-treated rats and were 0.23 and 0.31 nM for the saline-treated animals and 0.23 and 0.20 nM for the AMPH-treated animals.

To determine how the equilibrium binding parameters were affected by AMPH treatment, the compiled saline and AMPH saturation curves (from 0.2 to 40 nM) were analyzed simultaneously by the SCAFIT program (Table 3). Initially the data were fit assuming there was no difference in either the K_D and B_{max} values between the saline and AMPH groups by requiring $K_S = K_A$ and $R_S = R_A$ during the analysis. This yielded a sum of squares value (Table 3, *fit 1*), which is the summation of the deviations of the theoretical curve from the individual data points and is a measure of how well the curve fits the data. To determine whether the B_{max} values were different between the two treatment groups, the same data were then refitted, but the constraint $R_S = R_A$ was removed. This resulted in an improvement in fit as evidenced by a decrease in the sum of squares value (Table 3, *fit 2*). To test whether fit 2 was significantly better than fit 1, the *F* ratio was used. This is the ratio of the sum of squares divided by the degrees of freedom. The calculated *F* ratio was then compared with the tabulated *F* statistic with the appropriate degrees of freedom. A significant improvement in fit suggests the value of the parameter is different between the saline and AMPH groups. Allowing the B_{max} values to be estimated independently significantly improved the fit (Table 3, *fit 2*). This was also the case when the K_D values were allowed to vary (Table 3, *fit 3*). In fit 4 no constraints were placed on the analysis. The comparison of fits 2 and 3 with fit 4, which is shown in the *last column* of Table 3, revealed that the fit obtained when only the B_{max} is allowed to vary could not be improved upon by relaxing both constraints (fit 2 versus fit 4); however, this was not the case with the K_D for which fit 4 is significantly better than fit 3. Because the improvement in fit obtained when no constraints were placed on the analysis was the same as that seen when only the B_{max} was allowed to vary, these results suggest the change in agonist binding produced by AMPH is a lowering of the B_{max} value for the high affinity state; however, it is not possible to state whether the K_D was changed by AMPH treatment. The B_{max} values obtained when no constraints are placed on the analysis were, in units of fmol/mg of protein, 149 for the saline group and 104 for the AMPH group; the K_D values were 1.24 nM for the saline group and 1.51 nM for the AMPH group. These numbers suggest the major change in agonist binding produced by AMPH is a 30% decrease in B_{max}.

When each curve was fit separately using the SCAFIT program and the individual K_D and B_{max} values were averaged, a similar result was obtained. There was a statistically significant 44% decrease in the B_{max} for the AMPH group (80 ± 24 fmol/mg of protein) as compared with the saline group (143 ± 23 fmol/mg of protein), $n = 5$, $p < 0.05$, and no detectable change in the K_D between the AMPH group [1.14 (0.963–1.34) nM] and the saline group [1.08 (0.85–1.37) nM]. The average low affinity K_D values in units of nM (standard error), were 17.8 (16.2–19.5) and 19.5 (12.3–30.9) for the saline and AMPH groups, respectively; the B_{max} values, in units of fmol/mg of protein, were 350 ± 55 for the saline group and 428 ± 117 for the AMPH group. These results suggest that the loss in high affinity agonist binding was accompanied by an increase in the number of low affinity binding sites in tissue from the AMPH treatment group; however, due to the large standard error in the low affinity parameter determinations, this was not statistically significant.

A decrease in the number of receptors in the high affinity state can imply a modification in the coupling between the receptor and N_s. Guanyl nucleotides such as Gpp(NH)p are believed to decrease high affinity agonist binding by destabilizing receptor/N_s coupling. Therefore, we measured the ability of Gpp(NH)p to modify agonist binding in membranes from control- and AMPH-treated rats. Saturation analysis of [^{3}H]DA binding performed in the presence and absence of 100 μM Gpp(NH)p were also best fit by a two-site model. The Scatchard plots are shown in Fig. 3. Simultaneous fitting of the compiled control and Gpp(NH)p curves under the various constraints described in the previous analysis yielded a pattern similar to that discussed above for the saline/AMPH comparison. The improvement in fit produced by allowing both the K_D and B_{max} to vary between control and Gpp(NH)p could be entirely accounted for by allowing only the B_{max} to vary. Allowing the K_D values to be estimated independently during the analysis did significantly improve the fit; however, this could be further improved upon by allowing both parameters to vary. Therefore, it is not possible to state that Gpp(NH)p treatment altered the affinity of the receptor for [^{3}H]DA, but it can be concluded that the B_{max} was decreased. The individual parameters determined by SCAFIT analysis when no constraints were applied are shown in Table 4. Standard error values are not included in this table because the parameters are the result of a single analysis of the compiled saturation curves, but it is stated that the B_{max} values are different between the control and GppNHp groups because the fit of the data is significantly improved if this parameter is not constrained to be equal between the two groups during the SCAFIT analysis. The values included in Table 4, along with the low affinity parameters that were determined in the same analysis, were used to generate the theoretical curves that are included in Fig. 3. Gpp(NH)p pro-

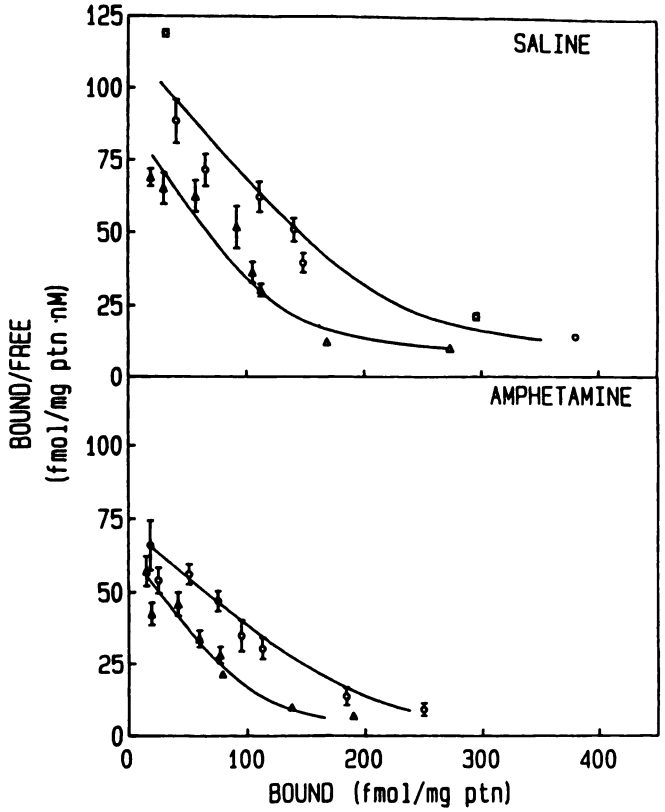

Fig. 3. Scatchard analysis of [^{3}H]DA binding to striatal membranes from saline- and AMPH-treated rats. Data *points* represent the average ± standard error for four determinations. The binding was carried out in the presence (Δ) or absence (○) of 100 μM Gpp(NH)p, and the [^{3}H]DA concentration ranged from 0.2 to 30 nM. The data were best fit to a two-site model, and the reduction in binding produced by Gpp(NH)p could be explained by a decrease in B_{max}. See Table 4 for details of analysis and equilibrium binding constants for the four curves.

TABLE 4

Effects of Gpp(NH)p on high affinity [^{3}H]DA binding

SCAFIT analysis was performed on [^{3}H]DA saturation binding data to striatal membranes from saline and AMPH-pretreated rats measured in the presence and absence of 100 μM Gpp(NH)p. The analyses were performed under various constraints as described in Materials and Methods. For both saline and AMPH, the improvement in fit obtained by allowing both K_D and B_{max} to be estimated independently could be entirely accounted for by allowing only the B_{max} to vary, suggesting that Gpp(NH)p lowers the B_{max} in both saline and AMPH groups. The K_D and B_{max} values listed below were obtained with no constraints on the analysis.

Treatment	K_D	B_{max}
	nM	*fmol/mg of protein*
Saline		
Control	2.0	214
+Gpp(NH)p	1.5	125
AMPH		
Control	2.7	184
+Gpp(NH)p	1.7	101

duced a 40% and 52% decrease in the high affinity B_{max} value for saline and AMPH, respectively. This suggests that the ability of Gpp(NH)p to modify agonist binding is not altered in the desensitized state. It should be noted that the difference in the B_{max} values between the saline and AMPH saturation curves in the absence of GppNHp is not as robust as reported in Table 3. However, the data obtained in Table 4 are the result of a simultaneous comparison between control and +GppNHp curves. The results obtained depend on the two groups that are being simultaneously fitted. If the saline and AMPH control

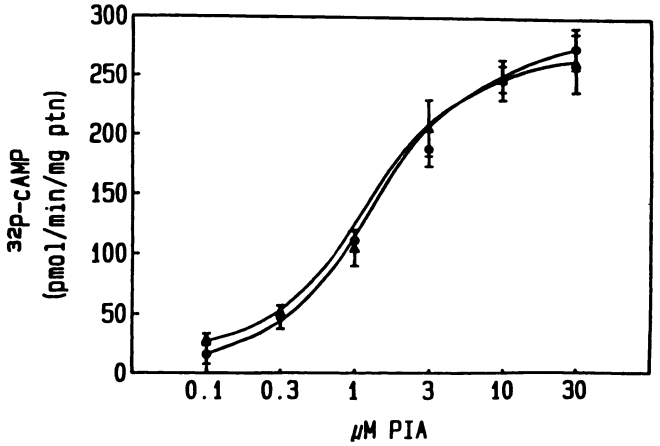

Fig. 4. Activation of rat striatal adenylate cyclase activity through the adenosine A_2 receptor by PIA. The data *points* represent the average ± standard error for four determinations. Striatal membranes were prepared from rats that received an acute injection of either saline (●) or 7.5 mg/kg AMPH (▲) 30 min before decapitation. Stimulation of activity by PIA was calculated as activity in the presence of drug plus 1 μM GTP minus that measured in the presence of GTP alone. Activity in the presence of 1 μM GTP, in pmol/min/mg of protein, ± standard error, was 280 ± 15 for saline and 270 ± 24 for AMPH. The V_{max} values for PIA stimulation, in units of pmol/min/mg of protein ± standard error, were 297 ± 24 for the saline group and 287 ± 25 for the AMPH group (four experiments); the EC_{50} values were, in units of μM (standard error range), 1.6 (1.5–1.7) for the saline group and 1.7 (1.4–2.2) for the AMPH group.

curves are analyzed simultaneously, the resultant B_{max} values are 175 and 111 fmol/mg of protein for saline and AMPH, respectively. This difference is similar to that seen in Table 3.

Effects of AMPH on adenosine A_2 receptor-mediated stimulation of adenylate cyclase activity. In the presence of 1 μM GTP, the adenosine analogue PIA produced a dose-dependent increase in the activity of adenylate cyclase. AMPH treatment that resulted in a desensitization of D1 receptor-coupled cyclase activity did not alter the dose-response curve for PIA stimulation of adenylate cyclase (Fig. 4). Neither the V_{max} nor the EC_{50} were significantly different between the saline- and AMPH-treated groups (see legend to Fig. 4).

Effects of AMPH on D2 receptor-mediated inhibition of adenylate cyclase activity and D2 receptor binding. The ability of DA to inhibit forskolin-stimulated adenylate cyclase activity through the D2 receptor was measured in a crude membrane preparation in the presence of the D1 antagonist SCH 23390 (100 nM). Forskolin, at a concentration of 1 μM, stimulated activity 12-fold above basal, and the inclusion of 50 μM GTP increased this activity by 13%. Activities in the presence of either forskolin or GTP were not significantly different between saline- or AMPH-treated animals (see legend to Fig. 5). The large standard error for forskolin plus GTP stimulation indicates that the baseline values were highly variable; however, in each experiment DA inhibited a relatively constant percentage of this activity. In membranes prepared from saline-treated animals, DA inhibited forskolin-stimulated activity in a dose-dependent manner. Inhibition reached a maximum at 100 μM DA and represented an 11% decrease from the activity seen with 1 μM forskolin plus 50 μM GTP (Fig. 5A). There was a significant difference in the percent of inhibition of forskolin-stimulated adenylate cyclase activity produced by 100 μM dopamine in membranes prepared from saline- (10.8 ± 1.2%) and AMPH- (5.6 ± 1.5%) treated rats, $p < 0.05$,

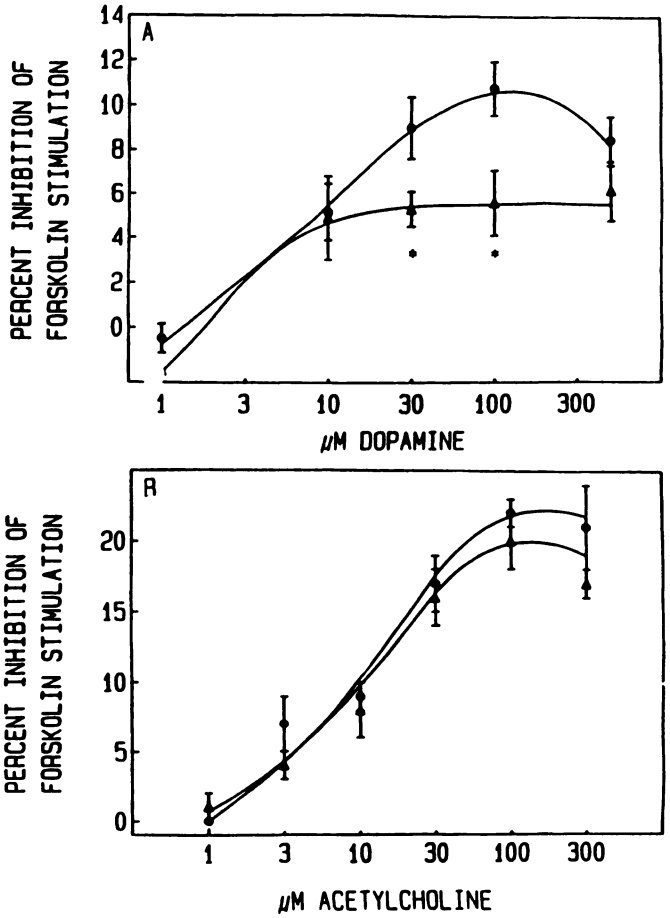

Fig. 5. Inhibition of forskolin-stimulated adenylate cyclase activity by ACh and DA in striatal membranes from rats that received a single injection of either saline (●) or 7.5 mg/kg AMPH (▲) 30 min before sacrifice. A, DA D2 receptor-mediated inhibition is expressed as per cent decrease from activity measured in the presence of 50 μM GTP plus 1 μM forskolin. The data *points* represent the average ± standard error for eight determinations. *, Significantly different, $p < 0.05$, from the saline group as determined by Student's *t* test. In the absence of added DA, the activity in saline- and AMPH-pretreated rats was 3727 ± 408 and 3611 ± 373 pmol/min/mg of protein ± standard error, respectively, eight rats. B, Inhibition by ACh of activity in the presence of 100 μM GTP plus 0.30 μM forskolin was calculated as the per cent decrease in activity from that produced by GTP plus forskolin. Data *points* represent the average ± standard error for three determinations. The activity of GTP plus forskolin, in pmol/min/mg of protein, ± standard error, was 2008 ± 844 for saline and 1815 ± 751 for AMPH. The maximum per cent inhibition produced by ACh, ± standard error, was 24 ± 3 for the saline group and 19 ± 1 for the AMPH group; the EC_{50} values, in units of μM (standard error), were 8.5 (6.4–11.2) for the saline group and 12.0 (9.6–15.1) for the AMPH group.

$n = 8$. Therefore, the D2 inhibitory pathway appears to be desensitized by AMPH.

In a separate experiment, binding of the D2 receptor-selective antagonist [^{3}H]spiroperidol was measured in membranes prepared from striatal homogenates. The binding was found to be saturable and Scatchard analysis revealed a single affinity state. In contrast to AMPH effects on D2-inhibited adenylate cyclase activity, there was no significant alteration in either the K_D or B_{max} for [^{3}H]spiroperidol binding 30 min after a 7.5 mg/kg injection of AMPH as compared with saline (Table 2).

Effects of AMPH on muscarinic cholinergic receptor-mediated inhibition of adenylate cyclase activity. ACh produced a dose-dependent inhibition of forskolin-stimulated adenylate cyclase activity in rat striatal membranes similar to that reported by others (27). The maximum inhibition was 20% (Fig. 5B). In contrast to D2 inhibition, AMPH pretreatment did not alter the dose-dependent inhibition of adenylate cyclase activity produced by ACh in striatal membranes as compared with saline. The kinetic constants are listed in the legend to Fig. 5B.

Discussion

The goal of the present study was to further characterize the dose- and time-dependent desensitization of rat striatal DA receptor-coupled adenylate cyclase activities in response to a single IP injection of AMPH. In order to determine the site of modification in the D1 receptor-coupled adenylate cyclase complex, various ligands that interact at each of the three components were utilized. The desensitization was not accompanied by a change in the activation of the catalytic subunit by $MnCl_2$ or calmodulin. Similarly, the V_{max} and EC_{50} values for GTP and Gpp(NH)p stimulation were not significantly different between the two treatment groups, and stimulation by NaF was unaltered. Therefore, it is unlikely that the desensitization is the result of a modification in the coupling between N_s and the catalytic subunit. However, inasmuch as the D1 receptor-coupled G protein and adenylate cyclase activity represent only a fraction of the total, it can not be concluded unambiguously that there is no change in these components. The K_D and B_{max} for the binding of the antagonist [^{3}H]SCH 23390 did not differ between the two groups; Barnett *et al.* (28) have also reported no change in the binding of [^{3}H](*cis*) flupenthixol to rat striatal D1 receptors after acute AMPH pretreatment. These results imply that the desensitization is not associated with an alteration in total receptor number. In contrast, the number of high affinity [^{3}H]DA binding sites was decreased by 30% in membranes from the AMPH group as compared with the saline controls.

High affinity agonist binding is believed to represent binding to receptors that are coupled to N_s (29). Guanyl nucleotides destabilize receptor-N_s coupling and decrease high affinity binding. In the present study, inclusion of 100 μM Gpp(NH)p in the assay produced a 40% decrease in the number of high affinity [^{3}H]DA binding sites. This is in agreement with indirect binding studies measuring the competition of DA for D1 receptor-specific [^{3}H]SCH 23390 binding. Hess *et al.* (30) have reported that DA competition curves are best fit by a two-site model composed of a high and a low affinity component. In the presence of guanyl nucleotides, they reported a 50% decrease in the proportion of receptors in the high affinity state. The interconversion of agonist binding states has been reported in several monoaminergic receptor systems (see Ref. 31 for review) and has led to the proposal of a generalized ternary complex model modified from the original models of Boeynaems and Dumont (32) and Jacobs and Cuatrecasas (33). In brief, the model predicts that both agonist and antagonist bind to the receptor recognition site but that only agonist promotes the interaction between the receptor and N_s. High affinity agonist binding is thought to represent binding to this formed ternary complex. Therefore, the present desensitization may be explained by a decrease in receptor-N_s coupling. This hypothesis agrees well with the current theory on the underlying molecular mechanism of homologous desensitization in the β-adrenergic

receptor system. The initial event in homologous desensitization is thought to be an uncoupling of the receptor from N_s (34), which is evidenced by a decrease in the amount of high affinity binding in agonist/[^{3}H]antagonist competition studies (35). The present desensitization could be explained by the following scheme. A decrease in the number of receptors that could couple to N_s would result in a decrease in the maximal number of N_s molecules that are activated. This in turn would decrease the number of catalytic components that could enter into cAMP production and would lead to a lowering of the maximal hormone receptor-mediated response.

It is interesting to note that Gpp(NH)p can produce an equivalent decrease in B_{max} in control and desensitized membranes. A possible explanation for this may be that AMPH treatment may reduce the number of receptors that are available to form the ternary complex, but those that remain are still sensitive to the destabilizing effects of Gpp(NH)p. The current theory on homologous desensitization is that it is due to a receptor modification (see below). Therefore, the ability of guanyl nucleotides to decrease agonist binding, which is believed to occur through an interaction with N_s (29), would not be expected to change in the desensitized state.

We report here that D2 receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity is desensitized after AMPH treatment. Additionally, total receptor number, as measured by the binding of the antagonist [^{3}H]spiroperidol, was unaffected. In contrast, the dose-response relationships for two ligands that affect adenylate cyclase through adenosine and muscarinic receptors were not modified by administration of AMPH. This suggests the desensitization is specific for DA receptors coupled to adenylate cyclase and may be termed homologous.

Recently Kelly and Nahorski (5) and Azzaro *et al.* (6) have shown that endogenous DA released by AMPH coactivates both D1 and D2 receptors in slices of rat striatum. The results presented here suggest that, after *in vivo* AMPH treatment, this coactivation results in a desensitization of both receptor subtypes. The net result of coactivation of D1 and D2 receptors in response to AMPH in rat striatal slices is an increase in the concentration of cAMP (5, 6). If the signal for desensitization were a rise in the cAMP levels, AMPH administration would be expected to desensitize D1 receptor-stimulated adenylate cyclase activity as a means to return cAMP levels to baseline. This does not explain the desensitization of the inhibitory D2 pathway. However, work in the β-adrenergic system has suggested homologous desensitization is mediated by a cAMP-independent protein kinase termed β-adrenergic receptor kinase (34). Homologous desensitization in amphibian erythrocytes is associated with the translocation of β-adrenergic receptor kinase from the cytoplasm to the plasma membrane (36), which is followed by a phosphorylation of the receptor, resulting in a change in its subcellular distribution (37). This kinase appears to only phosphorylate the agonist-occupied form of the receptor. Lefkowitz and co-workers (36) have hypothesized that this kinase is not specific for the β-adrenergic receptor but may phosphorylate other receptors types when they are occupied by agonist. It has been shown in kin$^-$ S49 lymphoma cells that translocation of the receptor kinase is also associated with the desensitization of somatostatin receptor-mediated inhibition of adenylate cyclase (38). The fact that this kinase has been shown to be present in bovine striatum (39) suggests the intriguing possibility that the D1 and D2 receptors, when activated by DA during the behavioral response to AMPH, could both serve as substrates for this receptor kinase. This could explain why both the stimulatory and inhibitory pathways are desensitized by AMPH treatment.

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