

# Inverse Agonist Properties of Dopaminergic Antagonists at the D<sub>1A</sub> Dopamine Receptor: Uncoupling of the D<sub>1A</sub> Dopamine Receptor from G<sub>s</sub> Protein

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## ABSTRACT

The interaction of dopaminergic antagonists with the D<sub>1A</sub> dopamine receptor was assessed in PC2 cells that transiently express this receptor. The maximal binding and dissociation constants for the D<sub>1A</sub> dopamine receptor, using the ligand [<sup>125</sup>I]SCH23982 were  $0.38 \pm 0.09$  nM and 1 to 4 pmol/mg, respectively, when assessed 48 h after transfection with cDNA encoding the rat D<sub>1A</sub> receptor. Basal adenylyl cyclase activity increased 50 to 60% in membranes of transfected PC2 cells compared with control membranes. The dopaminergic antagonists clozapine, *cis*-flupenthixol, (+)-butaclamol, haloperidol, chlorpromazine, and fluphenazine inhibited constitutive adenylyl cyclase activity in membranes of cells expressing the D<sub>1A</sub> receptor. SCH23982, a selective D<sub>1</sub> dopamine receptor antagonist, and (–)-butaclamol did not alter basal cyclase activity, whereas dopamine increased enzyme activity in membranes

expressing the D<sub>1A</sub> dopamine receptor. The coupling of D<sub>1A</sub> receptors with G<sub>s</sub> proteins was examined by immunoprecipitation of membrane G<sub>sα</sub> followed by immunoblotting with a D<sub>1A</sub> dopamine receptor monoclonal antibody. Clozapine, *cis*-flupenthixol, (+)-butaclamol, haloperidol, and fluphenazine but not SCH23982 or (–)-butaclamol decreased D<sub>1A</sub> receptor-G<sub>sα</sub> coupling by 70 to 80%, and SCH23982 was able to prevent the receptor-G<sub>sα</sub> uncoupling induced by haloperidol or clozapine. These results indicate that some dopaminergic antagonists suppress basal signal transduction and behave as inverse agonists at the D<sub>1A</sub> dopamine receptor. This action of the dopamine receptor antagonists may contribute to their antidopaminergic properties that seem to underlie their clinical actions as antipsychotic drugs.

Dopaminergic neuronal systems play important roles in brain function (Kalivas and Stewart, 1991; Williams and Goldman-Rakic, 1995). Dysfunctional dopaminergic neurotransmission has been suggested to be involved in such neurological and psychiatric disorders as Parkinson disease, Tourette syndrome, and schizophrenia (Albin et al., 1989; Knable and Weinberger, 1997). Pharmacological, biochemical, and largely molecular biological studies have identified five different dopamine receptors (Sokoloff and Schwartz, 1995). These receptors have seven transmembrane domains and couple to G proteins. Although the D<sub>1A</sub> and D<sub>1B</sub> dopamine receptors stimulate adenylyl cyclase, the D<sub>2</sub>-like dopamine receptors (D<sub>2</sub>, D<sub>3</sub>, D<sub>4</sub>) inhibit this pathway (Civelli et al., 1993; Seeman and Van Tol, 1994). Several studies have also shown that in brain, activation of phospholipase C is mediated by an as-yet unidentified D<sub>1</sub>-like dopamine recep-

tor (Undie and Friedman, 1990; Wang et al., 1995; Pacheco and Jope, 1997).

Inhibition of D<sub>2</sub> dopamine receptors has frequently been considered as the mechanism by which dopaminergic antagonists exert their therapeutic actions in schizophrenia and Tourette syndrome (Creese et al., 1976; Seeman et al., 1976; Richelson and Nelson, 1984). However, several studies have also shown that the antipsychotic drugs bind to D<sub>1A</sub> dopamine receptors (Kanba et al., 1994). This effect has been suggested to contribute to their ability to ameliorate the negative symptoms of schizophrenia (Lynch, 1992; Reynolds and Czudek, 1995). D<sub>1</sub> receptor antagonists may also be expected to produce fewer neurological adverse effects (Chipkin et al., 1988; Waddington, 1988; Lynch, 1992). Furthermore, an interaction between the D<sub>1</sub> and D<sub>2</sub> dopamine receptors has been suggested to be of functional importance in determining the output of dopaminergic neurotransmission; thus, inhibition of either receptor by the antipsychotic drugs may contribute to their therapeutic action in schizophrenia (Seeman et al., 1989).

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**ABBREVIATIONS:** bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; PBST, phosphate-buffered saline/Tween 20; Gpp(NH)p, 5'-guanylylimidodiphosphate.

Ligands that bind to G protein-coupled receptors may be classified as agonist, neutral antagonist, or inverse agonist, according to the outcome of their interaction with their receptor (Kenakin et al., 1995). Receptor agonists enhance receptor activity, neutral antagonists block the action of agonists without exerting receptor-mediated effects, and inverse agonists negatively regulate constitutive receptor activity and signaling of G protein-coupled receptors (Schutz and Freissmuth, 1992; Lefkowitz et al., 1993; Kenakin et al., 1995; Milligan et al., 1995; Tiberi and Caron, 1995). Inverse agonists may be particularly useful as therapeutic agents in treating disturbances that are characterized by elevated tonic, agonist-independent, G protein-coupled receptor activity (Bond et al., 1995). In the present study, we attempted to test whether dopaminergic antagonists behave as inverse agonists at the D<sub>1A</sub> dopamine receptor. For that purpose, we determined the nature of the interaction of dopaminergic antagonists with D<sub>1A</sub> dopamine receptors by monitoring their effect on adenylyl cyclase activity in membranes obtained from PC2 cells that transiently express D<sub>1A</sub> dopamine receptors. We also directly assessed the effect of dopaminergic antagonists on D<sub>1A</sub> dopamine receptor-G<sub>s</sub> protein coupling, because this association may determine the functional outcome of the interaction between a ligand and its receptor.

## Experimental Procedures

**Materials.** [<sup>125</sup>I]SCH23982 (2200 Ci/mmol), [ $\alpha$ -<sup>32</sup>P]ATP (800 Ci/mmol), and Anti-G<sub>s $\alpha$</sub>  protein antiserum (RM/1) were obtained from DuPont NEN (Boston, MA). Anti-D<sub>1A</sub> receptor monoclonal antibody was obtained from RBI (Natick, MA). [<sup>3</sup>H]adenosine 3',5'-cyclic monophosphate (cAMP) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). RPMI 1640, Lipofectin, and OPTI-MEM I were obtained from GIBCO BRL (Gaithersburg, MD). Pansorbin was purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). All other chemicals were obtained from Sigma (St. Louis, MO).

**PCR Amplification of Rat D<sub>1A</sub> Dopamine Receptor.** A full-length D<sub>1A</sub> dopamine receptor cDNA was amplified from rat genomic DNA using oligonucleotide primers corresponding to base pair (bp) -6 to +18 (5'-AGGAAGATGGCTCCTAACACTTCT-3') and the reverse complement of bp +1324 to +1347 (5'-GCGAGTTCAAGTGGAATGCTGTCC-3') of the rat D<sub>1A</sub> dopamine receptor sequence (Monsma et al., 1990; Zhou et al., 1990). The conditions for PCR was 48 s at 94°C, 2 min at 63°C, and 48 s at 72°C, 35 cycles followed by a 7-min extension at 72°C. The reaction products were purified and size-fractionated on a 1% agarose gel. The band of interest was excised, electroeluted, concentrated, and cloned into the pTarget Mammalian Expression System (Promega, Madison, WI). High-efficiency JM109 competent cells (Promega) were transformed and mini-preparations of plasmid DNA were prepared for insert sequencing.

Sequencing was performed by the Sanger dideoxynucleotide chain termination method using TaqTrack Sequencing System (Promega) on denatured double-stranded DNA. Both strands of the full-length cDNA were entirely sequenced using vector-specific primers or those derived from prior sequencing.

**Cell Culture and Expression of Rat D<sub>1A</sub> Dopamine Receptor.** PC2 cells were plated at a density of approximately 100 to 200/mm<sup>2</sup> on collagen coated dishes in RPMI 1640 supplemented with 10% horse serum, 5% fetal calf serum, 50 pg/ml streptomycin, 50 U/ml penicillin. The cells were cultured at 37°C in a water saturated atmosphere of 95% air and 5% CO<sub>2</sub> and used for transfection when they reached 40 to 60% confluence.

The rat D<sub>1A</sub> dopamine receptor cDNA was inserted into the polylinker region of the pTarget vector (Promega). For transient

expression of the D<sub>1A</sub> dopamine receptor, PC2 cells were transfected using positively charged liposomes and Lipofectin reagent. Lipofectin was mixed with an equal volume of plasmid DNA in buffer containing 10 mM Tris, 1 mM EDTA, pH 8.0, and allowed to stand for 20 min at room temperature. Cells were washed twice and then incubated with 3 ml of OPTI-MEM I, a reduced serum medium. The DNA-Lipofectin mixture was added to cultured cells and incubated for 24 h. The DNA-containing medium was removed and replaced with 3 ml of RPMI 1640 supplemented with 20% fetal bovine serum and incubated for an additional 24 h.

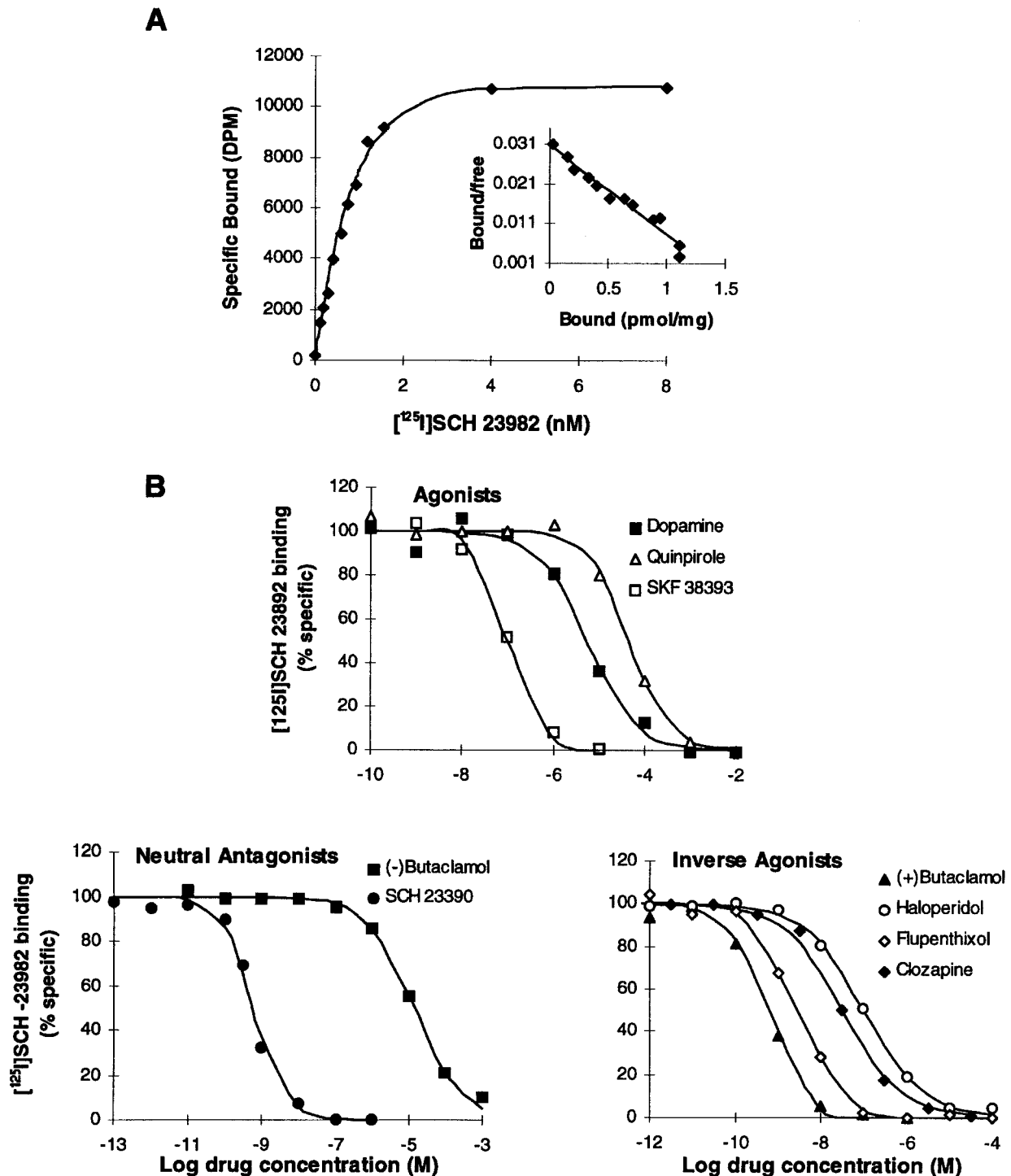
**Adenylyl Cyclase Assay.** PC2 cells were harvested after washing with a phosphate-buffered solution. The cells were homogenized in 10 volumes (w/v) of chilled buffer containing 50 mM Tris · HCl, pH 7.4, 2 mM EGTA, and 10% sucrose. The homogenate was centrifuged at 800g for 5 min and the supernatant centrifuged at 49,000g for 20 min. The pellet was washed twice and suspended in 50 mM Tris · HCl, pH 7.4. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard. Adenylyl cyclase activity was measured by a modified method of Salomon (1979). Each assay was performed in 250  $\mu$ l of solution containing 100 mM Tris · HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 0.1 mM ATP, 10 mM creatine phosphate, 0.2 mM EGTA, 100  $\mu$ M 3-isobutyl-1-methylxanthine, 1  $\mu$ M GTP, 1 mM dithiothreitol, 5 units of creatine phosphokinase, and 1  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]ATP. Reaction mixture was preincubated at 30°C for 5 min. The assay was initiated by adding 50  $\mu$ g of membrane protein and carried out at 30°C for 20 min. The reaction was terminated by adding 300  $\mu$ l of stopping solution containing 2% SDS, 25 mM ATP, and 1.3 mM cAMP. [<sup>32</sup>P]cAMP was separated from [<sup>32</sup>P]ATP by Dowex and alumina chromatography. [<sup>3</sup>H]cAMP was added to each reaction mixture to allow calculation and correction for column recovery. Radioactivity in each sample was determined by liquid scintillation spectroscopy.

**Radioligand Binding Assays.** PC2 cells were harvested and homogenized by sonication in buffer containing 5 mM Tris · HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 14  $\mu$ g/ml aprotinin. The homogenate was centrifuged at 49,000g for 20 min and the pellets resuspended in binding buffer containing 50 mM Tris · HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EDTA. For saturation binding experiments, 0.01 to 8 nM [<sup>125</sup>I]SCH23982 were used. In competition binding experiments, 0.6 nM [<sup>125</sup>I]SCH23982 was employed. Nonspecific binding was defined as the binding of the radioligand in the presence of 1  $\mu$ M *cis*-flupenthixol. The reaction was carried out at 25°C for 120 min and terminated by rapid filtration using a Brandel cell harvester with Whatman GF/C filter followed by washing with ice-cold binding buffer. The radioactivity on the filter was determined in a Beckman gamma counter. Saturation and competition binding curves were analyzed by nonlinear least-square regression using the LIGAND program (Munson and Rodbard, 1980).

**Coimmunoprecipitation of D<sub>1A</sub> Dopamine Receptor with G<sub>s $\alpha$</sub>  Protein.** Determination of the linkage between receptor and G protein was carried out as described previously by Wang et al. (1995). Transfected PC2 cells were homogenized in 10 volumes of buffer containing 25 mM HEPES, pH 7.5, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% 2-mercaptomethanol, 50  $\mu$ g/ml leupeptin, 25  $\mu$ g/ml pepstatin A, 5  $\mu$ g/ml aprotinin, 0.01 U/ml soybean trypsin inhibitor, and 0.04 mM PMSF. The homogenate was centrifuged at 800g for 5 min and the supernatant was centrifuged for 10 min at 49,000g. The resulting pellet was washed and resuspended in immunoprecipitation buffer containing 100 mM Tris · HCl, pH 7.5, 200 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% 2-mercaptomethanol, 50  $\mu$ g/ml leupeptin, 25  $\mu$ g/ml pepstatin A, 0.01 U/ml soybean trypsin inhibitor, and 0.04 mM PMSF. Membrane proteins (50  $\mu$ g) were solubilized in 1 ml of the immunoprecipitation buffer supplemented with 0.2% cholate and 0.5% digitonin. Solubilized tissues were precleaned by the addition of normal rabbit serum (1:100 dilution) at 4°C for 60 min followed by a 30-min incubation with 100  $\mu$ l of a 10% suspension of protein A-bearing *Staphylococcus aureus* cells (Pansorbin cells). The suspension was centrifuged and the supernatant was combined with antisera

(1:1000 dilution) raised against a specific G<sub>sa</sub> peptide and incubated for 3 h followed by an additional 30 min with 100  $\mu$ l of Pansorbin. After centrifugation, the pellet was suspended in 100  $\mu$ l of sample preparation buffer and boiled for 5 min. The D<sub>1A</sub> dopamine receptors in the immunoprecipitates were assessed by immunoblotting using a monoclonal antibody that recognizes D<sub>1A</sub> dopamine receptor [originally produced and characterized by Hersch et al. (1995)].

**Immunoblot Analysis.** Membrane proteins (25  $\mu$ g) were solubilized in sample preparation buffer and were separated by SDS-polyacrylamide gel electrophoresis (12%). Proteins were transferred electrophoretically to nitrocellulose membrane. The completeness of the transfer was checked by Coomassie blue staining of the gel. The membranes were incubated at 4°C overnight with 10% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (0.1%



**Fig. 1.** Specific D<sub>1</sub> dopamine receptor binding in PC2 cell membranes expressing D<sub>1A</sub> dopamine receptor. A, saturation binding was assessed by incubating 50  $\mu$ g of PC2 cell membrane protein with 0.01 to 8 nM [<sup>125</sup>I]SCH23982 at 25°C for 120 min. Nonspecific binding was defined as binding in the presence of 10  $\mu$ M *cis*-flupenthixol. B, displacement of specific [<sup>125</sup>I]SCH23982 binding was tested by incubating 50  $\mu$ g of PC2 cell membrane protein with 0.6 nM [<sup>125</sup>I]SCH23982 in the presence of increasing concentrations of the indicated dopaminergic ligands. The assay was carried out 25°C for 120 min. Reaction was terminated by rapid filtration. The results shown are representative of three independent experiments.



PBST) to block nonspecific sites. In some cases, 1  $\mu\text{g}/\text{ml}$  of goat antirabbit IgG was added to the blocking solution to reduce immunostaining of the IgG band. The membrane was washed with 0.1% PBST and incubated for 2 h either with  $G_{s\alpha}$  antiserum at 1:2000 dilution or with specific  $D_{1A}$  dopamine receptor antibody at 1:1000 dilution. The unbound antibody was washed out with 0.1% PBST. The blot was incubated for 60 min with 1:10,000 dilution of horseradish peroxidase-conjugated antirabbit IgG (for  $G_{s\alpha}$  protein blot) or anti-mouse IgG (for  $D_{1A}$  dopamine receptor blot) followed by washing in 0.3% PBST and in 0.1% PBST, respectively. The immunoreactive proteins were detected by the enhanced chemiluminescence Western blot detection system and visualized by exposure to X-ray film.

**Data Analysis.** Data are presented as mean  $\pm$  S.E. Two-tailed analysis of variance (ANOVA) was employed to compare the data among the groups followed by Newman-Keuls test. Significance was considered at  $P < .05$ .

## Results

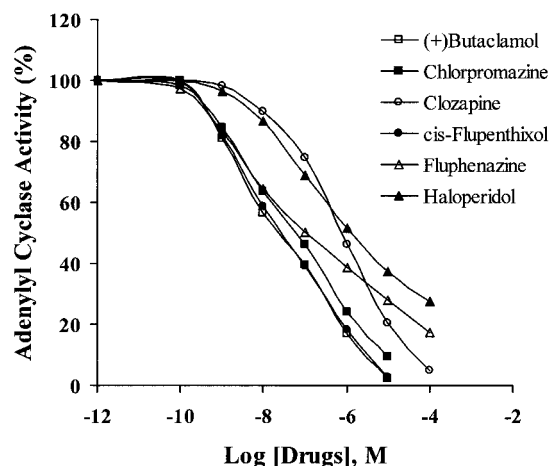
**Functional Expression of  $D_{1A}$  Dopamine Receptors in PC2 Cells.** The expression of  $D_{1A}$  dopamine receptors was determined in membranes obtained from transfected PC2 cells by saturation binding using the specific  $D_1$  dopamine receptor antagonist, [ $^{125}\text{I}$ ]SCH23392, as ligand. Specific [ $^{125}\text{I}$ ]SCH23392 binding was not detected in membranes of untransfected PC2 cells. After 48 h of transfection with rat  $D_{1A}$  dopamine receptor cDNA, [ $^{125}\text{I}$ ]SCH23392 binding was saturable and the dissociation constant ( $K_d$ ) was calculated to be  $0.38 \pm 0.09$  nM, with a maximal binding constant ( $B_{\text{max}}$ ) of 1 to 4 pmol/mg protein (Fig. 1A).

Functional expression of  $D_{1A}$  dopamine receptors in PC2 cells was also assessed by measuring basal and dopamine-stimulated adenylyl cyclase activity. Basal adenylyl cyclase activity in membranes of transfected cells was significantly increased compared with untransfected PC2 cells ( $22.2 \pm 0.7$  versus  $14.5 \pm 0.6$  pmol/min/mg). Dopamine ( $100 \mu\text{M}$ ) stimulated adenylyl cyclase 2-fold in transfected PC2 cell membranes, whereas the production of cAMP in membranes of untransfected cells was not affected by dopamine. The dopamine-induced stimulation of adenylyl cyclase was inhibited by the specific  $D_1$  dopamine receptor antagonist, SCH23390 (Table 1).

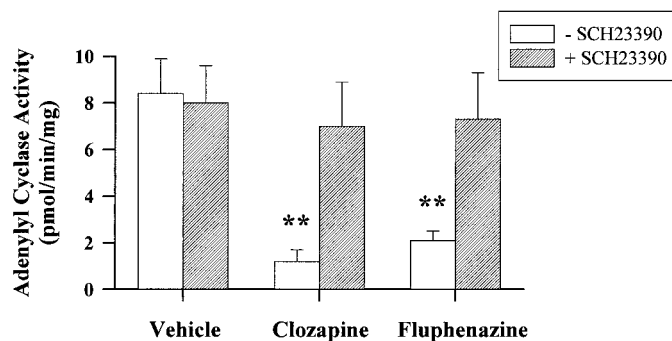
**Effects of Dopaminergic Antagonists on Constitutive Adenylyl Cyclase Activity.** Inverse antagonist activity of dopaminergic antagonists was measured by monitoring the effect of compounds on  $D_{1A}$  receptor-dependent constitutive

adenylyl cyclase activity, which was defined by subtracting basal adenylyl cyclase activity in wild-type PC2 cells from that in PC2 cells expressing  $D_{1A}$  receptors. Although previously classified as dopaminergic antagonists, (+)-butaclamol, chlorpromazine, clozapine, fluphenazine, *cis*-flupenthixol, and haloperidol inhibited constitutive adenylyl cyclase activity in a dose-dependent fashion (Fig. 2A). (+)-Butaclamol, chlorpromazine, clozapine, and *cis*-flupenthixol completely inhibited constitutive activity at concentrations between 10 to  $100 \mu\text{M}$ , whereas fluphenazine and haloperidol achieved maximal inhibitions of 78.9% and 76.9%, respectively (Table

A



B



**Fig. 2.** Effect of dopaminergic antagonists on constitutive adenylyl cyclase activity in PC2 cell membranes. The constitutive activity was defined by subtracting basal adenylyl cyclase activity in wild-type PC2 cells from that in PC2 cells expressing  $D_{1A}$  receptors. A, membrane protein (50  $\mu\text{g}$ ) obtained from PC2 cells expressing  $D_{1A}$  dopamine receptors was incubated at  $30^\circ\text{C}$  for 10 min with dopaminergic antagonists in the presence of 1  $\mu\text{Ci}$  [ $\alpha$ - $^{32}\text{P}$ ]ATP and formed [ $^{32}\text{P}$ ]cAMP was determined. The antagonists (+)-butaclamol, chlorpromazine, clozapine, fluphenazine, *cis*-flupenthixol, and haloperidol, in doses ranging from 0.1 nM to  $100 \mu\text{M}$ , inhibited constitutive adenylyl cyclase activity. SCH23390, (-)-butaclamol, and *l*-sulpiride, tested at  $10 \mu\text{M}$  each, were ineffective in inhibiting cyclase activity. B, PC2 cell membranes were preincubated with  $10 \mu\text{M}$  SCH23390 at  $30^\circ\text{C}$  for 10 min before addition of buffer or  $10 \mu\text{M}$  clozapine or fluphenazine and adenylyl cyclase activity was assessed. The  $D_1$  dopamine receptor antagonist SCH23390 inhibited clozapine- or fluphenazine-mediated inhibition of constitutive adenylyl cyclase activity. The results are the mean  $\pm$  S.E. obtained from four to six independent experiments. \*\*,  $p < .01$  compared with the vehicle treatment.

TABLE 1

Dopamine-stimulated adenylyl cyclase activity in PC2 cells expressing  $D_{1A}$  dopamine receptors

Membranes prepared from PC2 cells transfected with or without plasmids containing  $D_{1A}$  dopamine receptors cDNA were incubated with  $100 \mu\text{M}$  dopamine at  $30^\circ\text{C}$  for 10 min in the presence of 1  $\mu\text{Ci}$  [ $\alpha$ - $^{32}\text{P}$ ]ATP. Antagonism of dopamine-stimulated adenylyl cyclase activity was carried out by preincubating membranes with  $10 \mu\text{M}$  SCH23390 for 10 min. The results are presented as mean  $\pm$  S.E. obtained from four to six independent experiments.

	Adenylyl Cyclase Activity	
	Untransfected	Transfected
	pmol/min/mg	
Basal	$14.5 \pm 0.6$	$22.2 \pm 0.7^a$
Dopamine $100 \mu\text{M}$	$15.1 \pm 0.9$	$45.4 \pm 6.5^{b,c}$
SCH23390 $10 \mu\text{M}$ + Dopamine $100 \mu\text{M}$	$14.9 \pm 0.8$	$23.8 \pm 1.9^{a,d}$

<sup>a</sup>  $p < .05$  compared with the untransfected group.

<sup>b</sup>  $p < .01$  compared with the untransfected group.

<sup>c</sup>  $p < .01$  compared with basal activity.

<sup>d</sup>  $p < .05$  compared with dopamine alone.

2). The potencies of clozapine and haloperidol were lower than that of the other antipsychotic drugs in inhibiting constitutive adenylyl cyclase activity (Table 2). Constitutive adenylyl cyclase activity was not affected by the inactive isomer of butaclamol, (–)-butaclamol, by the selective D<sub>1</sub> dopamine receptor antagonist SCH23390, or by the selective D<sub>2</sub> dopamine receptor antagonist *l*-sulpiride. The selective D<sub>1</sub> dopamine receptor antagonist SCH23390 (Fig. 2B) inhibited the effects of fluphenazine and clozapine on constitutive adenylyl cyclase activity. In contrast, the dopaminergic antagonists clozapine, *cis*-flupenthixol, and haloperidol did not alter adenylyl cyclase activity in membranes obtained from untransfected wild-type PC2 cells (Fig. 3).

**Binding of Dopaminergic Antagonists to D<sub>1A</sub> Dopamine Receptor.** The binding affinities of the dopaminergic compounds for the D<sub>1A</sub> dopamine receptor were assessed by their ability to compete for [<sup>125</sup>I]SCH23982 binding sites in PC2 cell membranes expressing D<sub>1A</sub> dopamine receptors. (+)-Butaclamol, haloperidol, *cis*-flupenthixol, fluphenazine,

chlorpromazine, and clozapine inhibited [<sup>125</sup>I]SCH23982 binding in a dose-dependent manner, yielding inhibitory dissociation constants (*K<sub>i</sub>*) in the range of 0.8 to 60 nM (Fig. 1B, Table 3). On the other hand, (–)-butaclamol and SCH23390 displayed a large difference in their affinities for the D<sub>1A</sub> dopamine receptor (*K<sub>i</sub>* = 9 μM and 0.28 nM, respectively). Binding properties of these dopaminergic compounds at the D<sub>1A</sub> dopamine receptor were consistent with those reported in previous studies on brain membranes (Dearry et al., 1990; Kanba et al., 1994; Sugamori et al., 1994).

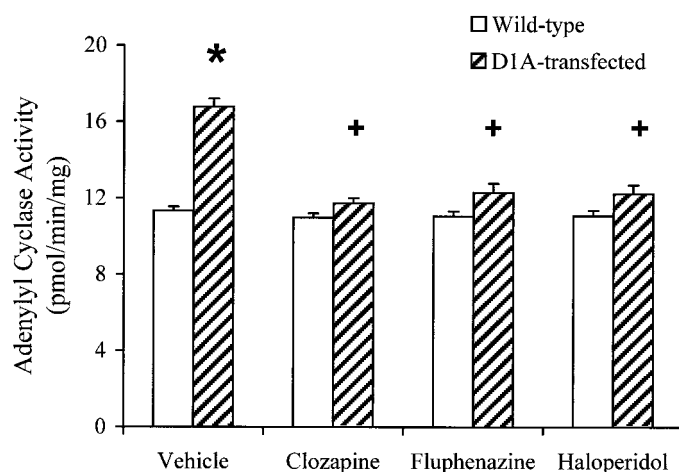
**Effects of Dopaminergic Antagonists on D<sub>1A</sub> Dopamine Receptor-G Protein Coupling.** The coupling of D<sub>1A</sub> dopamine receptors to G<sub>s</sub> protein was assessed by monitoring D<sub>1A</sub> dopamine receptor protein that coimmunoprecipitated with G<sub>sα</sub> protein. The D<sub>1A</sub> dopamine receptor protein was detected in immunoblots using a monoclonal D<sub>1A</sub> dopamine

TABLE 2

The potencies and efficacies of dopaminergic antagonists in inhibiting constitutive adenylyl cyclase activity

Membrane protein (50 μg) obtained from PC2 cells expressing D<sub>1A</sub> dopamine receptors was incubated at 30°C for 10 min with the test drugs in the presence of 1 μCi [<sup>α-32</sup>P]ATP and formed [<sup>32</sup>P]cAMP was assessed. The effects of the dopaminergic antagonists (+)-butaclamol, chlorpromazine, clozapine, fluphenazine, *cis*-flupenthixol, and haloperidol on constitutive adenylyl cyclase activity were tested at concentrations ranging from 0.1 nM to 100 μM. The potencies and efficacies for inhibition of constitutive adenylyl cyclase activity were calculated from the data presented in Fig. 2A using nonlinear regression analysis.

Compound	EC <sub>50</sub>	Maximal Inhibition
	μM	%
(+)-Butaclamol	0.021 ± 0.007	96.1 ± 5.9
Chlorpromazine	0.099 ± 0.059	99.3 ± 1.2
Clozapine	0.821 ± 0.406	95.5 ± 6.2
Fluphenazine	0.026 ± 0.012	78.9 ± 6.8
<i>cis</i> -Flupenthixol	0.035 ± 0.025	96.5 ± 3.6
Haloperidol	0.408 ± 0.169	76.9 ± 6.8



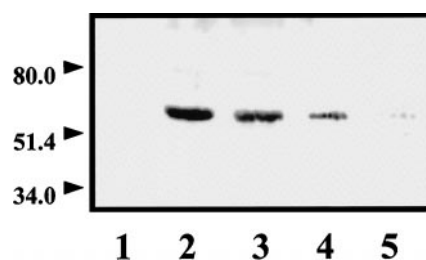
**Fig. 3.** Effect of dopaminergic antagonists on basal adenylyl cyclase activity in membranes of wild-type PC2 cells and D<sub>1A</sub> dopamine receptor-expressing PC2 cells. Membrane proteins (50 μg) were incubated at 30°C for 10 min with dopaminergic antagonists in the presence of 1 μCi of [<sup>α-32</sup>P]ATP and formed [<sup>32</sup>P]cAMP was determined. Clozapine, fluphenazine, and haloperidol, in doses of 10 μM decreased basal adenylyl cyclase activity in PC2 cells expressing the D<sub>1A</sub> dopamine receptor but not in the untransfected wild-type PC2 cells. Each bar represents the mean ± S.E. obtained from four individual experiments. \*, *p* < .05 compared with the basal adenylyl cyclase activity in untransfected wild-type PC2 cells. +, *p* < .05 compared with the vehicle treatment.

TABLE 3

*K<sub>i</sub>* values for ligands in competing for [<sup>125</sup>I]SCH23982 binding to D<sub>1A</sub> dopamine receptors in transfected PC2 cell membranes

Membranes prepared from PC2 cells expressing D<sub>1A</sub> dopamine receptors were incubated with 0.6 nM [<sup>125</sup>I]SCH23982 with or without increasing concentrations of the listed ligands. Specific D<sub>1A</sub> dopamine receptor binding was assessed and the inhibitory dissociation constants (*K<sub>i</sub>*) were calculated using the LIGAND program. The results were obtained from three independent experiments.

Compound	<i>K<sub>i</sub></i>
	nM
<b>Agonists</b>	
Dopamine	3,800
SKF39393	76
Quinpirole	19,000
<b>Neutral Antagonists</b>	
SCH23390	0.28
(–)Butaclamol	9,000
<b>Inverse Agonists</b>	
(+)-Butaclamol	0.8
Chlorpromazine	25
Clozapine	20
Fluphenazine	3
<i>cis</i> -Flupenthixol	1.2
Haloperidol	60



**Fig. 4.** D<sub>1A</sub> dopamine receptor immunoblots of wild-type PC2 cells and D<sub>1A</sub> dopamine receptor-expressing PC2 cells. Membrane proteins (50 μg) obtained from wild-type PC2 cells (lane 1) and D<sub>1A</sub> dopamine receptor-expressing PC2 cells (lane 2) were immunoblotted with an anti-D<sub>1A</sub> dopamine receptor antibody. In the immunoprecipitation experiment, 200 μg of membrane protein obtained from PC2 cells expressing D<sub>1A</sub> dopamine receptors was incubated at 30°C for 5 min in the absence (lane 3) or presence (lane 4) of 1 μM dopamine. Membrane protein (200 μg) incubated at 30°C for 15 min with 100 μM Gpp(NH)p was shown in lane 5. Membranes were solubilized, immunoprecipitated with Gas anti-serum, and 50 μg of original membrane proteins were immunoblotted with an anti-D<sub>1A</sub> dopamine receptor monoclonal antibody. The data indicate the absence of detectable D<sub>1A</sub> dopamine receptor protein in wild-type PC2 cell membrane, whereas D<sub>1A</sub> dopamine receptor cDNA-transfected cells show a clear 60-kDa band. D<sub>1A</sub> dopamine receptor protein was also detected in G<sub>sα</sub> immunoprecipitates of transfected cell membranes; the amount of receptor protein coprecipitated with G<sub>sα</sub> protein was increased by stimulation with dopamine and reduced by exposure to Gpp(NH)p.

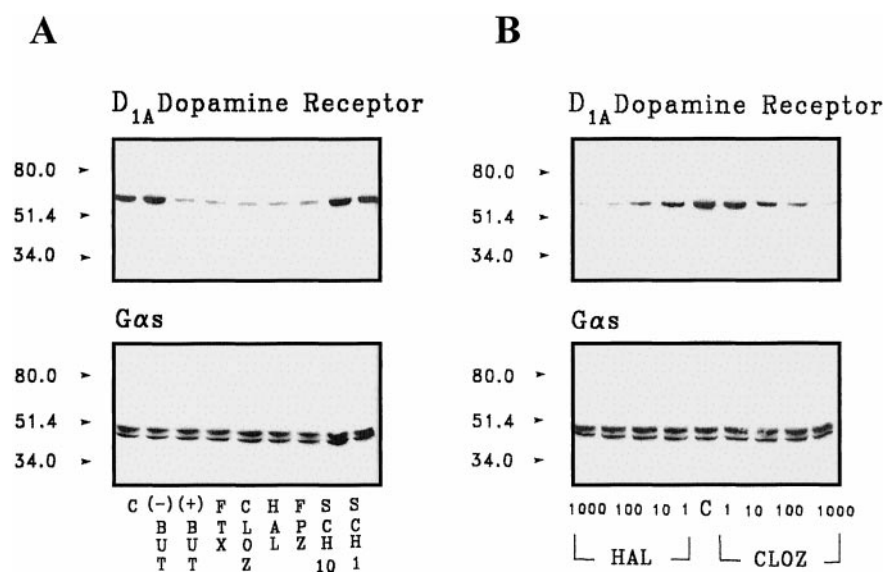
receptor antibody. The specificity of this antibody was supported by the fact that a single 60-kDa band was observed in membranes of D<sub>1A</sub> dopamine receptor-transfected PC2 cells but not in membranes of untransfected wild-type PC2 cells (Fig. 4). Significant basal coupling of receptor with G<sub>sα</sub> protein was found in membranes of PC2 cells expressing D<sub>1A</sub> dopamine receptors. Receptor-G<sub>sα</sub> coupling was enhanced by exposing the membranes to 1 μM dopamine. On the other hand, treatment with 100 μM 5'-guanylylimidodiphosphate [Gpp(NH)p] reduced D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> coupling (Fig. 4). (+)-Butaclamol, *cis*-flupenthixol, clozapine, haloperidol, and fluphenazine at concentrations of 1 μM reduced D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling by 70 to 80%. In contrast, no apparent effect on coupling was found when 1 μM (-)-butaclamol or 1 to 10 μM SCH23390 were tested (Fig. 5A). Furthermore, as shown in Fig. 5B, clozapine and haloperidol reduced D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling in a dose-dependent manner. The effects of these compounds on coupling were not attributable to immunoprecipitation efficiency because comparable G<sub>sα</sub> protein levels were detected when the immunoblots were probed with G<sub>sα</sub> antiserum (Fig. 5, A and B, bottom).

The coupling of D<sub>1A</sub> dopamine receptor with G<sub>sα</sub> protein was enhanced by incubating membranes with dopamine, reflecting an increase in receptor-stimulated transmem-

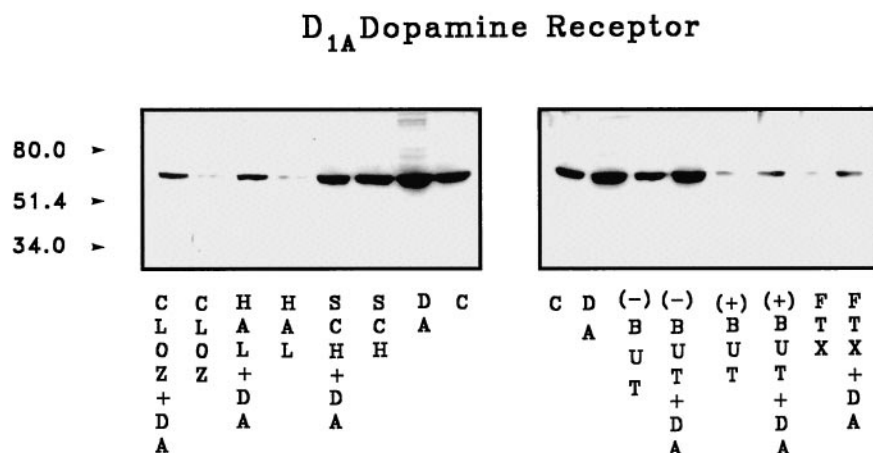
brane signaling. Clozapine, haloperidol, (+)-butaclamol, *cis*-flupenthixol, and SCH23390 blocked dopamine-enhanced D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling. Furthermore, the combination of dopamine and the inverse agonists resulted in coupling at a level lower than basal. However, the neutral antagonist SCH23390 prevented the dopamine-induced effect on D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling without reducing coupling to a level below control (Fig. 6).

## Discussion

Overexpression of G protein-coupled receptors enhances basal receptor activity, thus facilitating the assessment of the nature of the interaction of a ligand with its receptor-linked signal transduction pathway (Adie and Milligan, 1994; Barker et al., 1994; Chidiac et al., 1994; Bond et al., 1995; Barr and Manning, 1997). In the present experiments, the introduction of D<sub>1A</sub> dopamine receptors into PC2 cells, which ordinarily do not express this receptor, resulted in increased agonist-independent constitutive adenylyl cyclase activity. In this system, adenylyl cyclase activity was enhanced by receptor stimulation with dopamine and was inhibited, in a receptor-specific manner, by several dopaminergic antagonists, including (+)-butaclamol, chlorpromazine,



**Fig. 5.** Effect of dopaminergic antagonists on D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling. A, membrane protein (200 μg) obtained from PC2 cells expressing D<sub>1A</sub> receptors was incubated at 30°C for 5 min without (C) or with 1 μM (-)-butaclamol [(-)BUT], (+)-butaclamol [(+)BUT], *cis*-flupenthixol (FTX), clozapine (CLOZ), haloperidol (HAL), fluphenazine (FPZ), or with 1 or 10 μM SCH23390 (SCH). Membranes were solubilized, immunoprecipitated with G<sub>sα</sub> antiserum and the immunoprecipitates subjected to immunoblotting with an anti-D<sub>1A</sub> dopamine receptor monoclonal antibody (top) or with G<sub>sα</sub> antiserum (bottom). The effects of increasing concentration (1–1000 nM) of haloperidol or clozapine are shown in B. The results indicate that (+)-butaclamol, *cis*-flupenthixol, clozapine, haloperidol, and fluphenazine reduce D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> coupling by 70–80%, whereas (-)-butaclamol and SCH23390 do not affect the coupling. The results are representative of four independent experiments.



**Fig. 6.** Effects of dopaminergic antagonists on dopamine-induced increase in D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> protein coupling. Membrane protein (200 μg) obtained from PC2 cells expressing D<sub>1A</sub> dopamine receptors was incubated at 30°C for 5 min with 1 μM (-)-butaclamol [(-)BUT], (+)-butaclamol [(+)BUT], *cis*-flupenthixol (FTX), clozapine (CLOZ), haloperidol (HAL), and SCH23390 (SCH) in the absence (C) or presence of 1 μM dopamine (DA). Membranes were solubilized, immunoprecipitated with G<sub>sα</sub> antiserum and the immunoprecipitates subjected to immunoblotting with an anti-D<sub>1A</sub> dopamine receptor monoclonal antibody. The results indicate that (+)-butaclamol, *cis*-flupenthixol, clozapine, haloperidol, and SCH23390 inhibit DA-induced increase in D<sub>1A</sub> dopamine receptor-G<sub>sα</sub> coupling, whereas (-)-butaclamol does not show this inhibitory action. The results are representative of four independent experiments.



clozapine, fluphenazine, *cis*-flupenthixol, and haloperidol. The D<sub>1A</sub> dopamine receptor-dependent constitutive adenylyl cyclase activity was, however, not affected by other dopaminergic antagonists such as the selective D<sub>1</sub> dopamine receptor antagonist SCH23390, the inactive isomer of butaclamol, (–)-butaclamol, or the D<sub>2</sub> dopamine receptor antagonist *l*-sulpiride. The selective action of these dopaminergic antagonists on constitutive adenylyl cyclase activity was further supported by the fact that these antagonists did not affect adenylyl cyclase activity in untransfected wild-type PC2 cells. This profile of action of the tested compounds at the D<sub>1A</sub> dopamine receptor supports their classification as agonist (dopamine), inverse agonist [chlorpromazine, (+)-butaclamol, clozapine, fluphenazine, *cis*-flupenthixol, and haloperidol], or neutral antagonist (SCH23390). These compounds demonstrated a range of affinities for the D<sub>1A</sub> dopamine receptor that did not parallel their action on cyclase activity. Nonetheless, the inverse agonist activity of the dopaminergic agents is related to their affinity for the D<sub>1A</sub> dopamine receptor, because the specific and neutral D<sub>1</sub> dopamine receptor antagonist SCH23390 prevented their actions. Thus, the antipsychotic dopaminergic antagonists do not behave as simple competitive antagonists but also exert inverse agonist activity at the D<sub>1A</sub> dopamine receptor. These drugs, therefore, may be expected to be highly effective in inhibiting signals transduced by D<sub>1A</sub> dopamine receptors even at low levels of receptor occupancy compared with the simple competitive receptor blockers. A recent study has demonstrated that some dopaminergic antagonists also act as inverse agonists at D<sub>2</sub> dopamine receptors (Hall and Strange, 1997). It is possible, therefore, that the therapeutic actions of antipsychotic drugs may be mediated by inverse agonist activity at either the D<sub>1A</sub> or D<sub>2</sub> dopamine receptor or both. Thus, inverse agonist properties of dopaminergic antagonists should be considered together with their affinities for the dopamine receptors to more fully understand the mechanism of action of these drugs in the pharmacotherapy of schizophrenia and other disorders that may be related to a hyperdopaminergic state.

The two-state model of receptor activation suggests that G protein-coupled receptors exist in active (R\*) and inactive (R) forms that are in dynamic equilibrium (Leff, 1995). The active form may exist in the absence of receptor occupancy, reflecting spontaneous or constitutive receptor activity (Lefkowitz et al., 1993). The active form of G protein-coupled receptors has a high binding affinity for receptor agonists and is believed to be precoupled to G proteins (Lefkowitz et al., 1993). Agonists elicit increased receptor-G protein coupling, whereas inverse agonists might be expected to affect a reduction in receptor-G protein coupling. In the present study, a coimmunoprecipitation technique was used to directly assess the effect of dopamine, Gpp(NH)p, and potential inverse agonists on D<sub>1A</sub> dopamine receptor-G protein coupling. Although the agonist, dopamine, increased the coupling of D<sub>1A</sub> dopamine receptors to G<sub>sα</sub> protein, Gpp(NH)p and the dopaminergic inverse agonists were found to reduce the basal association of D<sub>1A</sub> dopamine receptors with G<sub>sα</sub> protein, and the neutral D<sub>1A</sub> dopamine receptor antagonist SCH 23390 failed to directly influence this coupling, although it prevented the interaction of inverse agonists with the D<sub>1A</sub> receptors. Furthermore, the inverse agonists blocked the dopamine-

induced increase in D<sub>1A</sub> receptor-G protein coupling, resulting in a coupling level lower than that found under basal conditions, suggesting that the uncoupling induced by the inverse agonists can not be easily surmounted by receptor stimulation. When we consider these results in the framework of the two-state receptor model, in which active and inactive conformations of a receptor exist in equilibrium, the compounds that bind to the D<sub>1A</sub> dopamine receptors are classified as agonists if they increase the receptors that are in the active state. Inverse agonists shift the equilibrium in favor of the inactive form of the receptor, and neutral antagonists do not change the ratio of active to inactive receptors. Thus, an increase in receptors that are in the active state by dopamine enhances the coupling of D<sub>1A</sub> dopamine receptors to their associated G protein; inverse agonists, on the other hand, are more likely bound to the inactive form of the D<sub>1A</sub> receptor and shift the equilibrium toward the inactive form of the receptor, dissociating precoupled receptors from G<sub>s</sub> protein. Neutral antagonists do not alter the ratio of active and inactive receptors or the coupling state of the D<sub>1A</sub> receptor to G<sub>s</sub> protein. Westphal and Sanders-Bush (1994) have previously demonstrated that inverse agonists bind to the uncoupled form of the 5-HT<sub>2C</sub> receptor with a higher affinity than to the G protein-coupled form of the receptor, and agonists have a high affinity for the coupled rather than the uncoupled form of the receptor. The present results directly demonstrate that D<sub>1A</sub> dopamine receptor inverse agonists negatively regulate the coupling of the D<sub>1A</sub> dopamine receptor with G<sub>s</sub> proteins. This is probably mediated by their binding to the inactive form of the receptor and shifting the equilibrium of receptors from the active to the inactive form.

In conclusion, the results of this study indicate that some classical dopaminergic antagonists inhibit constitutive adenylyl cyclase activity and behave as inverse agonists of D<sub>1A</sub> dopamine receptors. The mechanism for the apparent inverse agonist activity of these dopaminergic antagonists is related to their ability to reduce D<sub>1A</sub> dopamine receptor-G<sub>s</sub> protein coupling. This newly described action of the antipsychotic drugs should be considered as an alternative mechanism or an additional action of these agents that contributes to the therapeutic action of these drugs in the treatment of neuropsychiatric disorders.

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