

# Constitutive Activity of a Chimeric D<sub>2</sub>/D<sub>1</sub> Dopamine Receptor

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## SUMMARY

Chimeric D<sub>1</sub>/D<sub>2</sub> receptors were constructed to identify structural determinants of drug affinity and efficacy. We previously reported that chimeras that had D<sub>1</sub> receptor transmembrane domain VII together with amino-terminal sequence from the D<sub>2</sub> receptor were nonfunctional. D<sub>2</sub>/D<sub>1</sub> chimeras were constructed that contained D<sub>2</sub> receptor sequence at the amino- and carboxyl-terminal ends and D<sub>1</sub> receptor sequence in the intervening region. Chimeric receptors with D<sub>2</sub> sequence from transmembrane domain 7 to the carboxyl terminus together with D<sub>2</sub> receptor sequence from the amino terminus through transmembrane helix 4 (D<sub>2</sub>[1-4,7]) and 5 (D<sub>2</sub>[1-5,7]) bound [<sup>3</sup>H]spiperone with high affinity, consistent with the hypothesis that D<sub>2</sub> receptor transmembrane domain I or II is incompatible with D<sub>1</sub> receptor transmembrane domain VII. D<sub>2</sub>[1-4,7] and D<sub>2</sub>[1-5,7] had affinities similar to D<sub>1</sub> and D<sub>2</sub> receptors for most nonselective dopamine antagonists and had affinities for most of the selective antagonists that were intermediate between those of the parent receptors. D<sub>2</sub>[1-4,7] and D<sub>2</sub>[1-5,7] mediated dopamine receptor agonist-induced stimulation and inhibition, respec-

tively, of cAMP accumulation. The more efficient coupling of D<sub>2</sub>[1-5,7] to inhibition of cAMP accumulation, compared with the coupling of D<sub>2</sub>[5-7] and D<sub>2</sub>[3-7], supports the view that multiple D<sub>2</sub> receptor cytoplasmic domains acting in concert are necessary for receptor activation of G<sub>i</sub>. In contrast, D<sub>2</sub>[1-4,7], which contains only one cytoplasmic loop (the third) from the D<sub>1</sub> receptor, is capable of activating G<sub>s</sub>. D<sub>2</sub>[1-4,7] exhibited several characteristics of a constitutively active receptor, including enhanced basal (unliganded) stimulation of cAMP accumulation, high affinity for agonists even in the presence of GTP, and blunted agonist-stimulated cAMP accumulation. A number of dopamine receptor antagonists were inverse agonists at D<sub>2</sub>[1-4,7], inhibiting basal cAMP accumulation. Some of these drugs were also inverse agonists at the D<sub>1</sub> receptor. Interestingly, several antagonists also potentiated forskolin-stimulated cAMP accumulation via D<sub>2</sub>[1-5,7] and via the D<sub>2</sub> receptor, which could reflect inverse agonist inhibition of native constitutive activity of this receptor.

The dopamine receptor family comprises D<sub>1</sub>-like (D<sub>1</sub> and D<sub>5</sub>) and D<sub>2</sub>-like (D<sub>2L</sub>, D<sub>2S</sub>, D<sub>3</sub>, and D<sub>4</sub>) receptors (1). The D<sub>1</sub>-like dopamine receptors have a shorter IC3 and a longer carboxyl terminus than the D<sub>2</sub>-like receptors. D<sub>1</sub>-like receptors have high affinity for benzazepine ligands, such as SCH23390, whereas D<sub>2</sub>-like receptors have high affinity for benzamide and butyrophenone ligands, such as sulpiride and spiperone. D<sub>1</sub>-like dopamine receptors couple to the G protein G<sub>s</sub>, stimulating adenylate cyclase, whereas the D<sub>2</sub>-like receptors couple to G<sub>i</sub> or G<sub>o</sub>, inhibiting adenylate cyclase. D<sub>1</sub>- and D<sub>2</sub>-like receptors both regulate phosphoinositide turnover, and D<sub>2</sub>-like dopamine receptors also modulate arachidonic acid release, Na<sup>+</sup>/H<sup>+</sup> exchange, K<sup>+</sup> currents, and Ca<sup>2+</sup> currents (2).

One approach to identification of the structural features of G protein-coupled receptors that determine function is to

construct mutant and chimeric receptors. Mutagenesis of single amino acids in dopamine receptors has identified a number of residues in the putative transmembrane helices that are involved in the binding of ligands and agonist activation of D<sub>1</sub> and D<sub>2</sub> receptors (3-8). Although point mutations often can identify residues that are critical for specific functions, some receptor properties are likely to be attributable to multiple, contiguous amino acid residues that cannot be identified by point mutations.

Our analysis of chimeric D<sub>1</sub>/D<sub>2</sub> receptors has suggested that structural determinants of selective potency and efficacy can differ, with TMVI of the D<sub>2</sub> dopamine receptor implicated in selective efficacy of some agonists and TMVII of D<sub>1</sub> and D<sub>2</sub> receptors involved in the selective potency of some ligands. We demonstrated that there are determinants of receptor down-regulation in TMV and IC3 and confirmed the importance of IC3 in stimulation of adenylate cyclase by D<sub>1</sub> receptors, although inhibition of adenylate cyclase via D<sub>2</sub> receptors seems to require both IC2 and IC3 (9, 10).

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**ABBREVIATIONS:** ΔG°, change in free energy; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; BCS, bovine calf serum; DHX, dihydrexidine; G<sub>i</sub>, G protein that inhibits adenylate cyclase; G<sub>s</sub>, G protein that stimulates adenylate cyclase; ICX, intracellular loop, where X is the loop number; K<sub>H</sub>, high affinity binding state; K<sub>L</sub>, low affinity binding state; NPA, N-propylnorapomorphine; TM, transmembrane domain; HEK, human embryonic kidney.

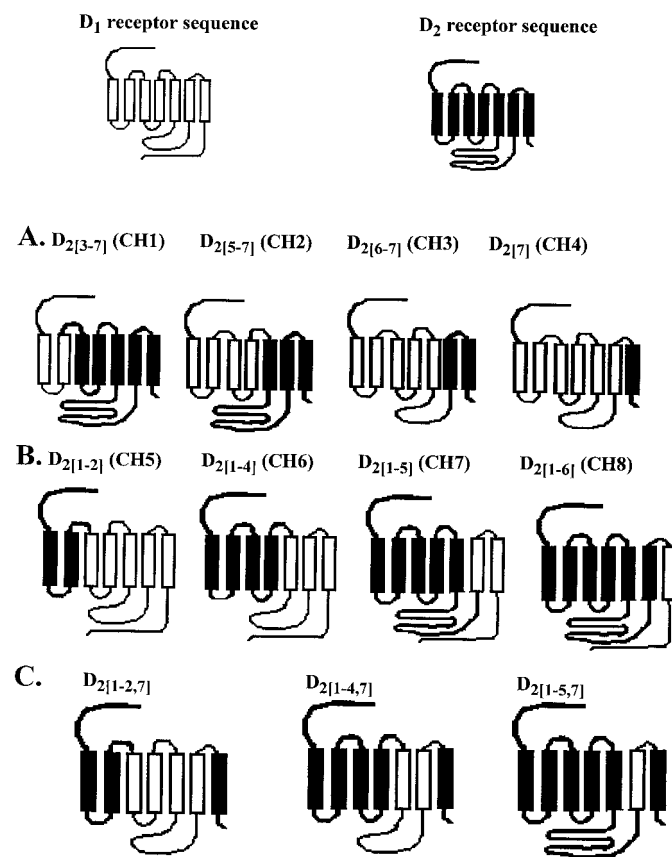
It often is observed that the function of certain chimeric receptors is dramatically impaired due to incompatibilities between adjacent transmembrane regions (11). Such chimeras can be used to identify intramolecular interactions in the parent receptors (12, 13). When we constructed chimeric receptors containing the D<sub>2</sub> receptor TMI and TMII and D<sub>1</sub> receptor TMVII, the chimeras were nonfunctional (9). Because three-dimensional models of G protein-coupled receptors and mutagenesis data suggest that TMI, TMII, and TMVII are adjacent to one another and that interactions between pairs of amino acids in these adjacent helices may stabilize the receptor structure (12–15), we hypothesized that incompatibilities between D<sub>1</sub> TMVII and D<sub>2</sub> TMI and TMII produced the nonfunctional state of these chimeric receptors. We now report that replacement of the D<sub>1</sub> TMVII in the nonfunctional chimeras with D<sub>2</sub> TMVII restored function. Interestingly, one of the chimeric receptors exhibited several characteristics of a constitutively active receptor. Constitutive activity results from mutations that increase the probability of spontaneous isomerization of the receptor to its active conformation, which increases the affinity of the receptor for agonists and enhances “basal,” or unliganded, stimulation of effectors by the receptor (16).

## Experimental Procedures

**Materials.** [<sup>3</sup>H]Spiperone (80 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [<sup>3</sup>H]SCH23390 (70 Ci/mmol) and [<sup>3</sup>H]cAMP (30 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). SCH23390, SKF-38393, spiperone, chloro-APB (SKF 82958), 6-chloro-PB, quinpirole (LY17155), apomorphine, bromocriptine, lisuride, and forskolin were purchased from Research Biochemicals (Natick, MA). DHX (Dr. Richard Mailman, University of North Carolina, Chapel Hill, NC), epidepride (NCQ 219; Dr. Tomas de Paulis, Vanderbilt University, Nashville, TN), fenoldopam (SKF82526; Dr. Richard Wilcox, University of Texas at Austin), and pergolide (Eli Lilly, Indianapolis, IN) were generous gifts. Dopamine (3-hydroxytyramine), 3-isobutyl-1-methylxanthine, and most other reagents were purchased from Sigma Chemical (St. Louis, MO).

**Construction of chimeric receptor cDNAs.** Eight chimeric cDNAs (previously referred to as CH1–8; new nomenclature depicted in Fig. 1, A and B) were constructed by *trans*-polymerase chain reaction and cloned into *Hind*III and *Eco*RI sites of pcDNA-1. The construction, expression in C<sub>6</sub> glioma cells, and characterization of these receptors were described previously (9). The D<sub>1</sub> TMVII and cytoplasmic tail was removed from D<sub>2[1-2]</sub> (CH5), D<sub>2[1-4]</sub> (CH6), and D<sub>2[1-5]</sub> (CH7) by digestion with *Cla*I and *Eco*RI and replaced with a fragment containing D<sub>2</sub> TMVII and cytoplasmic tail from D<sub>2[7]</sub> (CH4), creating D<sub>2[1-2,7]</sub>, D<sub>2[1-4,7]</sub>, and D<sub>2[1-5,7]</sub> (Fig. 1C).

**Expression of recombinant receptors.** D<sub>1</sub>, D<sub>2L</sub>, and chimeric cDNAs (D<sub>2[1-2,7]</sub>, D<sub>2[1-4,7]</sub>, and D<sub>2[1-5,7]</sub>) were stably expressed in HEK 293 cells by electroporation. HEK 293 cells (2.2 × 10<sup>7</sup>/ml) were resuspended with the appropriate cDNA (15 μg) and pBabe Puro (2 μg), to confer resistance to puromycin (17), in Dulbecco's modified Eagle's medium supplemented with 10% BCS and 5 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid in a total volume of 400 μl. With a 0.4-cm cuvette gap, the electroporator settings were 0.17 kV and 950 μF, yielding time constants between 40 and 50 msec. The cells were split into four 10-cm-diameter tissue culture plates and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% BCS, penicillin G (50 units/ml), and streptomycin (50 μg/ml) in a humidified incubator at 37° in the presence of 10% CO<sub>2</sub>. After 48 hr, the medium was replaced with growth medium containing puromycin (2 μg/ml). Puromycin-resistant colonies were

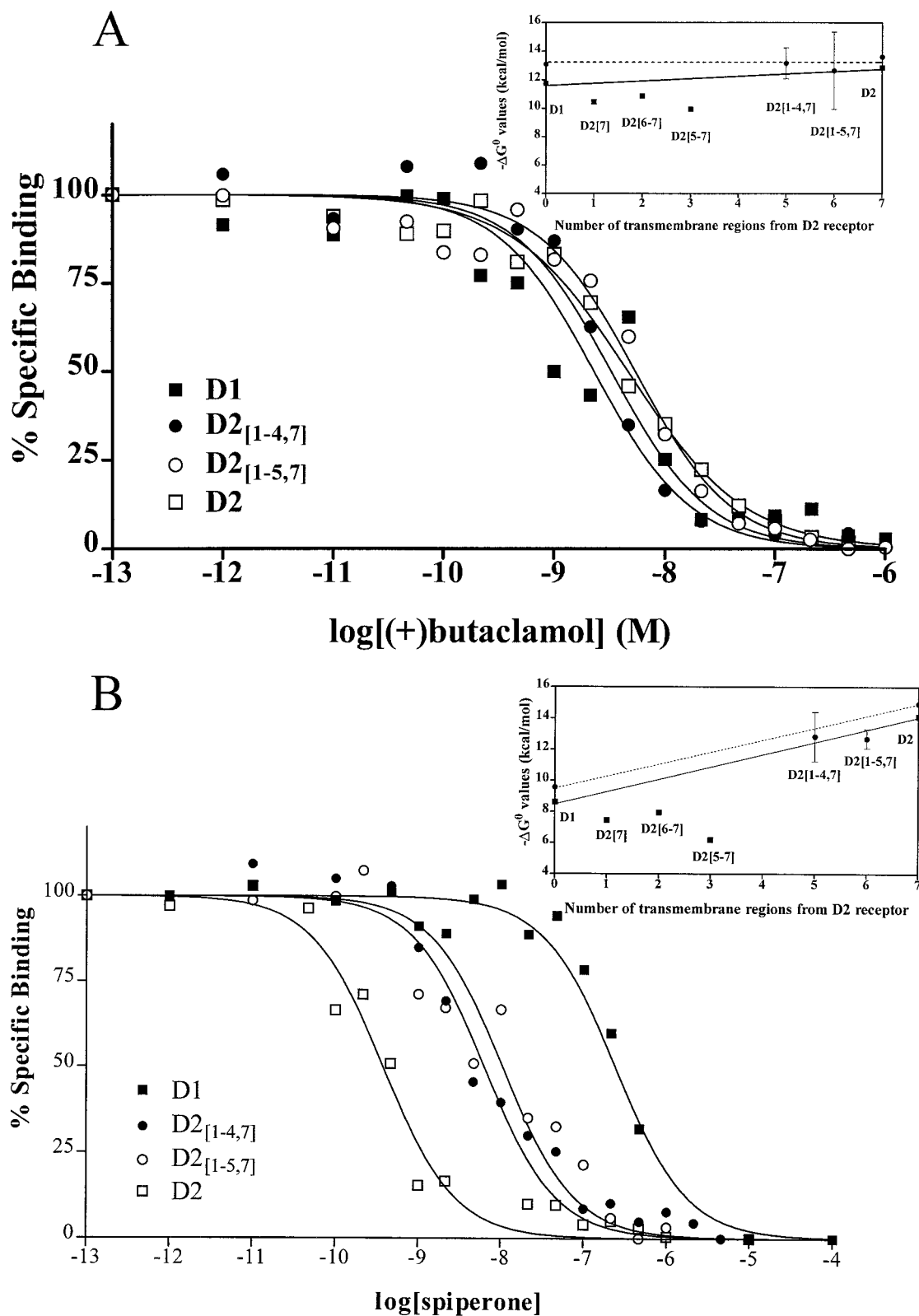


**Fig. 1.** Structure of the chimeric dopamine D<sub>2</sub>/D<sub>1</sub> receptors. D<sub>1</sub> sequence shows putative TM helices (*open rectangles*) and intracellular and extracellular hydrophilic regions (*thin lines*). D<sub>2</sub> sequence shows putative TM helices (*filled rectangles*) and hydrophilic regions (*thick lines*).

transferred to duplicate wells and tested for binding of D<sub>1</sub>- and D<sub>2</sub>-selective radioligands.

**Radioligand binding assay.** Confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na<sup>+</sup> HEPES, pH 7.4, 2 mM EDTA). After swelling for 10–15 min, the cells were scraped off the plate and centrifuged at 24,000 × *g* for 20 min. The crude membrane fraction was resuspended in Tris-buffered saline with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 10 sec and used for radioligand binding assays. For determinations of *K<sub>i</sub>* values, the affinity of D<sub>2[1-4,7]</sub>, D<sub>2[1-5,7]</sub>, and D<sub>2</sub> receptors for ligands was assessed by inhibition of the binding of [<sup>3</sup>H]spiperone, whereas the affinity of the D<sub>1</sub> receptor for ligands was quantified by inhibition of the binding of [<sup>3</sup>H]SCH23390. Aliquots of the membrane preparation (5–100 μg of protein) were added to duplicate assay tubes containing (final concentrations): 50 mM Tris-HCl, pH 7.4, with 155 mM NaCl (Tris-buffered saline), 0.001% bovine serum albumin, radioligand, and appropriate drugs. (+)-Butaclamol (2 μM for wild-type or 20 μM for chimeras) was used to define nonspecific binding. Incubations for binding studies were carried out at 30° for 1 hr and terminated by filtration through glass-fiber filters on a 96-well Tomtec cell harvester. The filters were dried before the addition of BetaPlate scintillation fluid, and radioactivity on the filters was determined with a Wallac (Gaithersburg, MD) 1205 BetaPlate scintillation counter.

For competition binding studies in which displacement of radioligand binding by agonists was assessed, the crude membrane fraction was resuspended in Tris-buffered saline containing 4 mM MgCl<sub>2</sub> before addition to assay tubes containing Tris-buffered saline and (final concentrations) 4 mM MgCl<sub>2</sub>, 1 mM EDTA, 200 μM GTP,



**Fig. 2.** Binding of antagonists. Data are shown from one of three or more independent experiments in which inhibition of radioligand binding was determined for the indicated drugs. Data are plotted as a percentage of the specific binding in the absence of inhibitor versus the logarithm of the concentration of (A) (+)-butaclamol or (B) spiperone. *Insets*, Averaged data from all experiments are expressed as the free energy change of binding (in Kcal/mol) plotted versus the number of transmembrane regions from the D<sub>2</sub> receptor. *Error bars*, 95% confidence intervals. ■, Data from our previous report using chimeric and wild-type receptors expressed in C<sub>6</sub> glioma cells (9). *Solid line*, line drawn between D<sub>1</sub> and D<sub>2</sub> receptor ΔG° values from our previous report. ●, Data from the current study using receptors expressed in HEK 293 cells. *Dotted line*, line drawn between D<sub>1</sub> and D<sub>2</sub> ΔG° values from the current study.

TABLE 1

**Affinity of D<sub>1</sub>, D<sub>2</sub>, and chimeric dopamine receptors for antagonists**

$K_i$  values for inhibition by the indicated antagonists of the binding of [<sup>3</sup>H]spiperone to chimeric and D<sub>2</sub> dopamine receptors, and the binding of [<sup>3</sup>H]SCH23390 to D<sub>1</sub> receptors, determined as described in Experimental Procedures. Affinity values represent geometric mean values from three or more independent experiments with the limits defined by the asymmetrical standard error given in parentheses.

Drug	$K_i$			
	D <sub>1</sub>	D <sub>2</sub> [1-4.7]	D <sub>2</sub> [1-5.7]	D <sub>2</sub>
		<i>nm</i>		
(+)-Butaclamol	2.2 (1.8–2.8)	2.0 (1.5–2.6)	4.4 (2.4–7.8)	1.0 (0.40–2.6)
Clozapine	200 (160–250)	9.5 (7.2–13)	98 (88–110)	240 (170–350)
cis-Flupenthixol	3.5 (2.0–6.2)	3.3 (3.0–3.7)	15 (14–16)	1.2 (0.87–1.6)
Epidepride	12,000 (11,000–14,000)	26 (20–33)	15 (12–19)	0.15 (0.12–0.17)
Haloperidol	120 (116–124)	4.6 (3.7–5.6)	6.2 (4.9–8.0)	2.1 (1.7–2.6)
Spiperone	470 (250–890)	3.3 (2.2–4.9)	4.0 (3.5–4.6)	0.13 (0.08–0.22)
Sulpiride	50,000 (39,000–65,000)	2,600 (1,800–3,900)	7,200 (3,600–14,000)	160 (130–210)
SCH23390	1.1 (0.9–1.3)	570 (340–930)	3,100 (2,900–3,400)	2,600 (2,000–3,400)

0.0025% ascorbic acid, 0.001% bovine serum albumin, radioligand, and appropriate drug concentrations. For competition binding studies in which dopamine displacement of binding in the absence and presence of GTP (200  $\mu$ M) was assessed, the crude membrane fraction was added to assay tubes containing 20 mM HEPES, pH 7.5, with 6 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.0025% ascorbic acid, and 0.001% bovine serum albumin, together with radioligand and dopamine concentrations as indicated. Incubations were carried out and filtered as detailed above.

**cAMP accumulation assay.** Cells were plated at a density of ~100,000 cells/well in 48-well tissue culture clusters. After 2–4 days, when the cells were confluent, the plates were used for adenylate cyclase stimulation or inhibition experiments. The rates of division of the clones were similar, so that each had 300,000–400,000 cells/well at the time of assay. The cAMP accumulation assay of adenylate cyclase activity was carried out essentially as described previously (18). Cells were rinsed on ice two times with 200  $\mu$ l of assay buffer (Earle's balanced salt solution, containing 0.02% ascorbic acid, 2% BCS, and 500  $\mu$ M 3-isobutyl-1-methylxanthine) for 5 min followed by the addition of assay buffer with or without forskolin (10  $\mu$ M final concentration) and appropriate drugs. Incubations were carried out at 37° for 10 min, except for agonist stimulation and inverse agonist inhibition of D<sub>2</sub>[1-4.7], which was carried out at 40°, a temperature that produced more consistent results. The assays were terminated by decanting the buffer, placing the plates on ice, and lysing the cells with 3% trichloroacetic acid. The plates were centrifuged at 1000  $\times$  *g* for 15 min and stored at 4° for  $\geq$ 1 hr before quantification of cAMP.

**Quantification of cAMP.** cAMP was quantified using a competitive binding assay (19) as described previously (18). Samples of the cell lysate from each well (5–20  $\mu$ l) were added to duplicate assay tubes. [<sup>3</sup>H]cAMP (~1 pmol) in cAMP assay buffer (100 mM Tris-HCl, pH, 7.4, 100 mM NaCl, 5 mM EDTA) was added to each tube, followed by cAMP-binding protein (100  $\mu$ g of crude bovine adrenal extract in cAMP assay buffer) for a final volume of 500  $\mu$ l. The reaction tubes were incubated on ice for 2–5 hr. The contents of the tubes were harvested by filtration (Whatman GF/C filters or Wallac Filter Mat A) using a 96-well Tomtec cell harvester. Filters were dried, and BetaPlate scintillation fluid was added to each sample. Radioactivity on the filters was determined using a Wallac BetaPlate scintillation counter. The cAMP concentration in each sample was estimated from a standard curve ranging from 0.1 to 100 pmol of cAMP.

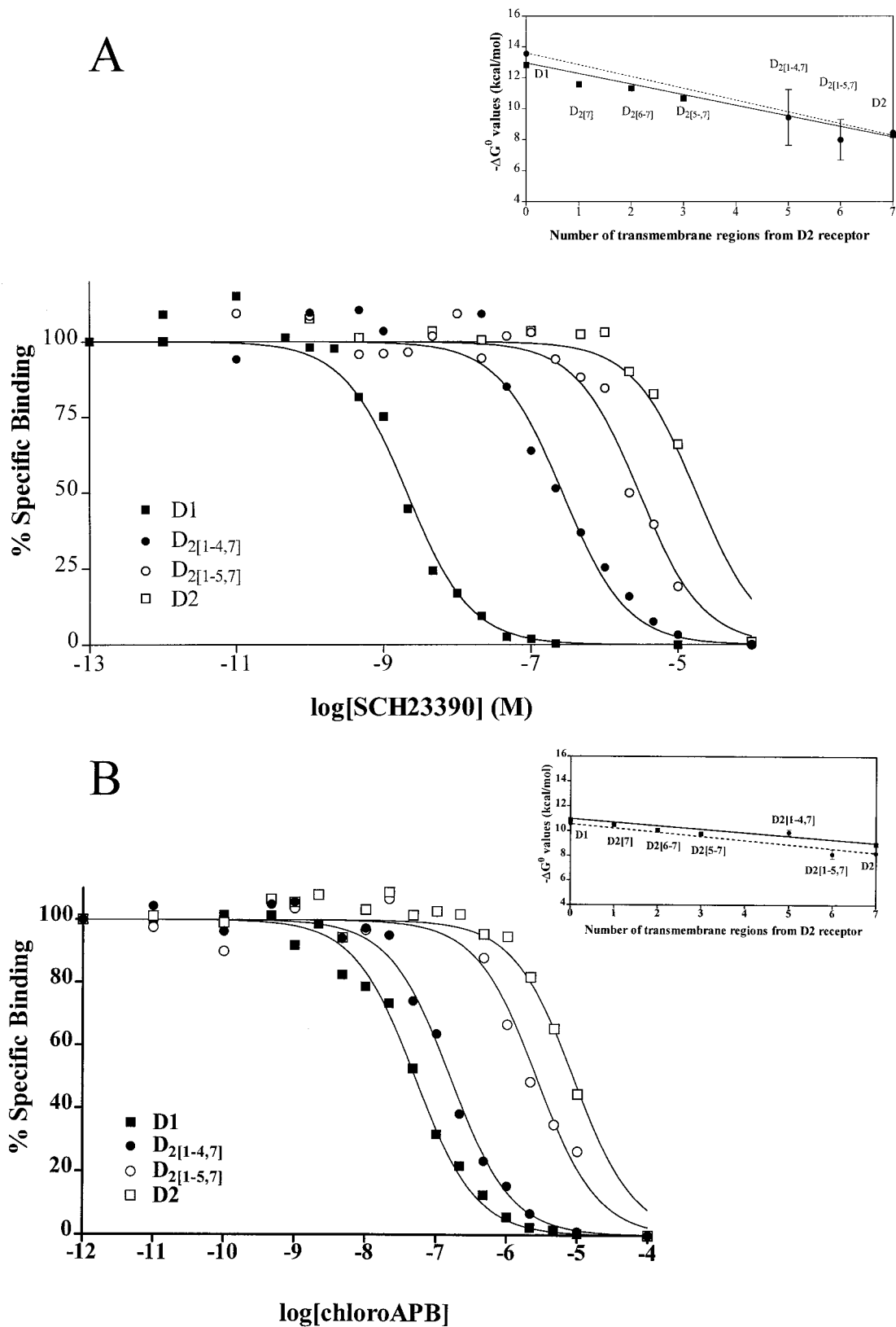
**Data analysis.** Saturation isotherms, radioligand displacement curves, and dose-response curves for cAMP accumulation were analyzed by nonlinear regression using the program GraphPAD Prism (GraphPAD Software, San Diego, CA).  $K_i$  values are geometric mean values from three or more independent experiments  $\pm$  the asymmetrical standard error. For dopamine, the goodness of fit for one- and two-site analyses was compared using an *F* test. At a value of *p* < 0.05 for improvement of the fit assuming two classes of binding sites, data were analyzed in terms of two classes of binding sites. Other statistical comparisons were made using Student's paired *t* test (two-tailed) as indicated. The change in free energy of binding of a drug was calculated using the equation  $\Delta G^\circ = -RT \ln(1/K_i)$  where

TABLE 2

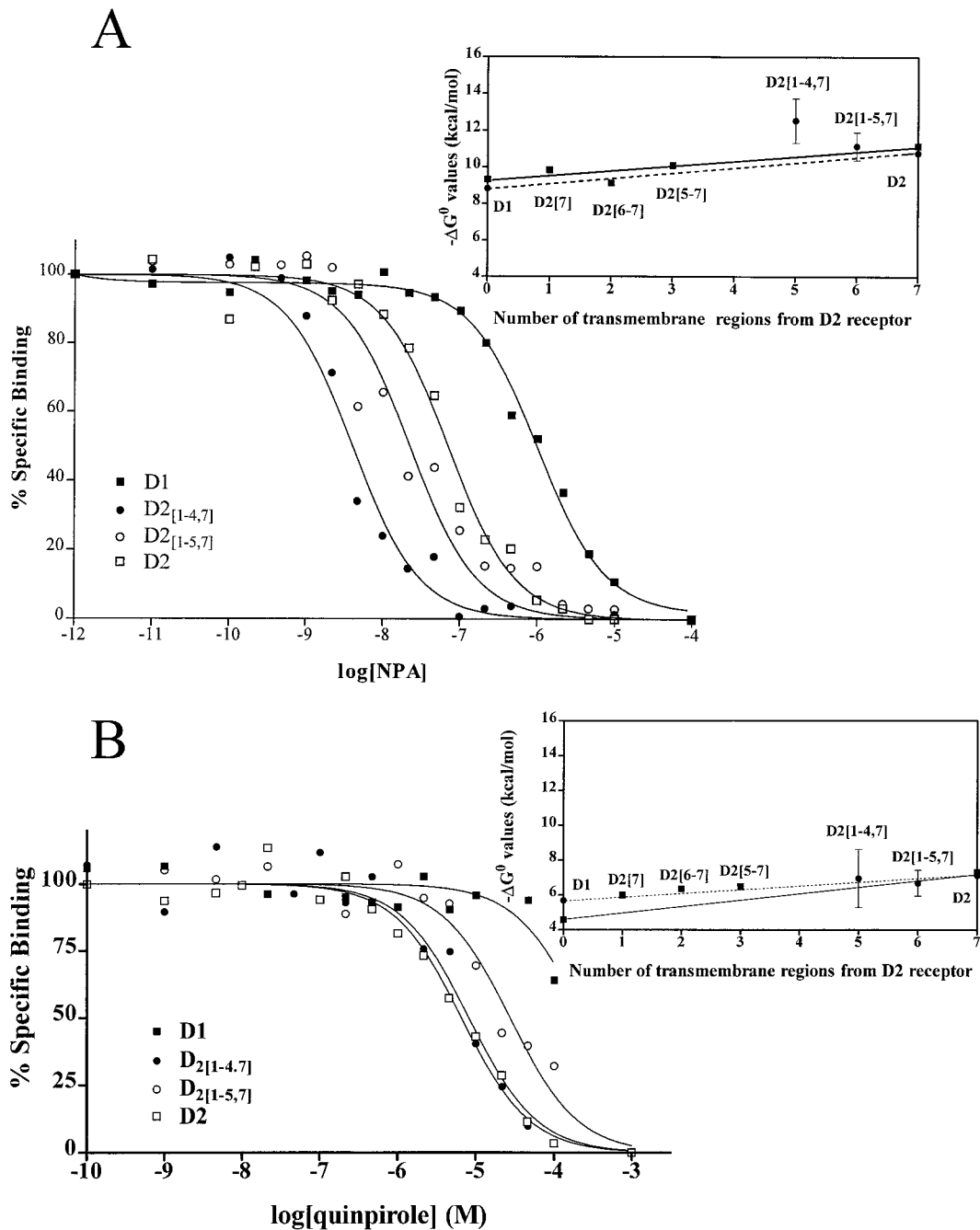
**Affinity of D<sub>1</sub>, D<sub>2</sub>, and chimeric dopamine receptors for agonists**

$K_i$  values for inhibition by the indicated agonists of the binding of [<sup>3</sup>H]spiperone to chimeric and D<sub>2</sub> receptors and the binding of [<sup>3</sup>H]SCH23390 to D<sub>1</sub> receptors determined as described in Experimental Procedures. All experiments were carried out in the presence of 200  $\mu$ M GTP. Affinity values (nm except where indicated) represent geometric mean values from three or more independent experiments with the limits defined by the asymmetrical standard error given in parentheses.

Drug	$K_i$			
	D <sub>1</sub>	D <sub>2</sub> [1-4.7]	D <sub>2</sub> [1-5.7]	D <sub>2</sub>
6,7-ADTN	15,000 (12,000–20,000)	41 (38–46)	500 (310–820)	600 (370–970)
Apomorphine	430 (400–470)	2.5 (2.1–3.1)	110 (87–130)	530 (410–680)
NPA	620 (430–880)	1.5 (1.1–2.0)	14 (12–17)	26 (18–39)
Dihydropyridine ( $\mu$ M)	1.2 (1.0–1.4)	0.30 (0.29–0.31)	4.8 (3.4–6.7)	1.7 (1.5–1.9)
Dopamine ( $\mu$ M)	31 (21–44)	0.52 (0.39–0.70)	20 (14–29)	13 (11–15)
Bromocriptine	1,400 (900–1,900)	6.1 (4.8–7.8)	9.5 (9.3–9.7)	2.2 (2.1–2.4)
Lisuride	77 (63–94)	0.4 (0.2–0.7)	1.4 (0.9–2.3)	0.70 (0.59–0.82)
Pergolide	2,000 (1,500–2,800)	25 (19–34)	29 (27–31)	85 (67–110)
Fenoldopam	85 (59–120)	320 (210–460)	520 (400–690)	1,300 (900–1,800)
Quinpirole ( $\mu$ M)	100 (70–120)	13 (9–19)	20 (17–24)	10 (10–14)
6-Chloro-PB	58 (43–79)	800 (680–950)	850 (400–1400)	4,800 (3,600–6,500)
Chloro-APB	36 (33–38)	110 (110–120)	1,900 (1,700–2,000)	1,700 (1,600–1,800)
SKF38393 ( $\mu$ M)	0.53 (0.34–0.84)	10 (0.8–1.4)	20 (11–36)	21 (18–24)



**Fig. 3.** Binding of benzazepine. Data are shown from one of three or more independent experiments in which inhibition of radioligand binding was determined for the indicated drugs. Data are plotted as a percentage of the specific binding in the absence of inhibitor versus the logarithm of the concentration of (A) SCH23390 or (B) chloro-APB in the presence of GTP. *Insets*, averaged data from all experiments are expressed as free energy change of binding (in Kcal/mol) plotted versus the number of transmembrane regions from the D<sub>2</sub> receptor. *Error bars*, 95% confidence intervals. ■, Data from our previous report using chimeric and wild-type receptors expressed in C<sub>6</sub> glioma cells (9). *Solid line*, line drawn between D<sub>1</sub> and D<sub>2</sub> receptor  $\Delta G^\circ$  values from our previous report. ●, Data from the current study. *Dotted line*, line drawn between D<sub>1</sub> and D<sub>2</sub>  $\Delta G^\circ$  values from the current study.



**Fig. 4.** Binding of agonists. Data are shown from one of three or more independent experiments in which inhibition of radioligand binding was determined for the indicated drugs. Data are plotted as a percentage of the specific binding in the absence of inhibitor versus the logarithm of the concentration of (A) NPA or (B) quinpirole in the presence of GTP. *Insets.* Averaged data from all experiments are expressed as the free energy change of binding (in Kcal/mol) plotted versus the number of transmembrane regions from the  $D_2$  receptor. *Error bars,* 95% confidence intervals.  $\blacksquare$ , Data our previous report using chimeric and wild-type receptors expressed in  $C_6$  glioma cells (9). *Solid line,* the line drawn between  $D_1$  and  $D_2$  receptor  $\Delta G^\circ$  values from our previous report.  $\bullet$ , Data from the current study. *Dotted line,* line drawn between  $D_1$  and  $D_2$   $\Delta G^\circ$  values from the current study.

$\Delta G^\circ$  is the change in free energy,  $R$  is the gas constant,  $T$  is temperature in  $^\circ\text{K}$ , and  $K_i$  is the apparent affinity of the drug.

## Results

To test the hypothesis that incompatible interactions between  $D_1$  TMVII and  $D_2$  TMI and TMII resulted in nonfunctional chimeric receptors, we modified  $D_{2[1-2]}$ ,  $D_{2[1-4]}$ , and  $D_{2[1-5]}$  (Fig. 1), so both the amino- and carboxy-terminal ends were from the  $D_2$  receptor, with  $D_1$  receptor sequence in the

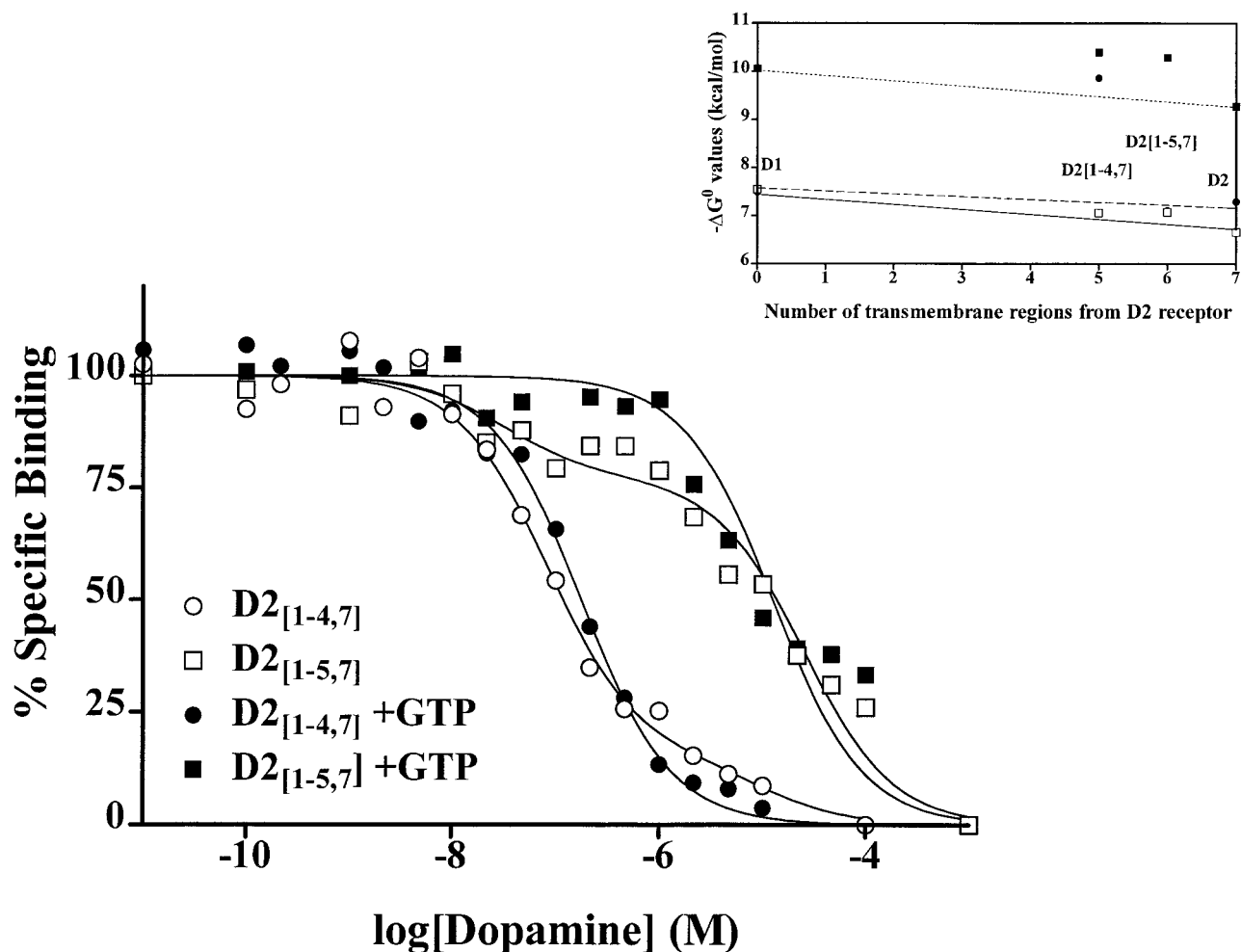
intervening region. The chimeric and wild-type  $D_1$  and  $D_2$  receptors were stably expressed in HEK 293 cells, and the binding of two radioligands was assessed. The chimeric receptors  $D_{2[1-4,7]}$  and  $D_{2[1-5,7]}$  bound [ $^3\text{H}$ ]spiperone, a  $D_2$ -selective ligand, with high affinity. Saturation analysis of radioligand binding to these receptors determined that the density of  $D_{2[1-4,7]}$  was  $730 \pm 70$  fmol/mg of membrane protein, with a  $K_D$  value for [ $^3\text{H}$ ]spiperone of  $0.60 \pm 0.04$  nM (11 experiments), the density of  $D_{2[1-5,7]}$  was  $300 \pm 30$  fmol/mg,

TABLE 3

**Affinity of D<sub>1</sub>, D<sub>2</sub>, and chimeric dopamine receptors for dopamine in the presence and absence of GTP**

$K_i$  values for the binding of [<sup>3</sup>H]spiperone to chimeric and D<sub>2</sub> dopamine receptors and binding of [<sup>3</sup>H]SCH23390 to D<sub>1</sub> receptors determined as described in Experimental Procedures. Affinity values ( $\mu$ M) represent geometric mean values for the number of independent experiments indicated ( $n$ ) with the limits defined by the asymmetrical standard error given in parentheses. The proportion of sites in the high and low affinity classes is also indicated (%).

	D <sub>1</sub>	%	$n$	D <sub>2</sub> [1-4,7]	%	$n$	D <sub>2</sub> [1-5,7]	%	$n$	D <sub>2</sub>	%	$n$
No GTP												
High	0.08 (0.05–0.13)	30	4	0.05 (0.03–0.07)	76	3	0.06 (0.04–0.09)	33	6	0.3 (0.2–0.4)	43	5
Low	4.9 (3.9–6.1)	70	11	11 (0.5–2.2)	24	11	11 (7.3–15)	67	21	21 (17–25)	57	
One site in absence of GTP	3.8 (3.1–4.7)	100	4	0.08 (0.07–0.09)	100	3						
200 $\mu$ M GTP	5.3		6	0.12		4	9.9 (7.6–13)		6	7.3 (5.7–9.4)		6
Addition of	(4.0–7.0)			(0.09–0.14)								



**Fig. 5.** GTP sensitivity of the binding of dopamine. Representative data are shown from one of three or more independent experiments in which inhibition of radioligand binding to D<sub>2</sub>[1-4,7] and D<sub>2</sub>[1-5,7] was determined for dopamine. Data are plotted as the percentage of the specific binding versus the logarithm of the concentration of dopamine. Inset, Averaged data from all experiments are expressed as the free energy change of binding (in Kcal/mol) plotted versus the number of transmembrane regions from the D<sub>2</sub> receptor. ■,  $\Delta G^\circ$  values for  $K_H$ . □,  $\Delta G^\circ$  values for  $K_L$  in the absence of GTP. ●,  $\Delta G^\circ$  values in the presence of GTP. Solid line, line drawn between D<sub>1</sub> and D<sub>2</sub> receptor  $\Delta G^\circ$  values in the presence of GTP. Dotted line, line drawn between high affinity states. Dashed line, line drawn between low affinity states of the D<sub>1</sub> and D<sub>2</sub> receptor  $\Delta G^\circ$  values in the absence of GTP.

with a  $K_D$  value of  $2.00 \pm 0.33$  nM (13 experiments), and the density of D<sub>2</sub> receptors was  $480 \pm 30$  fmol/mg, with a  $K_D$  value of  $0.071 \pm 0.018$  nM (9 experiments). Although we were able to detect specific binding of [<sup>3</sup>H]spiperone and [<sup>3</sup>H]clozapine to D<sub>2</sub>[1-2,7] and specific binding of [<sup>3</sup>H]SCH23390 to

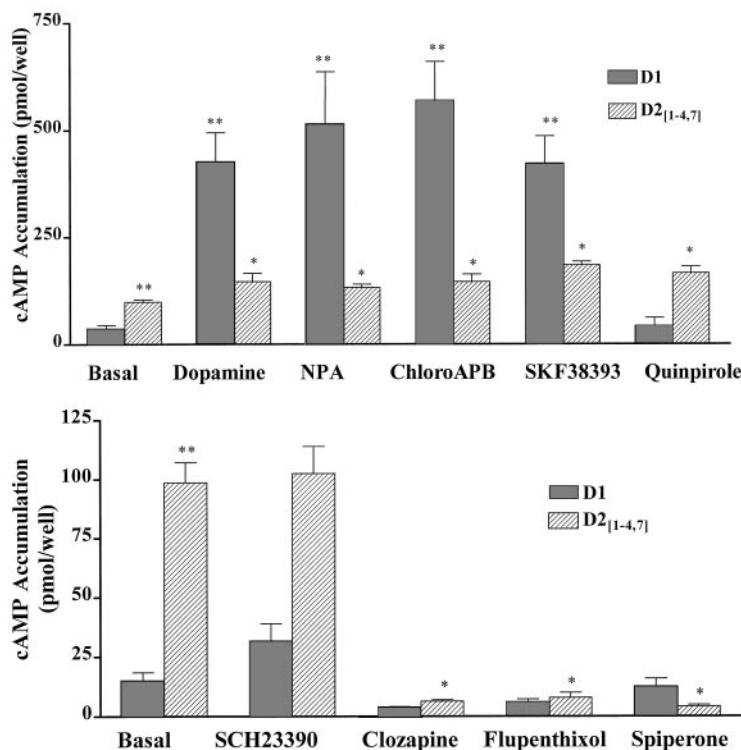
D<sub>2</sub>[1-2,7], D<sub>2</sub>[1-4,7], and D<sub>2</sub>[1-5,7], the binding was of such low affinity that accurate estimates of  $K_D$  and  $B_{max}$  values could not be obtained.

Three clonal lines of D<sub>1</sub> receptor-expressing HEK 293 cells were used for these experiments. The density of D<sub>1</sub> receptors

TABLE 4

**Basal levels of cAMP in nontransfected cells or cells expressing D<sub>1</sub>, D<sub>2</sub>, and chimeric dopamine receptors**Basal cAMP levels were determined as described in Experimental Procedures. Results shown are mean from  $\geq 10$  independent experiments  $\pm$  standard error.

Assay temperature	cAMP				
	HEK 293 cells	D <sub>1</sub>	D <sub>2[1-4,7]</sub>	D <sub>2[1-5,7]</sub>	D <sub>2</sub>
37°	4.3 $\pm$ 1.1	28.6 $\pm$ 3.2 <sup>a</sup>	101.5 $\pm$ 8.8 <sup>a</sup>	6.4 $\pm$ 1.0 <sup>b,c</sup>	6.3 $\pm$ 1.0 <sup>b,c</sup>
40°		34.3 $\pm$ 6.2	100 $\pm$ 6.2 <sup>c</sup>		

<sup>a</sup> Significantly different from nontransfected HEK 293 cells,  $p < 0.01$ .<sup>b</sup> Significantly different from D<sub>2[1-4,7]</sub>,  $P < 0.01$ .<sup>c</sup> Significantly different from D<sub>1</sub>,  $P \geq 0.02$ .

**Fig. 6.** Modulation of cAMP accumulation via D<sub>1</sub> and D<sub>2[1-4,7]</sub> receptors. Data shown are the average  $\pm$  standard error from three or more independent experiments in which (top) stimulation by agonists (1  $\mu$ M, except 10  $\mu$ M quinpirole) or (bottom) inhibition by antagonists (1  $\mu$ M) of cAMP accumulation via D<sub>1</sub> and D<sub>2[1-4,7]</sub> receptors was assessed. Data are expressed as pmol/well. Assays were performed at 37°, except for agonist stimulation and antagonist inhibition of D<sub>2[1-4,7]</sub>, which was at 40°. In other experiments, no stimulation of cAMP accumulation via the D<sub>1</sub> receptor was observed in the presence of 100  $\mu$ M quinpirole (data not shown). \*,  $p < 0.05$  compared with basal, Student's *t* test for paired means. \*\*,  $p < 0.05$  compared with D<sub>1</sub> basal, Student's *t* test for paired means.

on the clones used to assess stimulation of adenylate cyclase was  $2700 \pm 490$  fmol/mg (9 experiments) and  $1100 \pm 90$  fmol/mg (4 experiments) of membrane protein.

**Characterization of antagonist and agonist binding.**

For catecholamine receptors, structural determinants of ligand binding seem to be primarily in the conserved  $\alpha$  helical regions (20). Therefore, we analyzed the chimeras in terms of the number of transmembrane helices that were contributed by D<sub>1</sub> or D<sub>2</sub> receptors. As has been done previously (9, 21), we calculated the change in the free energy of binding of a ligand ( $\Delta G^\circ$ ) at each chimeric receptor. If each transmembrane region contributes an equal amount, or one seventh, of the difference between the affinity of a given ligand for the D<sub>1</sub> and D<sub>2</sub> receptors, then a plot of  $\Delta G^\circ$  at each chimeric and wild-type receptor versus the number of transmembrane regions that are from the D<sub>2</sub> receptor should be linear, with the values for the chimeric receptors falling on a line drawn between the wild-type receptors. The 95% confidence interval of the mean  $\Delta G^\circ$  was calculated for each drug. If the line drawn between  $\Delta G^\circ$  for the drug at the wild-type receptors did not intersect the 95% confidence interval for the  $\Delta G^\circ$  at a given chimera, then the affinity of the chimera for the drug was considered to be significantly different from the value

predicted by the null hypothesis that each transmembrane region contributes equally to the  $\Delta G^\circ$  of binding. A value of  $\Delta G^\circ$  for a chimeric receptor significantly above or below the line could indicate that a particular transmembrane region contributes more or less, respectively, than the average contribution of the other transmembrane regions to the selectivity of the ligand being tested.

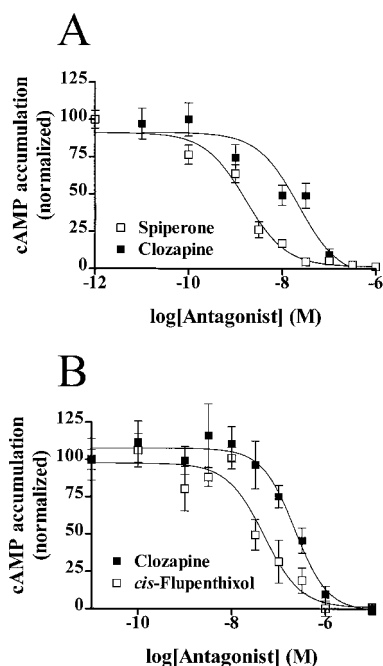
Three of the antagonists that were tested, (+)-butaclamol, clozapine, and *cis*-flupenthixol, were relatively nonselective for D<sub>1</sub> and D<sub>2</sub> dopamine receptors. (+)-Butaclamol had similar affinities for all four receptors (D<sub>1</sub>, D<sub>2</sub>, D<sub>2[1-4,7]</sub>, and D<sub>2[1-5,7]</sub>) (Table 1 and Fig. 2A), but the affinity of *cis*-flupenthixol for D<sub>2[1-5,7]</sub> was significantly below the line drawn between wild-type receptors. The affinity of D<sub>2[1-4,7]</sub> for clozapine however, was significantly greater than the affinity of either wild-type receptor for the drug (Table 1)

Although the mean  $\Delta G^\circ$  values for the binding of the D<sub>2</sub>-selective antagonists epidepride, haloperidol, spiperone, and sulpiride were slightly below the line drawn between values for each drug at D<sub>1</sub> and D<sub>2</sub> dopamine receptors, only for the binding of spiperone and epidepride to D<sub>2[1-5,7]</sub> did the line not intersect the 95% confidence interval of the mean (Table

1 and Fig. 2B). There was no difference in the affinity of D<sub>2[1-4,7]</sub> and D<sub>2[1-5,7]</sub> for these D<sub>2</sub>-selective ligands.

The affinity was assessed of D<sub>1</sub>-selective benzazepines SCH23390, SKF38393, and chloro-PB for D<sub>2[1-4,7]</sub> and D<sub>2[1-5,7]</sub>. The mean  $\Delta G^\circ$  values were not significantly different from the line drawn between D<sub>1</sub> and D<sub>2</sub> receptors (Table 2 and Fig. 3A). Also, the affinity of SCH23390 and SKF38393 for D<sub>2[1-5,7]</sub> and D<sub>2</sub> receptors was not significantly different. For another D<sub>1</sub>-selective benzazepine, chloro-APB, the affinity of D<sub>2[1-5,7]</sub> was significantly less than predicted, whereas the potency of chloro-APB for D<sub>2[1-4,7]</sub> was higher than would be predicted by the line drawn between D<sub>1</sub> and D<sub>2</sub> receptors (Fig. 3B).

For most agonists,  $\Delta G^\circ$  values for D<sub>2[1-5,7]</sub> were not significantly different from those predicted by the line drawn between D<sub>1</sub> and D<sub>2</sub> receptors. However, D<sub>2[1-4,7]</sub> receptors had significantly higher affinity for many dopamine agonists than did D<sub>1</sub> and D<sub>2</sub> receptors (Table 2). The affinity of D<sub>2[1-4,7]</sub> receptors was higher than either wild-type dopamine receptor for 6,7-ADTN, apomorphine, NPA, DHX, dopamine, lisuride, and pergolide. For every agonist tested, except the partial agonist SKF38393,  $\Delta G^\circ$  values for D<sub>2[1-4,7]</sub> were above the line drawn between D<sub>1</sub> and D<sub>2</sub> receptors (Table 2 and Fig. 4, A and B), indicating a higher-than-expected affinity of D<sub>2[1-4,7]</sub> for most agonists. Because one characteristic of constitutively active receptors is high affinity for agonists (16), these results suggested that D<sub>2[1-4,7]</sub> is constitutively active.



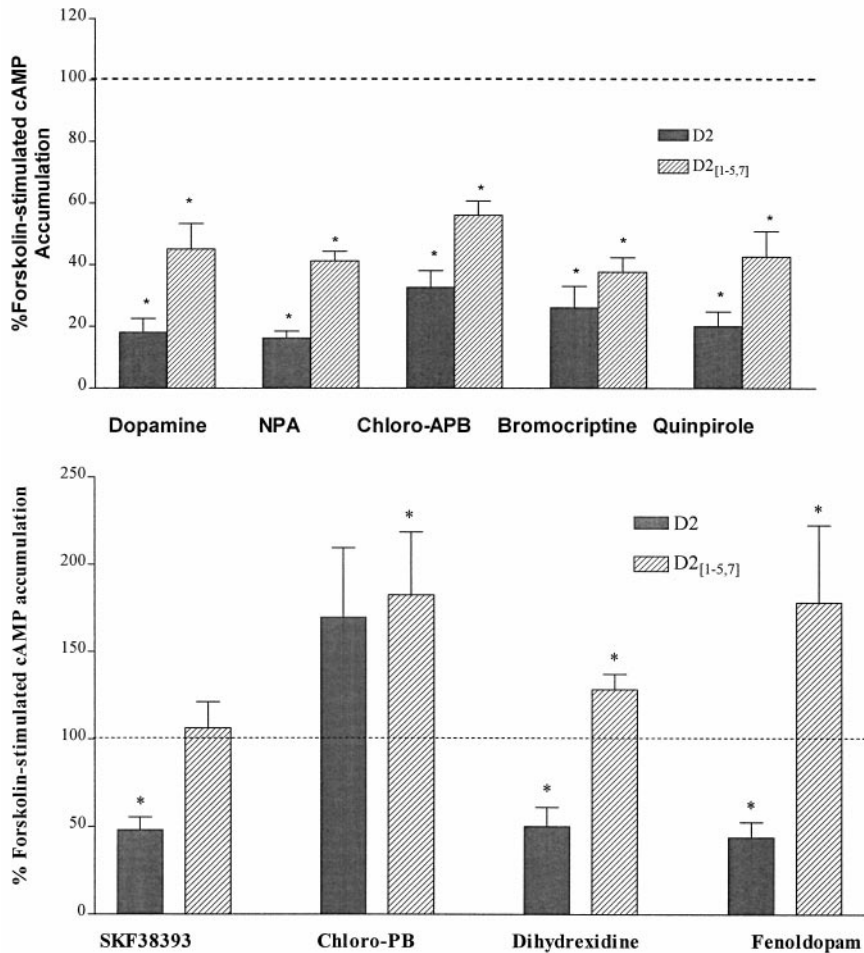
**Fig. 7.** Inhibition of forskolin-stimulated cAMP accumulation by inverse agonists. Data shown are representative of four independent experiments in which inverse agonist inhibition of cAMP accumulation in the presence of 100 nM forskolin was assessed in cells expressing (A) D<sub>2[1-4,7]</sub> or (B) D<sub>1</sub> receptors. Data are plotted as a percentage of forskolin-stimulated cAMP accumulation in the absence of inverse agonist versus the logarithm of the concentration of inverse agonist.

To assess the hypothesis that D<sub>2[1-4,7]</sub> was constitutively active, we examined agonist binding in the presence and absence of GTP. When a receptor is constitutively active, the equilibrium between active and inactive receptor conformations shifts spontaneously toward the active conformation, resulting in high affinity binding of agonists even in the presence of GTP (16). We were able to detect both high ( $K_H$ ) and low ( $K_L$ ) affinity states in the absence of GTP for D<sub>2</sub>, D<sub>1</sub>, D<sub>2[1-4,7]</sub>, and D<sub>2[1-5,7]</sub> receptors (Table 3 and Fig. 5). However, for D<sub>2[1-4,7]</sub>, >75% of the receptors were in a high affinity state, whereas for D<sub>2</sub>, D<sub>1</sub>, and D<sub>2[1-5,7]</sub>, <45% of the receptors were in a high affinity state (43%, 30%, and 33%, respectively). In several assays in the absence of GTP, the goodness of fit of the binding data for D<sub>1</sub> and D<sub>2[1-4,7]</sub> receptors was not significantly improved by assuming the presence of two classes of binding sites. For the D<sub>1</sub> receptor, the  $K_i$  value for dopamine when only one class of binding sites was detected was close to the  $K_L$  value when the best fit assumed two classes of binding sites. On the other hand, when only one class of binding sites could be detected for dopamine at D<sub>2[1-4,7]</sub>, the  $K_i$  value was similar to the value for  $K_H$  (Table 3). Furthermore, the  $K_i$  value for dopamine at D<sub>2[1-4,7]</sub> was unchanged by the addition of GTP, whereas for D<sub>1</sub>, D<sub>2</sub>, and D<sub>2[1-5,7]</sub> receptors, only the low affinity state  $K_L$  value could be detected in the presence of GTP.

**Stimulation of adenylate cyclase via D<sub>1</sub> and D<sub>2[1-4,7]</sub> receptors.** Consistent with the hypothesis that D<sub>2[1-4,7]</sub> was constitutively active, cells that expressed D<sub>2[1-4,7]</sub> receptors had basal cAMP levels that were significantly elevated compared with nontransfected HEK 293 cells and compared with cells expressing D<sub>1</sub>, D<sub>2[1-5,7]</sub>, and D<sub>2</sub> receptors (Table 4). Interestingly, basal cAMP accumulation in nontransfected HEK 293 cells was significantly lower than in cells expressing D<sub>1</sub> receptors (Table 4), suggestive of a low level of unliganded activity by D<sub>1</sub> receptors expressed in HEK 293 cells. Assays incubated at either 37° or 40° resulted in similar levels of basal cAMP accumulation (Table 4). Because agonist-stimulated accumulation of cAMP was more consistent at 40° for cells expressing D<sub>2[1-4,7]</sub> receptors, those assays were carried out at 40°.

Most dopamine receptor agonists stimulated cAMP accumulation via D<sub>2[1-4,7]</sub> and D<sub>1</sub> receptors (Fig. 6, top). Apomorphine, NPA, chloro-APB, SKF38393, DHX, lisuride, pergolide, and bromocriptine were as efficacious as dopamine via D<sub>2[1-4,7]</sub>. Fenoldopam and chloro-PB did not stimulate cAMP accumulation via D<sub>2[1-4,7]</sub>, although both drugs are agonists at D<sub>1</sub> receptors. Interestingly, the D<sub>2</sub> receptor agonist quinpirole stimulated adenylate cyclase via D<sub>2[1-4,7]</sub>, although quinpirole lacks efficacy at D<sub>1</sub> receptors. We were unable to detect stimulation of cAMP accumulation by dopamine via D<sub>2[1-2,7]</sub>.

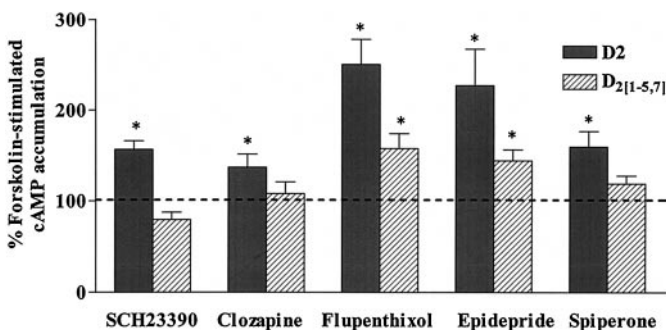
Stimulation of adenylate cyclase via D<sub>1</sub> receptors resulted in cAMP levels of >450 pmol/well for many of the agonists tested, whereas agonist-mediated stimulation of adenylate cyclase via D<sub>2[1-4,7]</sub> receptors was blunted, resulting in maximal cAMP levels that were only ~150 pmol/well (Fig. 6, top). To determine whether the reduced ability to stimulate cAMP accumulation was due to coupling of the D<sub>2</sub> cytoplasmic domains of D<sub>2[1-4,7]</sub> to G<sub>i/o</sub>, we treated cells with pertussis toxin (25 ng/ml for 18–22 hr). Treatment with pertussis toxin elevated cAMP accumulation stimulated by 1  $\mu$ M dopamine from 137  $\pm$  10 to 327  $\pm$  43 pmol/well (three experiments).



**Fig. 8.** Modulation of cAMP accumulation via  $D_2$  and  $D_{2[1-5,7]}$  receptors. The data shown are the average  $\pm$  standard error of four or more independent experiments in which (*top*) inhibition or (*bottom*) potentiation of forskolin-stimulated ( $10 \mu\text{M}$ ) adenylate cyclase activity by  $D_1$  receptor agonists ( $1 \mu\text{M}$ ) was assessed. Data are expressed as a percentage of forskolin-stimulated cAMP accumulation in the absence of receptor agonist. \*,  $p < 0.05$  compared with control, Student's  $t$  test for paired means.

The cAMP accumulation stimulated by  $10 \mu\text{M}$  forskolin in these cells was not altered by treatment with pertussis toxin (data not shown), indicating that the treatment did not non-specifically alter the responsiveness of adenylate cyclase.

**Inverse agonism at  $D_{2[1-4,7]}$  and  $D_1$  receptors.** Another characteristic of constitutively active receptors is the ability of inverse agonists to antagonize basal second messenger generation (22). A number of nonselective antagonists and



**Fig. 9.** Potentiation of forskolin-stimulated adenylate cyclase activity by antagonists. Data shown are the average  $\pm$  standard error of four or more independent experiments in which potentiation of forskolin-stimulated ( $10 \mu\text{M}$ ) adenylate cyclase activity by antagonists ( $1 \mu\text{M}$ ) was assessed. Data are expressed as a percentage of forskolin-stimulated cAMP accumulation in the absence of antagonist. \*,  $p < 0.05$  compared with control, Student's  $t$  test for paired means.

$D_2$ -selective antagonists seemed to be inverse agonists at  $D_{2[1-4,7]}$  receptors. (+)-Butaclamol, clozapine, epidepride, *cis*-flupenthixol, haloperidol, and spiperone decreased basal cAMP levels in the absence of agonist by  $\sim 70\%$  [Fig. 6, *bottom*; data for (+)-butaclamol, epidepride, and *cis*-flupenthixol not shown]. Inhibition of basal cAMP accumulation by clozapine was not altered by pretreatment with pertussis toxin (data not shown). Although not statistically significant, there was a trend for nonselective antagonists, such as clozapine and haloperidol, to decrease basal cAMP formation in cells expressing  $D_1$  receptors. The  $D_1$ -selective antagonist, SCH23390, did not inhibit basal levels of cAMP formation at either  $D_1$  or  $D_{2[1-4,7]}$  receptors. There was a tendency for SCH23390 to increase  $D_1$  receptor-mediated cAMP formation, consistent with its reported weak partial agonist activity at  $D_1$  receptors (23).

In other experiments, inhibition was assessed of forskolin-stimulated cAMP accumulation by antagonists. Spiperone and clozapine both inhibited forskolin-stimulated cAMP accumulation via  $D_{2[1-4,7]}$  in a dose-dependent manner, with  $EC_{50}$  values of  $1.9 \pm 0.2$  and  $24 \pm 10$  nM, respectively (Fig. 7A). To confirm that some antagonists are inverse agonists at the wild-type  $D_1$  receptor, we determined that clozapine, *cis*-flupenthixol, and haloperidol inhibited forskolin-stimulated activity via the  $D_1$  receptor with  $EC_{50}$  values of  $180 \pm 24$ ,  $61 \pm 5$ , and  $540 \pm 200$  nM, respectively (Fig. 7B).

**Agonist modulation of D<sub>2</sub> and D<sub>2[1-5,7]</sub> receptor-mediated cAMP accumulation.** Inhibition of adenylate cyclase activity was assessed in cells expressing wild-type and chimeric receptors. The nonselective agonists dopamine, NPA, and apomorphine inhibited ~80% of forskolin-stimulated adenylate cyclase activity via the D<sub>2</sub> receptor and 50–60% of activity via D<sub>2[1-5,7]</sub> (Fig. 8, *top*; data for apomorphine not shown). Similarly, the D<sub>2</sub>-selective agonists bromocriptine, lisuride, pergolide, and quinpirole inhibited adenylate cyclase activity via the D<sub>2</sub> receptor by 70–75% and via D<sub>2[1-5,7]</sub> by 40–50% (Fig. 8, *top*; data for pergolide and lisuride not shown). However, only one of the D<sub>1</sub>-selective agonists tested, chloro-APB, inhibited adenylate cyclase activity via D<sub>2[1-5,7]</sub> (60%; Fig. 8, *top*). At this chimera, other D<sub>1</sub>-selective agonists (SKF38393, 6-chloro-PB, DHX, and fenoldopam) either had no effect or potentiated forskolin stimulation of cAMP (Fig. 8, *bottom*). SKF38393, DHX, and fenoldopam inhibited adenylate cyclase via D<sub>2</sub> receptors by ~50%, whereas 6-chloro-PB did not inhibit adenylate cyclase. Although 6-chloro-PB was an agonist at D<sub>1</sub> receptors, it seemed to be an antagonist or an inverse agonist at D<sub>2</sub>, D<sub>2[1-4,7]</sub>, and D<sub>2[1-5,7]</sub> receptors.

**Inverse agonism at D<sub>2[1-5,7]</sub> and D<sub>2</sub> receptors.** We also evaluated the ability of antagonists to act as inverse agonists at D<sub>2</sub> receptors. The presence of (+)-butaclamol, epidepride, *cis*-flupenthixol, or haloperidol more than doubled forskolin-stimulated cAMP accumulation in cells expressing D<sub>2</sub> receptors (Fig. 8; data for (+)-butaclamol not shown). Clozapine, spiperone, and SCH23390 produced a more modest potentiation of forskolin-stimulated cAMP accumulation, ~40% over control values. Because the binding profile of D<sub>2[1-5,7]</sub> was similar to D<sub>2</sub> receptors for most antagonists, we also evaluated the effect of antagonists on forskolin-stimulated cAMP accumulation in cells expressing D<sub>2[1-5,7]</sub> receptors. Epidepride, *cis*-flupenthixol, and haloperidol significantly increased forskolin-stimulated cAMP accumulation in cells expressing D<sub>2[1-5,7]</sub> by 40–50% (Fig. 9). Antagonists had no effect on forskolin-stimulated cAMP accumulation in untransfected HEK 293 cells (data not shown).

## Discussion

We described previously the construction of eight chimeric receptors. Four D<sub>1</sub>/D<sub>2</sub> chimeras were functional (Ref. 9; Fig. 1A), whereas reciprocal D<sub>2</sub>/D<sub>1</sub> chimeras (Fig. 1B) were not. We hypothesized that the chimeric receptors were nonfunctional due to incompatible interactions between D<sub>1</sub> TMVII and D<sub>2</sub> TMI and TMII, regions that other mutagenesis and modeling data have suggested to be adjacent (12–14). Specifically, amino acid residues in TMII of the serotonin 5-hydroxytryptamine<sub>2A</sub>, gonadotropin-releasing hormone, and adrenergic receptors and TMI of the muscarinic m2 and m5 receptors interact with amino acid residues in TMVII (12, 14, 15, 24). For example, mutation of Asn87 in TMII to aspartic acid in the GnRH receptor resulted in a nonfunctional receptor with no detectable binding of ligands, whereas addition of the reciprocal mutation of Asp318 in TMVII to asparagine restored function to the mutant receptor (15). Also, mutation of Asn312 in β<sub>2</sub>-adrenergic receptors to the amino acid residue found in the homologous domain of the α<sub>2</sub>-adrenergic receptor (phenylalanine) resulted in a nonfunctional protein, whereas replacement of TMI and TMII of the β<sub>2</sub>-adrenergic

receptor mutant with α<sub>2</sub>-adrenergic receptor sequence restored function to the mutant receptor (12). These findings suggest that the low affinity of D<sub>2[5-7]</sub>, D<sub>2[6-7]</sub>, and D<sub>2[7]</sub> for a number of ligands (9) and the lack of detectable antagonist binding to D<sub>2[1-2]</sub>, D<sub>2[1-4]</sub>, D<sub>2[1-5]</sub>, and D<sub>2[1-6]</sub> are due to structural distortions of the chimeric receptors that are not observed when TMI, TMII, and TMVII are from the same receptor. The affinity of D<sub>2[1-4,7]</sub> and D<sub>2[1-5,7]</sub> for most antagonists was not significantly different from that predicted by the null hypothesis that all transmembrane regions contribute equally to ligand binding.

These experiments highlight the difficulty of distinguishing effects on ligand binding that are due to interactions of specific receptor domains with the ligands from effects that are due to disruption of helix packing or other nonspecific perturbations of structure. The latter is apparently the explanation for the lack of ligand binding to D<sub>2[1-2]</sub>, D<sub>2[1-4]</sub>, D<sub>2[1-5]</sub>, and D<sub>2[1-6]</sub>, and perhaps also the low affinity of D<sub>2[5-7]</sub>, D<sub>2[6-7]</sub>, and D<sub>2[7]</sub> for some ligands, but it is difficult to make firm conclusions regarding other effects, such as the high affinity of D<sub>2[1-4,7]</sub> and D<sub>2[1-5,7]</sub> for clozapine or the low affinity of D<sub>2[1-2,7]</sub> for the radioligands tested. One finding that was consistent for all the chimeras tested in these experiments and previous work (9) is that TMVI of the D<sub>1</sub> receptor apparently contributes little to the selective binding of most benzazepine ligands.

These difficulties of interpretation also apply to functional studies, in which it may be difficult to distinguish results due to the loss or addition of domains that interact specifically with G proteins from nonspecific structural effects. Still, the results presented here and in our previous work (9), such as the ability of D<sub>2[1-4,7]</sub> and D<sub>2[6-7]</sub> and the inability of D<sub>2[1-5,7]</sub> and D<sub>2[5-7]</sub> to stimulate adenylate cyclase, demonstrate consistently that the IC3 of D<sub>1</sub> receptors is necessary and sufficient for coupling to G<sub>s</sub>. Furthermore, the lack of inhibition of isoproterenol-stimulated cAMP accumulation by D<sub>2[5-7]</sub> and the modest inhibition by D<sub>2[3-7]</sub> indicated that both IC2 and IC3 from the D<sub>2</sub> receptor are required for inhibition of adenylate cyclase (9). Dopamine activation of D<sub>2[3-7]</sub> receptors inhibits cAMP accumulation by only ~20%, but dopamine was able to cause ~60% inhibition via the D<sub>2[1-5,7]</sub> receptor. These results suggest that the first cytoplasmic loop (IC1) of the D<sub>2</sub> receptor is also important for inhibition of adenylate cyclase activity via the D<sub>2</sub> receptor. If all three cytoplasmic loops are involved in coupling to G proteins that inhibit adenylate cyclase, it might be predicted that D<sub>2[1-4,7]</sub> would activate both G<sub>i/o</sub> and G<sub>s</sub>. Consistent with this, pertussis toxin treatment enhanced stimulation of adenylate cyclase by D<sub>2[1-4,7]</sub>.

The extended (allosteric) ternary complex model of receptor activation of G proteins proposes that receptors spontaneously isomerize between inactive (R) and active (R\*) conformations, with agonists having higher affinity for and stabilizing or inducing the active conformation (16). This model predicts that most receptors will have some ability to activate G proteins in the absence of agonist, with the extent of unliganded receptor activity depending on the density of the receptor and the constant (*J*) that describes the equilibrium between the active and inactive conformations of that receptor. For example, the D<sub>1</sub> and D<sub>5</sub> receptors both exhibit a receptor density-dependent ability to stimulate adenylate cyclase activity in the absence of agonist, but the slope of the

line that describes the relationship between receptor density and basal activity is much steeper for the  $D_5$  receptor than for the  $D_1$  receptor, indicating greater unliganded activity of the former (23). Constitutive activation of a receptor describes any manipulation that tends to increase the formation of  $R^*$  in the absence of agonist. In the current study, basal levels of cAMP accumulation were 4-fold higher in HEK cells expressing  $D_{2[1-4,7]}$  than in HEK cells expressing the wildtype  $D_1$  receptor. Basal cAMP accumulation was in turn higher in HEK cells expressing the  $D_1$  receptor than in untransfected cells or cells expressing the  $D_2$  receptor. In these experiments, the level of expression of  $D_{2[1-4,7]}$  was lower than that of the wild-type  $D_1$  receptor, indicating that the enhanced activity was due to mutation-enhanced formation of  $R^*$  rather than to a greater density of receptors. In addition to the enhanced basal levels of cAMP in cells expressing the  $D_{2[1-4,7]}$  receptor, other characteristics of constitutively active G protein-coupled receptors were observed, including increased affinity for agonists, attenuation of agonist-induced second messenger signaling, and the ability of inverse agonists to block basal second messenger generation (16, 22).

Although the affinity of most agonists for  $D_{2[1-5,7]}$  was approximately what would be expected if all transmembrane domains contribute equally to ligand binding, the affinity of most agonists for  $D_{2[1-4,7]}$  was much higher than would be predicted. The high affinity of the receptor for dopamine was resistant to addition of GTP, indicating that it did not result from coupling to G proteins.

Maximal agonist stimulation of adenylate cyclase via  $D_{2[1-4,7]}$  receptors was only ~30% of the maximal stimulation via the  $D_1$  receptor. Some of the reduced responsiveness was apparently due to coupling of  $D_{2[1-4,7]}$  to both  $G_{i/o}$  and  $G_s$  because pertussis toxin treatment more than doubled the stimulation of cAMP accumulation by dopamine to 72% of stimulation via the  $D_1$  receptor. In addition, stimulation may have been attenuated because even in the absence of agonist, the chimeric receptor was desensitized, decreasing the ability of the chimera to respond to agonist stimulation. Constitutive engagement of cellular desensitization mechanisms has been demonstrated for constitutively active mutants of the  $\beta_2$ -adrenergic receptor (25) and the  $\alpha_2$ -adrenergic receptor (26). On the other hand, because  $D_{2[1-4,7]}$  is a mutant receptor, stimulation might have been attenuated because the receptor is missing some structural elements required for full agonist efficacy or is globally perturbed in a way that modestly interferes with coupling to  $G_s$ . Agonist-mediated cAMP accumulation via  $D_{2[6-7]}$  and  $D_{2[7]}$  receptors was also attenuated compared with the  $D_1$  receptor (9).

Every  $D_1$  or  $D_2$  receptor antagonist tested at the  $D_{2[1-4,7]}$  receptor, with the exception of SCH23390, inhibited basal cAMP accumulation. In addition, clozapine and spiperone both caused dose-dependent decreases in forskolin-stimulated cAMP accumulation, with  $EC_{50}$  values similar to their affinities at  $D_{2[1-4,7]}$ . Although inverse agonism at a chimeric receptor does not necessarily mean that the drugs are also inverse agonists at the wild-type  $D_1$  receptor, we determined that three presumed dopamine receptor antagonists (clozapine, haloperidol, and *cis*-flupenthixol) dose-dependently inhibited forskolin-stimulated cAMP accumulation in HEK cells expressing the  $D_1$  receptor. Unliganded activity of  $D_1$  receptors and the inhibition of this activity by dopamine

receptor antagonists confirms the results of Tiberi and Caron (23).

A mutant  $D_1$  receptor (L286A) with enhanced unliganded stimulation of adenylate cyclase activity was recently described (8), but the characteristics of this receptor differ in several respects from the constitutively active chimeric receptor,  $D_{2[1-4,7]}$ . In particular, the affinity of L286A (1.2  $\mu$ M) for dopamine was similar to the affinity of the wild-type  $D_1$  receptor (1.9  $\mu$ M). The sensitivity to GTP of high affinity agonist binding, and the ability of antagonists to inhibit basal cAMP accumulation via L286A were not assessed.

It has been proposed that the structural instability of a mutant  $\beta_2$ -adrenergic receptor confers on the receptor its constitutive activity because it is able to isomerize more readily between the R and  $R^*$  conformations (27). The constitutive activity of  $D_{2[1-4,7]}$  may reflect enhanced formation of  $R^*$  due to a global effect on helix packing, as proposed for a chimeric m2/m5 muscarinic receptor that constitutively activates  $G_q$  (28). Because the IC3 (29) and peptides derived from the IC3 of G protein-coupled receptors (30–33) have an intrinsic ability to activate G proteins, we propose that this intrinsic activity is typically constrained by intramolecular interactions among the transmembrane helices. Disruption of helix packing in  $D_{2[1-4,7]}$  may mimic the agonist-induced relaxation of the receptor that permits functional coupling to G proteins (34).

We also observed evidence for unliganded activity of the wild-type  $D_2$  receptor. All the antagonists that we tested enhanced forskolin-stimulated cAMP accumulation in HEK 293 cells expressing the wild-type  $D_2$  receptor but not in untransfected cells, suggesting that the  $D_2$  receptor constitutively inhibits adenylate cyclase. The potentiation of cAMP accumulation at these receptors likely reflects inverse agonist inhibition of the constitutively active receptors, consistent with the haloperidol-induced enhancement of prolactin release from  $GH_4C_1$  cells expressing  $D_2$  receptors (35). It is noteworthy that the density of receptors on the cells used in the current study (480 fmol/mg) is similar to the density of  $D_2$  receptors in brain regions in which the receptors are expressed most abundantly (36).

In summary, restoration of function in  $D_2/D_1$  chimeric receptors that have  $D_2$  receptor sequence in TMI, TMII, and TMVII is consistent with a model in which interactions among these helices maintain receptor function. Our data support the view that multiple  $D_2$  receptor cytoplasmic domains acting in concert are necessary for receptor activation of  $G_i$ , whereas a chimeric receptor with only one cytoplasmic domain from the  $D_1$  receptor, IC3, was capable of activating  $G_s$ . Furthermore, this chimeric receptor constitutively activated adenylate cyclase, had high affinity for agonists, and revealed the inverse agonism of a number of antagonists. The wild-type  $D_2$  receptor may constitutively inhibit adenylate cyclase activity, as indicated by antagonist-induced potentiation of cAMP accumulation.

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