Mechanisms of Drug Efficacy and Potency at Dopamine Receptors: Elucidation with Chimeric and Mutant Receptors

by

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CERTIFICATE OF APPROVAL

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ABBREVIATIONS

6,7 ADTN	2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene
$CaCl_2$	calcium chloride
Chloro-APB	6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SKF 82958
cAMP	cyclic AMP; adenosine 3',5'-cyclic monophosphate
COMT	catechol-O-methyl-transferase
DHX	dihydrexidine; trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydro benzo[a]phenanthridine)
DOPAC	dihydroxyphenylacetic acid
EC50	concentration that produces 50% of the maximal effect
G_i	inhibitory guanine nucleotide binding protein
G_s	stimulatory guanine nucleotide binding protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5-HT	5-hydroxytryptamine (serotonin)
IBMX	isobutylmethylxanthine
K_D	affinity constant
K_H	affinity constant for high affinity state
K_i	apparent affinity constant
K_L	affinity constant for low affinity state
MAO	monoamine oxidase
NPA	propylnorapomorphine
SCH 23390	7-chloro-8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SKF 38393	7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
VTA	ventral tegmental area

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PREFACE

In accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health Sciences University of Portland, Oregon, I have prepared my dissertation, consisting of a general introduction, three chapters of original data, and a discussion and conclusions chapter. Each data chapter includes an abstract, introduction, materials and methods, results, discussion, and conclusion section. References are listed separately in the order in which they first appear, and follow the format of *Molecular Pharmacology*.

Chapter 2 contains data, figures and text as they appear in an original paper that has been published previously (Kozell et al., 1994), with some minor modifications to the figures and text. Chapters 3 represents an original manuscript that has been submitted, reviewed, revised and resubmitted for publication (Kozell and Neve 1997A), and Chapter 4 represents data that will be submitted for publication (Kozell and Neve 1997B) and data that are published (Starr, Kozell and Neve 1995).

Papers representative of this work

Kozell L. B., and K. A. Neve, Mutagenesis of potential PKA phosphorylation sites in the D_1 dopamine receptor. to be submitted (1997B).

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Abstracts representative of this work

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ABSTRACT

The goals of my dissertation research were to assess ligand interactions with dopamine receptors. Specifically, I wanted to elucidate regions of the receptor that were involved in the potency of selective ligand binding, the efficacy of signal transduction, and the regulation of the receptor.

To assess the contribution of particular transmembrane domains (TMs) in ligand binding and signal transduction, I characterized chimeric D_1/D_2 receptors. There are several advantages to analyzing ligand affinity for binding and signal transduction by chimeric receptors. Chimeras can be designed so that the inclusion of a region from one parent receptor results in a "gain" of function, conversely removal of that particular region from a chimera results in a "loss" of the function. Also, mutagenesis of single amino acid residues cannot be used to assess the interactions between the multiple, contiguous amino acid residues that are probably involved in many aspects of receptor function.

Analysis of chimeric D_1/D_2 dopamine receptors resulted in several interesting observations. Several D_1-selective ligands demonstrated a loss of potency with the replacement of D_1 TMVII with D_2 TMVII, whereas a D_2-selective agonist manifested a gain of potency. For several non-selective ligands and D_2-selective antagonists, D_2 TMI through TMIV appeared to be crucial for ligand binding, whereas for other ligands most TMs appeared to contribute equally to ligand binding.

We hypothesized that incompatible interactions between D_2 TMI and TMII and D_1 TMVII resulted in several nonfunctional chimeric receptors. The replacement of D_1

TMVII with D_2 TMVII in these chimeras restored receptor function for two of the chimeric receptors, concurring with this hypothesis.

Chimeric receptors with the D_1 third cytoplasmic loop (IC3) were able to stimulate adenylate cyclase, but inhibition of adenylate cyclase *via* G_i required other structural features in addition to the D_2 third cytoplasmic loop.

One chimeric receptor, $D_{2[1\text{-}4,7]}$ exhibited several characteristics of a constitutively active receptor, including enhanced basal (unliganded) stimulation of adenylate cyclase activity, high affinity for agonists even in the presence of GTP, blunted agonist-stimulated cyclic AMP accumulation, and inhibition of basal cyclic AMP accumulation by inverse agonists.

To investigate structural determinants of receptor desensitization and down-regulation, we focused on the D_1 receptor, because agonist treatment of D_1 receptors results in robust desensitization and down regulation of the receptor. We mutated potential PKA phosphorylation sites and assessed desensitization and down regulation of mutant and D_1 dopamine receptors. One amino acid residue, serine 380, appeared to be involved in desensitization. Mutation of potential PKA phosphorylation sites did not abolish or decrease the ability of agonists to down regulate the D_1 receptor, suggesting that agonist-induced changes in D_1 receptor number are not dependent on phosphorylation *via* PKA. However, one of the mutant D_1 receptors, S127A, was down regulated more quickly than the wildtype D_1 receptor. Interestingly, this receptor did not appear to uncouple from G proteins following agonist pretreatment, thus implicating serine 127 in uncoupling of the D_1 dopamine receptor from G proteins.

I. INTRODUCTION

Neurotransmission involving dopamine is an important component of many central nervous system (CNS) functions, and is also implicated in a number of neurological disorders. Dopamine acts by binding to and activating integral membrane proteins, dopamine receptors, which then transduce the signal across the cell membrane. An understanding of how these proteins function is important for understanding the role of dopamine in the CNS. To this end, the goal of my dissertation was to examine how ligands interact with dopamine receptors. Specifically, I wanted to elucidate regions of the receptor that are involved in the potency of selective ligand binding, the efficacy of signal transduction, and regulation of receptor responsiveness and density. Chimeric D_1/D_2 dopamine receptors were constructed to analyze the contribution of particular transmembrane regions to ligand affinity and to modulation (stimulation or inhibition) of adenylate cyclase. To investigate structural determinants of receptor desensitization and down-regulation, I focused on the D_1 receptor, because agonist treatment results in robust desensitization and down regulation of the receptor. This introductory section contains background information on dopamine as a neurotransmitter, but its primary purpose is to discuss specific differences in ligand selectivity, signal transduction, and regulation of D_1 and D_2 dopamine receptors. The introduction will also focus on the use of chimeric and mutant receptors as a tool for delineating structural determinants of receptor function.

DOPAMINE AS A NEUROTRANSMITTER

History

Dopamine (3,4-dihydrophenylethylamine) neurotransmission is involved in numerous physiological responses, including motor control, cardiovascular homeostasis, endocrine function, and cognition. Disease states that have been linked to dysfunction of dopamine neurotransmission include Alzheimer's disease, Huntington's chorea, Parkinson's disease, schizophrenia and affective disorders, and endocrine disturbances. A functional role for dopamine, however, was not hypothesized until the late 1950's. Prior to that time dopamine was only considered to be a precursor in the biosynthetic pathway of the catecholamines, norepinephrine and epinephrine. The demonstration that dopamine is present in brain tissue at levels similar to norepinephrine (1) suggested that it might have a similar physiological role to norepinephrine. This was followed by the finding of Bertler and Rosengren, and others, (2,3) that in many species (rat, rabbit, cow, guinea pig, cat, dog, sheep and pig) brain levels of norepinephrine and dopamine are similar. Importantly, the distribution of norepinephrine and dopamine differ markedly. Norepinephrine is concentrated in the hypothalamus, with small quantities found throughout other brain tissue, whereas dopamine is concentrated in nuclei within the basal ganglia. This differential distribution of dopamine and norepinephrine in the brain suggested that dopamine is not just a precursor of the neurotransmitter catecholamines, but that it might also have a role of its own. In 1960, Ehringer and Hornykiewicz (4) demonstrated that there is a marked decrease in dopamine content in the basal ganglia of patients with Parkinson's disease, suggestive of a role for dopamine in motor control.

The demonstration that dopamine is present in the CNS, where dopamine has a unique distribution, and the suggestion that dopamine might have a function in motor control, were the first indications of a role for dopamine as a neurotransmitter.

Dopaminergic neuroanatomy

The major dopaminergic pathways in the central nervous system are depicted in Figure 1-1 (For a review see Cooper et al., 1996) (5). These include the *tuberoinfundibular* pathway, in which dopamine-containing neurons from the arcuate and periventricular nuclei of the hypothalamus project to the pituitary and the median eminence, the *nigrostriatal* pathway linking the substantia nigra with the neostriatum (caudate and putamen), and the *mesolimbic* and *mesocortical* pathways, which project from the ventral tegmental area (VTA) to the olfactory tubercle, nucleus accumbens, amygdala, and link the VTA with regions of the frontal cortex (prefrontal, cingulate, and enterhinal cortex). Other dopaminergic pathways include the *incertohypothalamic* dopamine-containing neurons which form projections within the hypothalamus and connect the hypothalamus with the lateral septal nuclei, the *medullary periventricular* dopamine-containing cells, which are located near the vagus nerve, in the nucleus tractus solitarius, and scattered in the tegmental radiation of the periaqueductal grey matter, and the ultrashort projections, forming extremely localized connections between the inner and outer plexiform layers in the retina, and between adjacent glomeruli *via* mitral cell dendrites in the olfactory bulb. For the most part dopamine neurons project topographically, so that dopamine-containing cells located close to one another innervate regions close to one another.

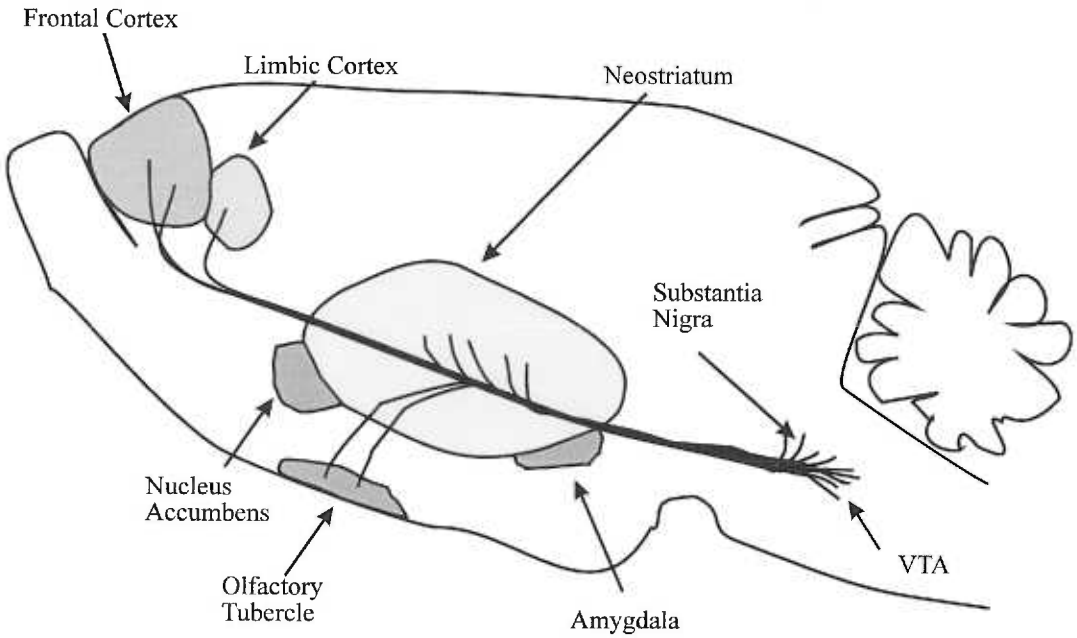

Figure 1-1 Major dopaminergic pathways in the rat.
Dopaminergic neurons in the substantia nigra project to and terminate in the caudate nucleus and putamen (Nigrostriatal pathway). There are two pathways for dopamine neurons projecting from the ventral tegmental area (VTA). Some axons terminate in the nucleus accumbens, olfactory tubercle or the amygdala forming the mesolimbic pathway, while axons projecting to the cortex comprise the mesocortical dopamine tract.

Dopamine biosynthesis and modulation of neuronal activity

Dopamine is synthesized from the amino acid, L-tyrosine, which is converted to L-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (Figure 1-2) (5). This is the rate limiting step in the synthesis of dopamine, as DOPA is very quickly converted to dopamine by the abundant enzyme L-aromatic amino acid decarboxylase (DOPA decarboxylase).

Dopamine synthesis can be modulated in a number of ways (Figure 1-3) (5). For example, interference with the transport of tyrosine across the blood-brain barrier may alter dopamine synthesis. Aside from the availability of the substrate, tyrosine, the rate of dopamine synthesis is mainly determined by modulation of tyrosine hydroxylase activity,

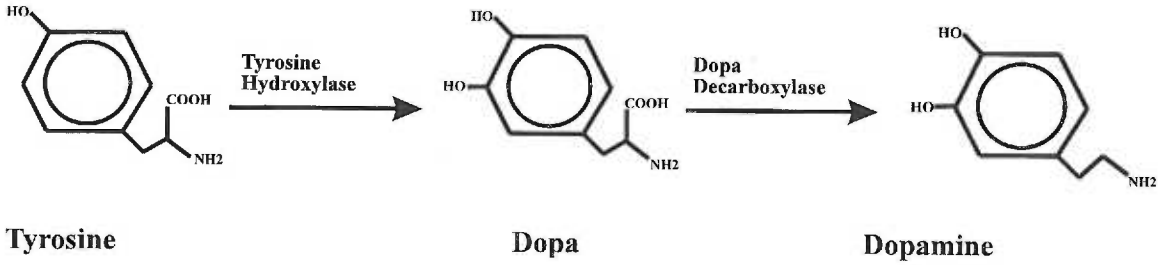

Figure 1-2 The synthesis of dopamine (3,4-dihydroxyphenylethylamine) from tyrosine.

which can occur in a number of ways. Pharmacologically, there are drugs, such as α-methyl-para-tyrosine, that can inhibit tyrosine hydroxylase. Dopamine and other catecholamines can also competitively inhibit binding of a co-factor necessary for activation of tyrosine hydroxylase, tetrahydrobiopterin (BH4). The activity of tyrosine hydroxylase can be enhanced by increases in neuronal activity, and by phosphorylation stimulated by increases in intracellular cAMP or Ca^{++} levels.

Once dopamine is released into the synaptic cleft it can interact with presynaptic (autoreceptors) and postsynaptic dopamine receptors (Figure 1-3). Nondopaminergic cells have postsynaptic dopamine receptors on their axon terminals, whereas autoreceptors may be located on the cell bodies or the axon terminals of dopamine-containing neurons. Stimulation of dopamine receptors modulates the cellular response to other stimuli (other transmitters or ion conductances). For example, dopamine receptors that stimulate adenylate cyclase activity raise the levels of cyclic AMP in the cell, activating protein kinase A (PKA) which can then phosphorylate proteins in the cell. Because the population of proteins that can be phosphorylated by PKA varies from cell to cell, the response to stimulation of dopamine receptors will also differ from cell to cell. Therefore it is not surprising that stimulation of these dopamine receptors has been shown

to both facilitate and inhibit the release of neurotransmitters from the cell. Other dopamine receptor subtypes have been shown to enhance or inhibit the ability of the cell to depolarize in response to an action potential.

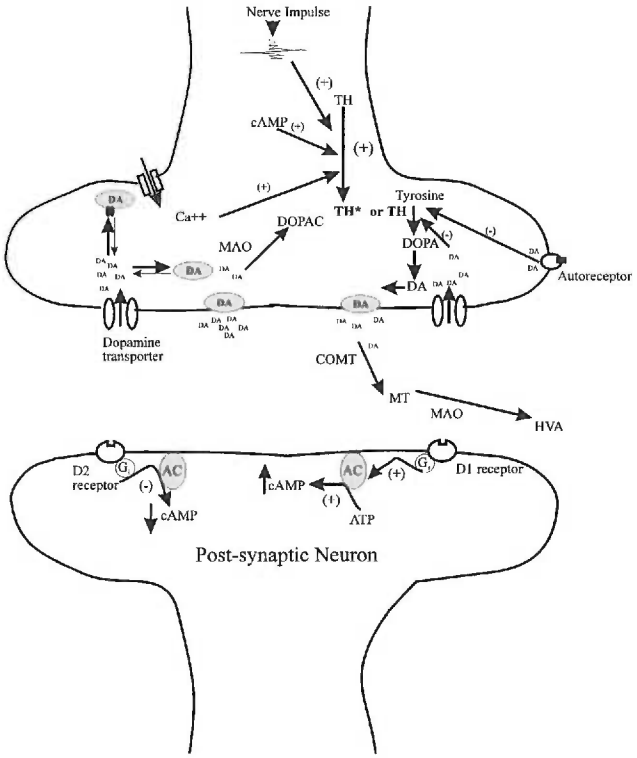

Figure 1-3 Schematic model of a prototypical dopamine synapse.
The conversion of tyrosine to L-DOPA by tyrosine hydroxylase is the rate limiting step in dopamine biosynthesis. Newly synthesized dopamine may be sequestered for subsequent release into storage vesicles. Presynaptic receptors (D_2 subtype), can modulate both synthesis and release of dopamine. Dopamine D_1 and D_2 receptors are found on post-synaptic cell membranes and exhibit a variety of functions. D_1 and D_2 receptors both couple to the enzyme adenylate cyclase(AC), stimulating and inhibiting the synthesis of cAMP, respectively. Dopamine neurotransmission is terminated by active transport into the pre-synaptic terminal by high affinity dopamine uptake sites (dopamine transporter). Dopamine can be degraded by MAO (inhibited by pargyline) intracellularly or by COMT (inhibited by tropolone) extracellularly.

The actions of dopamine are terminated by high affinity dopamine uptake by transporters (5). Transport of dopamine requires ATP and is both Na^+ and Cl^- dependent. High affinity dopamine transport can be inhibited by a number of drugs, including

benztropine, cocaine, nomifensine, and a series of GBR compounds. Other drugs, such as amphetamine, block dopamine uptake and induce dopamine release.

Dopamine is metabolized in the presynaptic neuron to dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO), or in the extracellular region by catechol-O-methyl-transferase (COMT). COMT metabolizes dopamine to 3-methoxytyramine (MT), which can be further metabolized by MAO in glial cells to homovanillic acid (HVA). The major metabolites of dopamine in the central nervous system are HVA and DOPAC.

MOLECULAR BIOLOGY OF DOPAMINE RECEPTORS

In the late 1970's, the existence of more than one class of dopamine receptors in the brain was proposed (6). This was based on pharmacological and biochemical evidence showing that drugs antagonizing dopamine function in the pituitary and CNS do not block dopamine-stimulated adenylate cyclase activity in the striatum. Two classes were proposed, the D_1 and the D_2 dopamine receptors. The D_1 dopamine receptors stimulate adenylate cyclase, have μM affinity for dopamine, and reduced affinity for butyrophenones and substituted benzamides, whereas the D_2 dopamine receptors do not stimulate adenylate cyclase, have nM affinity for dopamine and high affinity for butyrophenones and substituted benzamides.

In 1988, a cDNA encoding a 415 amino acid protein was isolated from a rat brain cDNA library (7). There were high levels of mRNA encoding this protein in the hypothalamus and basal ganglia, and hydrophobicity plots suggested that the protein had seven membrane spanning domains with a long third cytoplasmic loop (7). Transfection

into mammalian cell lines resulted in the expression of a receptor with D_2-like pharmacology. The receptor had high affinity for spiperone and sulpiride, and was able to mediate inhibition of adenylate cyclase in response to dopamine (7-9). This receptor also shared many common features with other G protein-coupled receptors, including a predicted amino acid sequence containing seven hydrophobic regions that putatively span the cell membrane (Figure 1-4). These transmembrane domains (TMs) are connected by intracellular and extracellular loops, with an extracellular amino terminal end and a carboxyl terminal tail that is intracellular (10,11). The third cytoplasmic loop of this family of receptors has been shown to be very important in G protein-coupling although other regions of specific G protein-coupled receptors may also be involved (12,13). Receptors that are coupled to G_i have long third cytoplasmic loops and short carboxyl tails like the D_2 receptor, whereas G_s-coupled receptors have shorter third cytoplasmic loops and much longer carboxyl tails.

The dopamine receptor sub-families

By the early 1990's, sequences for all of the currently known mammalian dopamine receptors had been published. There were multiple subtypes within the D_2-like receptor subclass. The second D_2-like receptor that was cloned, the D_{2L} receptor, is generated by alternative splicing of the same gene that encoded the first D_2 receptor (now called D_{2S}) (14-16). The D_{2S} and D_{2L} receptor sequences are identical, with the exception of a 29 amino acid insert in the third cytoplasmic loop (IC3) of D_{2L}. The D_{2L} receptor appears to be the predominant form of the D_2 receptor expressed in the brain (17,18).

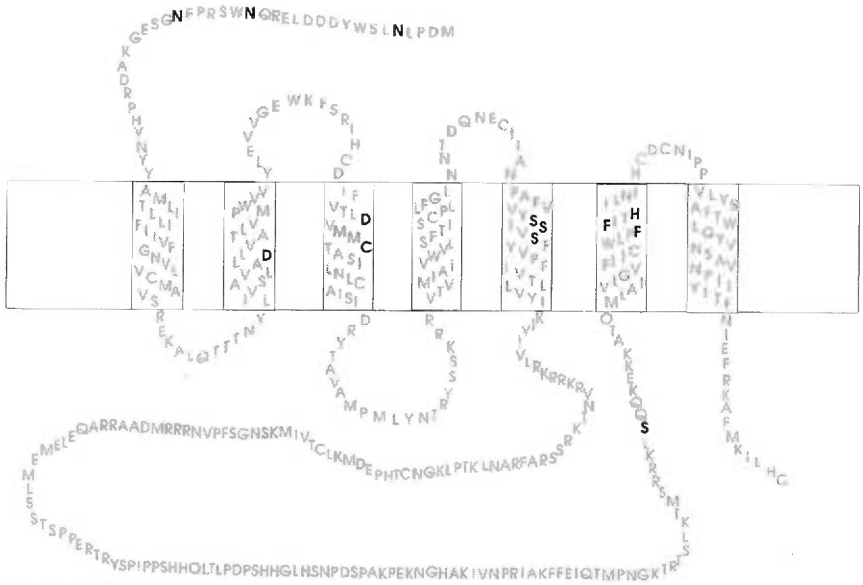

Figure 1-4 Predicted structure of the rat D_{2L} dopamine receptor.
*Several residues in the transmembrane regions that have been implicated in ligand binding or signal transduction are indicated by **BOLD** lettering. Asparagine residues in the extracellular region that can be glycosylated and serine and threonine residues in the intracellular loops that can be phosphorylated by PKA are also indicated by **BOLD** lettering.*

Based on pharmacological profiles, sequence homology with the D_2 receptor (53% and 41%, respectively), and gene structure, two other dopamine receptors (D_3 and D_4) fit into the D_2-subtype class (19-21). Both of these dopamine receptors exhibit high affinity for D_2-selective ligands, but D_3 appears to have higher affinity for many agonists (19,22-27), whereas D_4 has higher affinity for clozapine and lower affinity for many benzamide antagonists (20) than the D_2 receptor.

There are also multiple subtypes within the D_1-like dopamine receptor subclass. Four groups (28-31) nearly simultaneously isolated DNA encoding the D_1 receptor. The expression of the D_1 receptor in cell lines showed that it was pharmacologically indistinguishable from the D_1 class of receptors proposed by Kebabian and Calne (6,28,30,31). As expected, dopamine stimulated adenylate cyclase activity *via* this

receptor (6,31,32). The other D_1 receptor subtype, the D_5 or D_{1B} (rat) dopamine receptor exhibited high affinity for D_1-selective antagonists, and higher affinity for many agonists (33-35) than the D_1 receptor. Two novel non-mammalian D_1-like dopamine receptors are the D_{1C} (frog) (36) and D_{1D} receptors (chicken) (37); both with higher affinity for some agonists than the D_1 receptor.

Table 1-1 Distribution of dopamine receptor subtypes

Receptor Subtype	Receptor Localization	Receptor Enriched Brain Regions
D_1-like	Mostly post-synaptic	
D_1		striatum (C/P), nucleus accumbens (NA) olfactory tubercle (OT), amygdala and retina
D_5		hippocampus, thalamus, mammillary bodies
D_2-like	Pre- and post-synaptic	
D_{2S} and D_{2L}		C/P, OT, NA, substantia nigra (SN), globus pallidus (GP), VTA, pituitary
D_3		NA, OT, VTA, Islands of Calleja
D_4		Frontal cortex, hypothalamus, thalamus, olfactory bulb, amygdala

Localization of receptor mRNA

The predominant subclasses of dopamine receptor expressed in the brain are the D_1 and the D_2 subtypes. Expression of D_1 and D_2 receptor mRNA occurs in some of the same regions of the brain (Table 1-1) (5). Regions of the brain with the highest D_2 receptor mRNA content include the striatum (caudate nucleus and putamen), nucleus accumbens, and the olfactory tubercle (7), whereas mRNA for the D_3 receptor is most

abundant in the islands of Calleja, ventral forebrain, and nucleus accumbens (38), and mRNA for the D_4 receptor is highest in the frontal cortex, amygdala, and medulla (20,21,39). Similar to the D_2-like dopamine receptors, there is also very little overlap in the expression of mRNA for the D_1-like dopamine receptors (28,30). D_1 receptor mRNA is expressed at high levels in the caudate nucleus and putamen, and at low levels in the frontal cortex, whereas mRNA for the D_5 dopamine receptor is most abundant in the frontal cortex, hypothalamus, midbrain, and hippocampus (35).

Gene and protein structure

Like most G protein-coupled receptors, the D_1-like dopamine receptors do not have introns within the coding region (30,40). However, all of the D_2-like receptors contain several introns within the coding region, allowing for alternative splicing of a single gene, and resulting in multiple gene products such as D_{2L} and D_{2S}. The D_3 receptor gene also encodes splice variants, although only alternative splicing of the murine D_3 receptor yields two functional receptors (41). There are individual variations, or allelic variants, of the D_4 dopamine receptor in primates, but not rats. These D_4 receptor allelic variants differ in the number of copies (2 - 11 in humans) of an imperfect direct repeat of a 16-amino acid sequence in the third cytoplasmic loop (42,43).

The rat D_{2S} and D_{2L} receptors are 415 and 444 amino acids long, respectively, and contain one more amino acid than the human D_2 receptors. The rat D_3 receptor encodes a 446-amino acid protein, which is greater than 50% identical to the rat D_{2L} receptor. The human D_3 receptor, 400 amino acid residues long, is missing 46 amino acids that are present in the rat D_3 third cytoplasmic loop. The number of amino acids in

the D_4 receptor varies with the number of repeats in the third cytoplasmic loop. The shortest human D_4 receptor, $D_{4.2}$, has 387 amino acids, whereas the most common human D_4 receptor, $D_{4.4}$, has 419 amino acids. The D_4 receptor has relatively low homology, approximately 40%, to the other receptors in its subclass, D_2 and D_3 receptors. As for all the G protein-coupled receptors, the homology is highest within their transmembrane regions.

The D_1 receptor contains 446 amino acids. The human D_1 is about 44% identical with the human D_2 receptor. There are 477 amino acids in the D_5 dopamine receptor which is 60% homologous with the D_1 (~80% in the transmembrane regions) but only 30% homologous with the D_2 and D_3 receptors. Two human pseudogenes of the D_5 receptor have been identified (34,44).

All of the D_1-like and D_2-like receptors have potential sites for asparagine (N) linked glycosylation in the amino terminal end of the receptor (Figure 1-4). The D_2 receptor has three consensus sequences for N-linked glycosylation in the amino terminus, the D_3 receptor has two putative sites, with another site in the second extracellular loop (N-97), and the D_4 receptor has one consensus sequence. The D_1-like dopamine receptors each have one potential site for asparagine-linked glycosylation in the amino terminal end and one in the third extracellular loop. Not surprisingly, it has been demonstrated that both D_1-like and D_2-like receptors are glycosylated.

D_2-like dopamine receptors have at least one optimal consensus sequence (R-R/K-X-S/T) (45) for protein kinase A (PKA) phosphorylation in the third cytoplasmic loop (Figure 1-4). Most of the D_1-like receptors, have one potential PKA

phosphorylation site in the third cytoplasmic loop (not found in primate), and a second site in the second cytoplasmic loop. There are other serine and threonine residues in the D_1 receptor that may be phosphorylated by PKA, but the consensus sequences are less optimal (R-$X_{(1-2)}$-S/T) for phosphorylation. The D_1-like receptors have multiple serine and threonine residues in the long cytoplasmic tail in close proximity to acidic residues, making them potential sites of phosphorylation by the G protein-coupled receptor kinase family, (GRKs) (46,47), including the β-adrenergic receptor kinases, β-ARK1 and β-ARK2.

Pharmacology and signal transduction

The D_2-like dopamine receptors can be differentiated from one another by selective ligands (Table 1-2). Although many D_2-selective antagonists do not differentiate among the subtypes, some substituted benzamides have lower affinity for the D_4 receptor compared to the other D_2-like receptors. On the other hand, clozapine has a higher affinity for the D_4 receptor (20). Although there are slight differences in the affinity of the D_4 receptor allelic variants for clozapine (20), the functional significance of these differences has not been determined. Many agonists, including quinpirole, dopamine and 7-OH DPAT, have a higher affinity for the D_3 receptor subtype than for other D_2-like receptors. Selective ligands for the D_1-like receptors include a number of benzazepines, including the prototypical antagonist SCH 23390 (48), the partial agonist, SKF 38393, and the full agonist, SKF 82958 (chloroAPB). Other agonists that are selective for the D_1-like dopamine receptors include dihydrexidine (49) and A77636 (50).

The D_2-like dopamine receptors couple to the G proteins, G_i or G_o, to inhibit the activity of adenylate cyclase (Table 1-2). Although both D_2 receptor splice variants D_{2S} and D_{2L} couple to G proteins, there appear to be differences in which G-protein each receptor couples to (8,51,52). Another difference between the splice variants is the enhanced potency of agonists and greater inhibition of adenylate cyclase activity by the D_{2S} splice variant in some cell lines (53,54). The D_3 receptor inhibits adenylate cyclase activity, although this inhibition is very weak compared to inhibition *via* the D_2 receptor (19,25,27,55). The D_2-like dopamine receptors have also been shown to modulate arachidonic acid release, Na^+/H^+ exchange, K^+ currents, and Ca^{2+} currents. Activation of D_1-like receptors stimulates adenylate cyclase and modulates phosphoinositol turnover (56).

Table 1-2 Receptor pharmacology and signal transduction mechanisms

Receptor Subtype	Selective Agonist	Selective Antagonist	Modulation of Second Messenger
D_1	Dihydrexidine SKF-38393	SCH 23390	Stimulation of adenylate cyclase, stimulation of phosphoinositol(PI) turnover
D_5	SKF 38393	SCH 23390	
D_{2S} and D_{2L}	Bromocriptine, Quinpirole	Spiperone Sulpiride	Inhibition of adenylate cyclase, inhibition of Ca^{++} & enhancement of K^+ conductances, modulation of PI turnover
D_3	Quinpirole, 7-OH DPAT	UH232	
D_4	?	Clozapine	

Receptor regulation

Agonist treatment of cell lines expressing G protein-coupled receptors may result in a decrease in the ability of the receptor to modulate signal transduction (desensitization of the response). If agonist treatment is prolonged (hours to days), there is a decrease in the density of receptors localized to the membrane (down regulation). In response to agonist pretreatment, both D_1-like and D_2-like receptors undergo desensitization (57-60). More prolonged agonist pretreatment down regulates the D_1-like receptors (57), whereas the density of D_2-like receptors has been shown to both increase (61-64), and decrease (58,59,65).

STRUCTURE-FUNCTION ANALYSIS OF DOPAMINE RECEPTORS

The molecular cloning of the different dopamine receptor subtypes allows the expression of recombinant dopamine receptors in the same cell system, allowing a comparison of different recombinant receptors with one another. A receptor sequence can be altered by *in vitro* mutagenesis to create mutant or chimeric receptors, which can then be expressed on a cell background identical to that of the wild-type receptor. Mutation of a receptor involves changing one (or several) amino acids to look for alteration of function. The selective loss of a function in the mutant receptor may due to the involvement of the mutated amino acid residue(s) in the function.

Chimeric receptors are constructed using regions of two receptors (parents) which differ in specific characteristics. This technique can be used to evaluate the role of multiple, contiguous amino acid residues, or of specific amino acids within the region

that are different between the parents, in a particular function. One of the advantages of this technique is that reciprocal chimeras can be used to verify the importance of a region in a function. If removal of a region from one parent results in the loss of function, substitution of the region into another chimera should produce a gain of function. Mutation and construction of chimeric G protein-coupled receptors has provided a valuable tool for examining determinants of ligand binding and ligand-induced, receptor-mediated activation of cellular mechanisms leading to signal transduction or to regulation of the receptor.

Three dimensional models of G protein-coupled receptors and mutagenesis data have suggested that the transmembrane helices are in a barrel-like conformation, with TMI, TMII, and TMVII adjacent to one another, and interactions between pairs of amino acid residues in these adjacent helices stabilizing receptor structure (66-69). Analysis of mutant and chimeric G protein-coupled receptors has provided a basis for modifying and improving the three dimensional models (70). Mutation of amino acid residues that are conserved among all the catecholamine receptors, has suggested a role for some of these conserved amino acid residues in the binding of catecholamines. There is considerable evidence that a conserved aspartate in TMIII (D113 in β_2 adrenergic receptor) (71) is involved in ionic interactions with the positively charged amine of β_2-adrenergic receptor ligands and that two conserved serine residues (S204 and S207) in TMV are involved in interactions with the catechol ring hydroxyl groups (72). Mutation of these residues in the D_1 and D_2 dopamine receptors confirms that these residues are important for ligand

binding and receptor activation, but results with the dopamine receptors are not in total agreement with mutational data from the adrenergic receptors (73-79).

Chimeric receptors have already proven to be useful for the study of the structural basis of the function of adrenergic receptors. Analysis of chimeric α_2/β_2 adrenergic receptors demonstrated that TMVII has a major influence on subtype selectivity of agonists and antagonists (80). Further analysis of adrenergic receptor chimeras indicated that ligand interactions with chimeric β_1/β_2 adrenergic receptors are more complex. Several TM domains are involved in the subtype selectivity of all ligands, but other TM domains are important determinants for the selectivity of only some ligands (81,82).

Using α_2/β_2 adrenergic receptor chimeras, Kobilka *et al.* (80) also determined that TM V and VI and the third cytoplasmic loop of adrenergic receptors are involved in coupling to adenylate cyclase. Mutagenesis of receptors by many other groups has demonstrated that the third cytoplasmic loop is crucial for G protein coupling (83,84).

SPECIFIC AIMS OF THE DISSERTATION

For the first part of my thesis project, we constructed and expressed chimeric D_1/D_2 dopamine receptors to test the hypothesis that ligand binding to and functional coupling of dopamine receptors would be consistent with the α_2/β_2 chimeric adrenergic receptors; specifically that the third cytoplasmic loop is a primary determinant of G protein coupling and that a major contribution from TMVII determines the binding of selective agonists and antagonists.

There are several advantages to constructing chimeric receptors between D_1 and D_2 dopamine receptors. First, α_2- and β_2-adrenergic receptors share about 25% homology with one another, and the rhesus macaque D_1 and rat D_2 dopamine receptors also have about 25% homology with one another, suggesting that chimeric D_1/D_2 receptors would be functional. Second, there are a number of conserved amino acid residues thought to be involved in ligand binding to both D_1 and D_2 receptors, suggesting that these conserved residues would retain their ability to interact with ligands in the chimeric receptors. Third, the D_1 receptor couples to G_s, stimulating adenylate cyclase, whereas the D_2 dopamine receptor inhibits adenylate cyclase activity via G_i or G_o, so that it is possible to evaluate both the loss and gain of function by the chimeric receptors. And fourth, there are highly selective agonists and antagonists for both D_1 and D_2 receptors available for evaluating ligand binding to the chimeric receptors.

The second part of my thesis project involved examining the role of PKA phosphorylation in regulation of the D_1 receptors (desensitization and down regulation). Desensitization of the D_1 dopamine receptor is characterized by a decreased ability of the receptor to stimulate signal transduction, and occurs rapidly in the continuous presence of agonist (57,85-87). The β-adrenergic receptor also undergoes desensitization which is mediated by both cAMP-dependent protein kinase (PKA) and by the β-adrenergic receptor specific kinases, βARK1 and βARK2. The latter kinases are members of the G protein-coupled receptor kinase (GRK) family (46,88-91). Desensitization of the D_1 receptor has been shown to be associated with phosphorylation of the receptor (92) by GRKs (93) and PKA. Some studies suggest that PKA phosphorylation is involved in

desensitization (57,94) however other studies do not support a role for phosphorylation by PKA in desensitization of the D_1 receptor (86,87).

Longer, more prolonged treatment of cells or tissue expressing D_1 dopamine receptors leads to decreases in, or down regulates, the density of receptors (57,86). Down regulation of the D_1 receptor may be mediated by PKA phosphorylation (87,95). There are potential PKA phosphorylation sites (R-R/K-X-S/T) (45) in the second and third cytoplasmic loops and the carboxyl-terminal region. We wanted to examine the role of PKA phosphorylation in mediating desensitization and down regulation of the D_1 dopamine receptor by mutating potential PKA phosphorylation sites to determine their role in receptor regulation.

II. CHIMERIC D1/D2 DOPAMINE RECEPTORS: DISTINCT DETERMINANTS OF SELECTIVE EFFICACY, POTENCY, AND SIGNAL TRANSDUCTION

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ABSTRACT

D_1/D_2 chimeras were constructed that had D_1 dopamine receptor sequence at the amino terminal end, and D_2 dopamine receptor sequence at the carboxy terminal end. The chimeras with the first four, five and six transmembrane domains of the D_1 receptor ($D_{2[5-7]}$, $D_{2[6-7]}$, $D_{2[7]}$, respectively) bound the D_1 receptor antagonist [^{3}H]SCH 23390 with high affinity. Reciprocal chimeras constructed with D_2 receptor sequence at the amino terminal end displayed no detectable specific binding of [^{3}H]SCH 23390, [^{125}I]epidepride or [^{3}H]spiperone. $D_{2[5-7]}$, $D_{2[6-7]}$, and $D_{2[7]}$ had lower affinity than either D_1 or D_2 dopamine receptors for the non-selective antagonists and agonists and D_2-selective antagonists tested. The chimeric receptors had affinities for three D_1-selective ligands and the D_2-selective agonist, quinpirole, that were intermediate between D_1 and D_2 receptor affinities for the drugs. The substantial loss or gain of affinity for three ligands upon replacement of D_1 transmembrane VII with D_2 sequence ($D_{2[7]}$) suggests an important role for this region in the selectivity of these drugs.

Stimulation of adenylate cyclase activity by D_1 agonists occurred in cells expressing $D_{2[6-7]}$ and $D_{2[7]}$, both of which included the D_1 third cytoplasmic loop, but not in cells expressing $D_{2[3-7]}$ or $D_{2[5-7]}$, both with the D_2 third cytoplasmic loop. However, only $D_{2[6-7]}$ was able to mediate stimulation of adenylate cyclase by quinpirole, implying that D_2 receptor transmembrane domain VI was an important determinant of the selective efficacy of quinpirole. On the other hand, transmembrane domain VII was particularly important for the selective potency of quinpirole. Inhibition of β–adrenergic receptor-stimulated adenylate cyclase activity by dopamine was seen in

cells expressing D_2 receptors and $D_{2[3-7]}$, but not $D_{2[5-7]}$, $D_{2[6-7]}$ or $D_{2[7]}$. Thus, the third cytoplasmic loop of D_1 dopamine receptors was crucial for the coupling of the receptors to G_s, but inhibition of adenylate cyclase *via* G_i required structural features, such as the 2nd cytoplasmic loop of the D_2 receptor, in addition to the 3rd cytoplasmic loop.

INTRODUCTION

Dopamine receptors belong to the family of receptors that are coupled to guanine nucleotide-binding regulatory proteins (G proteins). G protein-coupled receptors have common structural features which include seven α-helical, presumably transmembrane (TM), domains containing 22 to 26 hydrophobic amino acid residues, an extracellular amino-terminus, an intracellular carboxy tail, and conserved amino acid sequences (10,11) Dopamine receptors have been classified into two subfamilies, D_1-like and D_2-like receptors. The D_1-like receptor subtypes are D_1 and D_5 receptors, which have about 50% sequence identity. The pharmacological profiles of these subtypes are similar, but the subtypes differ markedly in regional distribution in the brain (11). D_1 and D_5 receptors couple with the G protein G_s, causing stimulation of adenylate cyclase. The D_2-like receptor subtypes are D_2, D_3, and D_4 dopamine receptors. The D_2 subfamily of dopamine receptors share ~30% sequence identity (11). The D_2-like receptors have high affinity for many substituted benzamide and butyrophenone ligands. D_2 receptors inhibit adenylate cyclase activity (9), and also modulate a number of other signaling pathways, including phosphotidylinositol turnover, K^+ and Ca^{++} channels, and Na^+/H^+ exchange (96,97). Some of these signaling pathways are also modulated by D_3 and D_4 receptors (55,98,99).

To identify the structural features of G protein-coupled receptors that determine function, mutants of adrenergic, cholinergic, dopaminergic, and other G protein-coupled receptors have been constructed. By *in vitro* mutagenesis of single amino acids, a number of conserved residues have been found to be important for ligand binding and

signal transduction. However, receptor function probably involves the concurrent action of multiple, often contiguous, amino acid residues, that cannot always be identified by the mutation of single amino acids.

Exchanging regions between related receptors to create chimeras is another approach that has been used to identify sequence determinants of receptor function. With this approach, one determines the contributions of receptor domains, which may suggest regions where mutation of single amino acids would be fruitful. In addition, by constructing reciprocal chimeras it is often possible to measure both gain and loss of a function that is associated with a particular receptor domain. Analysis of chimeric receptors has already proven to be useful for the study of the structural basis of the function of adrenergic receptors. Kobilka *et al.* (80) used chimeras of α_2- and β_2-adrenergic receptors to show that TM V and VI and the third cytoplasmic loop of adrenergic receptors are determinants of coupling to adenylate cyclase, and also that the subtype selectivity of adrenergic receptor agonists and antagonists is strongly influenced by TM VII. One study of chimeric β_1/β_2 adrenergic receptors showed that TM IV was particularly important for the β_1-selectivity of norepinephrine, and that multiple TM regions contributed to the selectivity of two antagonists (81). A second study (82) found ligand interactions with chimeric β_1/β_2 adrenergic receptors to be more complex. Several TM domains were important determinants of subtype selectivity of all ligands, but other TM domains were involved in the selectivity of only some ligands.

D_1 and D_2 dopamine receptors are differentiated by primary structure, affinities for selective agonists and antagonists, and transduction mechanisms. Like α_2– and β_2–

adrenergic receptors, D_1 and D_2 receptors are similar in some respects, with about 25% amino acid sequence identity, primarily in the putative TM regions, and similar affinities for some ligands. On the other hand, the qualitative differences in second messenger coupling and the many quantitative differences in ligand binding indicated that analysis of D_1/D_2 receptor chimeras would be informative.

MATERIALS AND METHODS

Materials [^{3}H] SCH-23390 (80 Ci/mmol) was purchased from Du Pont-New England Nuclear, [^{3}H]adenine (30 Ci/mmol) from ICN, and [^{3}H]spiperone (70 Ci/mmol) from Amersham. (+)-Butaclamol, chloro-APB (SKF 82958), SKF 38393, sulpiride, and quinpirole (LY17155) were purchased from Research Biochemicals, Inc. Spiperone, haloperidol (Janssen), cis-flupentixol (Lundbeck), clozapine (Sandoz), and epidepride (NCQ 219; Dr. Tomas de Paulis, Vanderbilt University) were gifts. Most other drugs and reagents were purchased from Sigma Chemical Co.

Construction of chimeric receptor cDNAs---Chimeric cDNAs were constructed by trans-PCR, a procedure used to join DNA fragments that contain a region of overlap (17). D_1 sequence was amplified from the rhesus macaque D_1 receptor gene (60), whereas D_2 receptor sequence was amplified from a rat D_{2S} cDNA (7), generously supplied by Dr. Olivier Civelli (Hoffman La Roche LTD). To make a chimera, the fragments originating from each receptor were amplified in separate reactions, each containing one receptor as template, a vector primer, and a 36-mer primer comprised of 24 bases from the template sequence and an additional 12 bases from the other receptor. The two 36-mers used in

each reaction were complementary for 24 bases, with the junction between the D_1 and D_2 sequence in the center of the complementary region. The result of these reactions was the amplification of one fragment of D_1 receptor sequence with a 12-base D_2 end, and one fragment of D_2 receptor sequence with a 12-base D_1 end, yielding 24 bases of overlap between the two fragments. The fragments were gel-purified away from the full-length receptor templates and used as the template DNA in a third reaction that included only the two outer primers, one primer complementary to sequence within the SV40 polyadenylation signal and one primer corresponding to sequence within the T7 polymerase promoter. This reaction produced a full-length chimeric cDNA, presumably by the formation of heteroduplexes between complementary ends of the templates. The cDNA was flanked by the restriction sites present in the polylinker of pcDNA-1. Both the vector and the amplified cDNA were digested with appropriate restriction endonucleases, purified, and ligated. For example, to construct $D_{2[3\text{-}7]}$, D_2 sequence from the amino-terminal end of TM III to the carboxy terminus was amplified using an SV40 24-mer and the 36-mer, 5'-**GGG TCC TTC TGT / GAC ATC TTT GTC** ACT CTG GAT GTC-3'. D1 sequence from the amino terminus to the carboxy terminal end of the 2nd extracellular region was amplified using a T7 polymerase promoter 23-mer and a 36-mer that is the reverse complement of 5'-TTC TGG CCC TTT **GGG TCC TTC TGT / GAC ATC TTT GTC**-3'. The region of overlap in the two 36-mers is indicated by bold-faced type, D_1 sequence is underlined, and the junction between D_1 and D_2 receptor sequence is indicated by a slash. The PCR product was digested with *Hin*dIII and *Eco*RI and cloned into those sites of pcDNA-1.

The junction of $D_{2[3-7]}$ is between amino acid Cys-96 of the D_1 receptor and Asp-108 of the D_2 receptor, forming the sequence Gly-Ser-Phe-Cys/Asp-Ile-Phe-Val. The junction of $D_{2[5-7]}$ is between Arg-191 of the D_1 receptor and Ala-188 of the D_2 receptor, forming the sequence Ser-Leu-Ser-Arg/Ala-Phe-Val-Val. The junction of $D_{2[6-7]}$ is between Lys-271 of the D_1 receptor and Met-346 of the D_2 receptor, forming the sequence Lys-Val-Leu-Lys/Met-Leu-Ala-Ile. The junction of $D_{2[7]}$ is between Asp-309 of the D_1 receptor and Val-378 of the D_2 receptor, forming the sequence Phe-Cys-Ile-Asp/Val-Leu-Tyr-Ser.

Characterization of Recombinant Receptors---Chimeric cDNAs were stably expressed in C_6 glioma cells exactly as described previously (75). Confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, 2 mM EDTA). After 10 to 20 min., the cells were scraped off the plate into centrifuge tubes and centrifuged at 17,000 rpm for 20 min. The crude membrane fraction was re-suspended with a Brinkmann polytron homogenizer (setting 6 for 10 s) in assay buffer (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.001% bovine serum albumin). For competition binding studies in which agonist displacement of binding was assessed, the membranes were re-suspended in 50 mM Tris containing 4 mM $MgCl_2$, and incubated at 37° C for 30 min. The membranes were then re-centrifuged and re-suspended in assay buffer also containing 4 mM $MgCl_2$, 1 mM EDTA, and 0.025% ascorbic acid.

Binding of [^{3}H]SCH 23390 was carried out in an assay volume of 100 µL for the chimeric receptors, and either 100 µl or 250 µl for the D1 dopamine receptor. Assays included assay buffer, cell membranes, radioligand, and appropriate drugs.

(+)Butaclamol (2 μM for the D_1 receptor and 20 μM for the chimeric receptors) was used to define non-specific binding. The assays were incubated at 30° C for 1 h and stopped by the addition of 4 ml of ice-cold wash buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl). The samples were filtered through Whatman filters (GC FP-205) using a 48-well Brandel filtration apparatus and counted using a Beckman LS1701 scintillation counter. Binding of [^{125}I]epidepride or [^{3}H]spiperone was carried out as described previously (7,75). The stimulation or inhibition of cyclic AMP accumulation in intact cells was determined from the conversion of [^{3}H]adenine to [^{3}H]ATP and [^{3}H]cyclic AMP, as described previously (60,75). Data were analyzed by non-linear least-squares regression using GraphPAD InPlot. K_i values are geometric means from three or more independent experiments ± the asymmetrical standard error of the mean. The change in free energy was calculated using the equation $\Delta G = -RT\ln(1/K_i)$.

RESULTS AND DISCUSSION

As depicted in Figure 2-1, D_1/D_2 receptor cDNAs were constructed in such a way that the expressed chimeras had D_1 receptor sequence at the amino terminal end, and D_2 sequence at the carboxy terminal end. All of the chimeras and wild-type D_1 (60) and D_2 (75) receptors were stably expressed in C_6 glioma cells, and the binding of three radioligands was assessed. Three of the chimeras ($D_{2[5\text{-}7]}$, $D_{2[6\text{-}7]}$, $D_{2[7]}$) and D_1 receptors bound [^{3}H]SCH 23390, a D_1 receptor ligand (100,101), with high affinity.

Saturation analysis of radioligand binding to these receptors determined that the density of each receptor was between 200 and 500 fmol/mg of membrane protein.

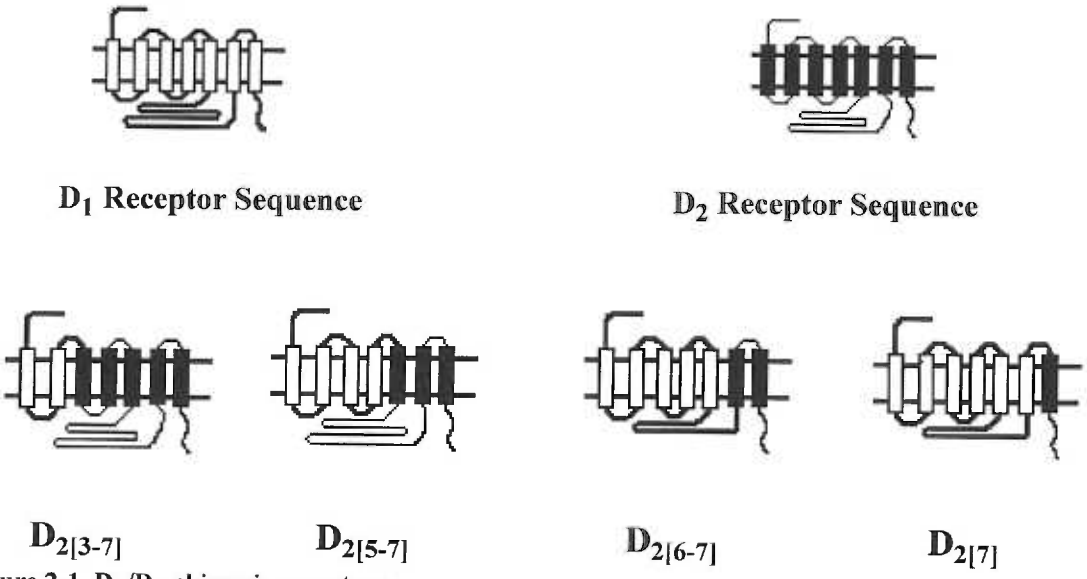


Figure 2-1 D_1/D_2 chimeric receptors.
Schematic representation of the structure of the chimeric receptors. D_1 sequence is denoted by open rectangles for putative TM domains and thick lines for intracellular and extracellular hydrophilic regions. D_2 sequence is denoted by filled rectangles for putative TM domains and thin lines for hydrophilic regions.

K_D values for the binding of [^{3}H]SCH 23390 were 21 ± 3 nM for $D_{2[5-7]}$ (n = 17), 10 ± 2 nM for $D_{2[6-7]}$ (n = 15), 8 ± 1 nM for $D_{2[7]}$ (n = 20), and 0.4 ± 0.1 nM for D_1 (n = 12). Although we were able to detect specific binding of [^{3}H]SCH 23390 to $D_{2[3-7]}$, the binding was of such low affinity that saturation analyses could not be performed reliably.

None of the chimeric or D_1 receptors had detectable specific binding of [^{125}I]epidepride, a substituted benzamide antagonist (102). To determine if the lack of affinity of the chimeric receptors was selective for this ligand, we also performed tests of the binding of the butyrophenone D_2 antagonist [^{3}H]spiperone, with similar results (data not shown). The average K_D value of wild-type D_2 receptors for [^{125}I]epidepride was 0.06 ± 0.01 nM (n = 13). In subsequent determinations of K_i values, the affinity of chimeric ($D_{2[5-7]}$ $D_{2[6-7]}$, and $D_{2[7]}$) and D_1 receptors for ligands was quantified by

inhibition of the binding of [^{3}H]SCH 23390, whereas the affinity of D_2 receptors for ligands was determined by inhibition of the binding of [^{125}I]epidepride.

We also constructed four chimeric receptors reciprocal to those depicted in Figure 1, creating receptors with D_2 sequence at the amino terminus and D_1 sequence at the carboxy terminus. None of these chimeras could be detected with any of the three radioligands used, although COS-7 cells transfected with the cDNAs expressed mRNA that hybridized specifically with a D_2 receptor cDNA (data not shown). We speculate that amino acids present in TM I or II of the D_2 receptor are incompatible with residues present in TM VII of the D_1 receptor. The juxtaposition of either TM I or TM II with TM VII in the membrane has been suggested by *in vitro* mutagenesis of G protein-coupled receptors. One result of the close spatial relationship between these helices is that mutations in one of these regions of a receptor can lead to loss of function due to the creation of an incompatibility between the helices (66,69). Alternatively, helices from different receptors may include incompatible amino acid residues, as is the case for TM I and TM VII of m5 and m2 muscarinic receptors, respectively (67). If TM I or II of D_2 receptors and TM VII of D_1 receptors are incompatible, it may be necessary to construct chimeric receptors that share D_2 receptor sequence in these regions.

Because the structural determinants of the binding of ligands to G protein-coupled receptors are thought to lie within the conserved α-helical regions, the chimeras were analyzed in terms of the number of TM regions that were from D_1 or D_2 receptors. We calculated the difference in the free energy change of binding of a ligand that resulted from substitution of a TM region from one subtype for that TM region of the other

subtype, as suggested by Catterall (103), and used by Marullo *et al.* (82). The difference between the free energy change of binding of a ligand to D_1 and to D_2 dopamine receptors is a quantitative measurement of the selectivity of the ligand for one of the subtypes. If we assume that each of the seven TM regions contributes equally to the selectivity of a ligand, then 1/7th of the difference would be contributed by each TM domain. For a ligand that fits this assumption, a plot of the free energy change of binding at each chimeric and wild-type receptor *vs.* the number of TM regions contributed by each receptor subtype should be linear, with the values for the chimeric receptors falling on a line drawn between the wild-type receptors. Values for chimeric receptors that fall above or below that line indicate a disproportionate contribution of particular TM regions to the selectivity of a ligand.

Three of the ligands that were tested, (+)-butaclamol, clozapine, and *cis*-flupentixol, are antagonists that are relatively non-selective for D_1 and D_2 dopamine receptors. The three chimeric receptors ($D_{2[5-7]}$, $D_{2[6-7]}$, and $D_{2[7]}$) had moderate reductions in affinity for each of these non-selective antagonists, with the greatest decrease in affinity observed for $D_{2[5-7]}$ (Table 2-1). This is graphically depicted in Fig. 2-2A, where the free energy change of binding of clozapine to each of the chimeras falls below the line drawn between the values for D_1 and D_2 receptors. Similar results were obtained for the binding of dopamine, a non-selective agonist (Table 2-2, Fig. 2-3A). Whereas the reduction in affinity of the chimeras for these ligands may reflect conformational changes that result from the non-physiological juxtaposition of TM regions, the fact that overall the affinity changes were modest suggests that these

nonspecific perturbations of receptor structure were also modest. The larger decrease in affinity of $D_{2[5\text{-}7]}$ for each of these ligands could be due to greater distortion of structure in this chimera, although results from other ligands (see below) are not consistent with this interpretation. An alternative explanation is that the binding pockets for these non-selective ligands are different for D_1 and D_2 receptors. For example, the K_i value of dopamine decreased markedly for $D_{2[6\text{-}7]}$, compared to $D_{2[7]}$ and D_1 receptors, which could result from the loss of important amino acids in TM VI of D_1 receptors. The implication is that TM VI of D_2 receptors is not as important as TM VI of D_1 receptors for the binding of dopamine.

Three D_2-selective antagonists, epidepride (104), spiperone, and haloperidol, had binding profiles similar to each other and to the non-selective ligands, but very different from the D_1-selective ligands. $D_{2[5\text{-}7]}$ had the lowest affinity of all of the receptors, even D_1, for each of these antagonists (Table 2-1, Fig. 2-2C). It might be expected that the three chimeras would resemble D_1 receptors more than D_2 receptors, because even $D_{2[5\text{-}7]}$, the least D_1-like of the three fully characterized chimeras, has 4 out of 7 D_1 TM regions. Nevertheless, the potencies of the D_2-selective antagonists for the chimeras were not intermediate between values for D_1 and D_2 receptors. It is possible that two processes combine to produce the strikingly low affinity of the chimeric receptors for D_2-selective antagonists. First, as hypothesized for the non-selective antagonists, it could be that nonspecific perturbations of the tertiary structure of the chimeras result in moderate decreases in affinity for D_2-selective antagonists. Second, TM I-IV of the D_2 receptor may be critical for the binding of these antagonists. Although we were unable to quantify

Table 2-1 Affinity of D_1, D_2 and chimeric dopamine receptors for antagonists.
K_i values (nM)

Antagonist	D2	$D_{2[5-7]}$	$D_{2[6-7]}$	$D_{2[7]}$	D1
(+) butaclamol	0.5	59	13	25	2.8
	(0.3-0.6)	(53-65)	(11-14)	(18-34)	(2.5-3.1)
clozapine	140	680	370	370	150
	(120-170)	(550-850)	(270-530)	(300-440)	(120-200)
epidepride	0.11	35000	13000	11000	5200
	(0.1-0.11)	(23000-52000)	(9000-16000)	(7900-15000)	(4500-5900)
cis-flupenthixol	1.1	76	24	19	7.4
	(1.0-1.1)	(55-110)	(19-30)	(13-26)	(7.0-7.7)
haloperidol	2.9	3100	2200	1900	110
	(2.8-3.1)	(2100-4400)	(1900-2400)	(1500-2300)	(78-170)
spiperone	0.05	31000	1700	3700	540
	(0.05-0.06)	(22000-45000)	(1300-2200)	(2800-5000)	(490-590)
SCH-23390	920	17	5.8	3.9	0.5
	(830-1020)	(12-25)	(4.7-7.1)	(1.6-9.6)	(0.4-0.5)

K_i values for antagonists for inhibition of the binding of [^{3}H]SCH 23390 to the chimeric and D_1 receptors, and the binding of [^{125}I]epidepride to D_2 receptors, were determined as described under "Materials and Methods". Results shown are geometric means from three or more independent experiments with the asymmetrical standard error of the means given in parentheses.

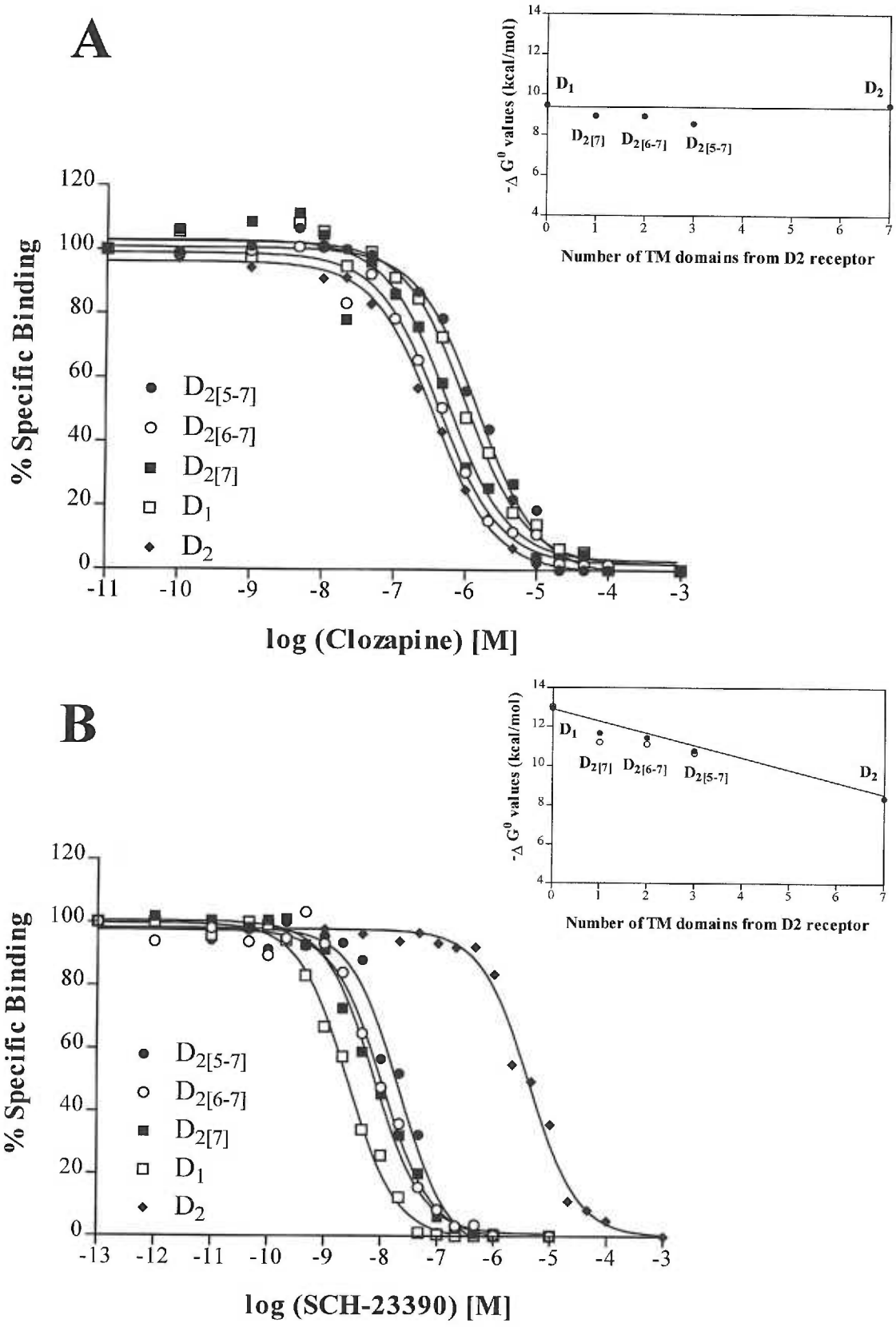
Table 2-2 Affinity of D_1, D_2, and chimeric receptors for agonists
K_i values (μM)

Agonist	D_2	$D_{2[5-7]}$	$D_{2[6-7]}$	$D_{2[7]}$	D_1
Chloro-APB	0.51	0.14	0.08	0.04	0.02
	(0.39-0.68)	(0.13-0.14)	(0.07-0.09)	(0.04-0.05)	(0.02-0.03)
SKF-38393	9.5	1.8	1.8	1.6	0.3
	(8.5-11)	(1.3-2.3)	(1.4-2.3)	(1.5-1.8)	(0.3-0.4)
quinpirole	2.6	51	53	59	530
	(1.5-4.5)	(40-64)	(32-86)	(31-110)	(370-740)
dopamine	8.8	700	710	34	18
	(6.7-12)	(590-830)	(510-1000)	(22-53)	(14-23)

K_i values for agonists for inhibition of the binding of [^{3}H]SCH 23390 to the chimeric and D_1 receptors, and the binding of [^{125}I]epidepride to D_2 receptors, in the presence of GTP, were determined as described under "Materials & Methods". Results shown are geometric means from three or more independent experiments with the asymmetrical standard error of the mean given in parentheses.

high affinity binding of [^{125}I]epidepride or [^{3}H]spiperone to $D_{2[3-7]}$ (data not shown), the affinity of $D_{2[5-7]}$ for these ligands is so low that even a 1000-fold increase in affinity for $D_{2[3-7]}$ resulting from the addition of TM III and IV from D_2 receptors would be difficult to detect.

Three of the ligands that were tested are D_1-selective benzazepines. SCH 23390 is an antagonist (48,105), whereas SKF 38393 (106) and chloro-APB (107) are agonists. $D_{2[7]}$ had a modest reduction in affinity for SCH 23390 compared to D_1 receptors (Table 2-1). The K_i value of SCH 23390 for $D_{2[6-7]}$ was similar to its value for $D_{2[7]}$, whereas the replacement of D_1 TM domain V with D_2 sequence in $D_{2[5-7]}$ caused an additional slight


A
% Specific Binding
log (Clozapine) [M]
$D_{2[5-7]}$
$D_{2[6-7]}$
$D_{2[7]}$
D_1
D_2
$-\Delta G^0$ values (kcal/mol)
Number of TM domains from D2 receptor
B
% Specific Binding
log (SCH-23390) [M]
$D_{2[5-7]}$
$D_{2[6-7]}$
$D_{2[7]}$
D_1
D_2
$-\Delta G^0$ values (kcal/mol)
Number of TM domains from D2 receptor

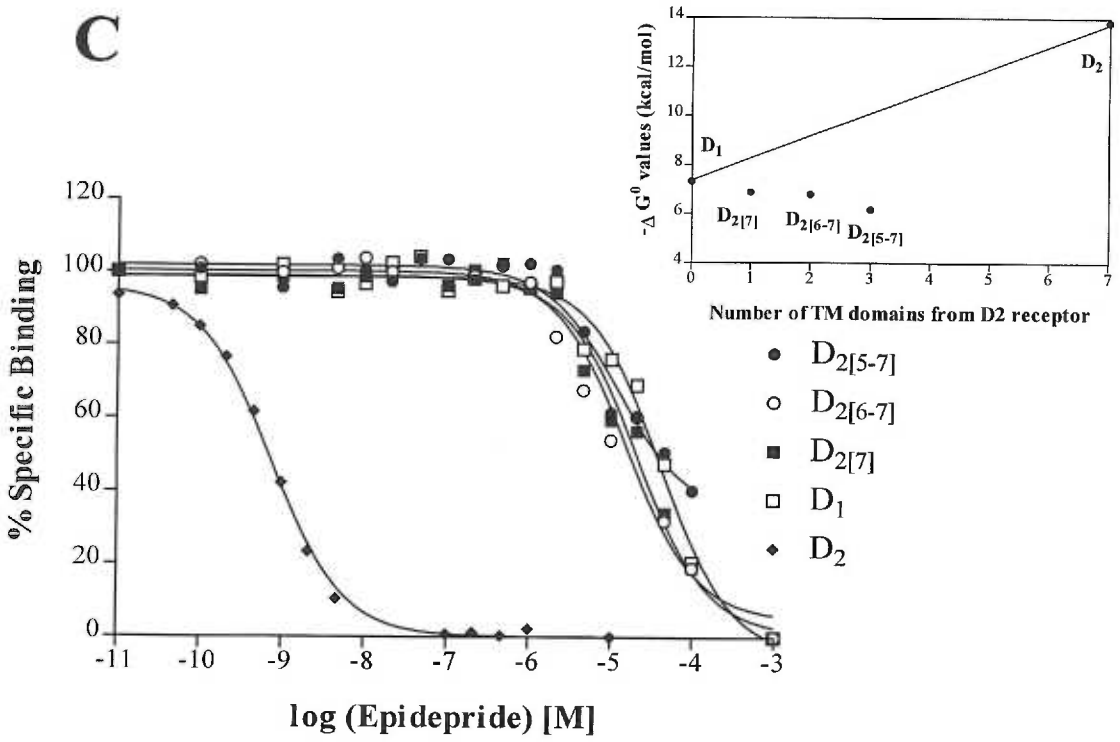


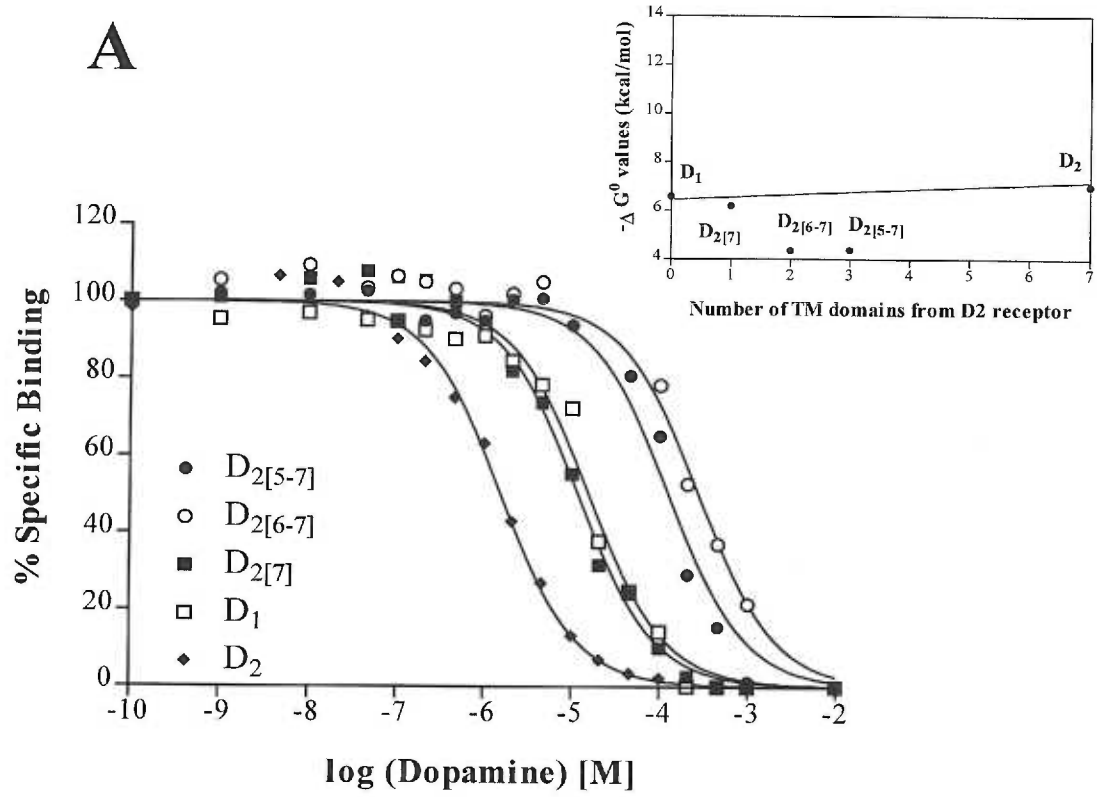
Figure 2-2 Binding of antagonists.

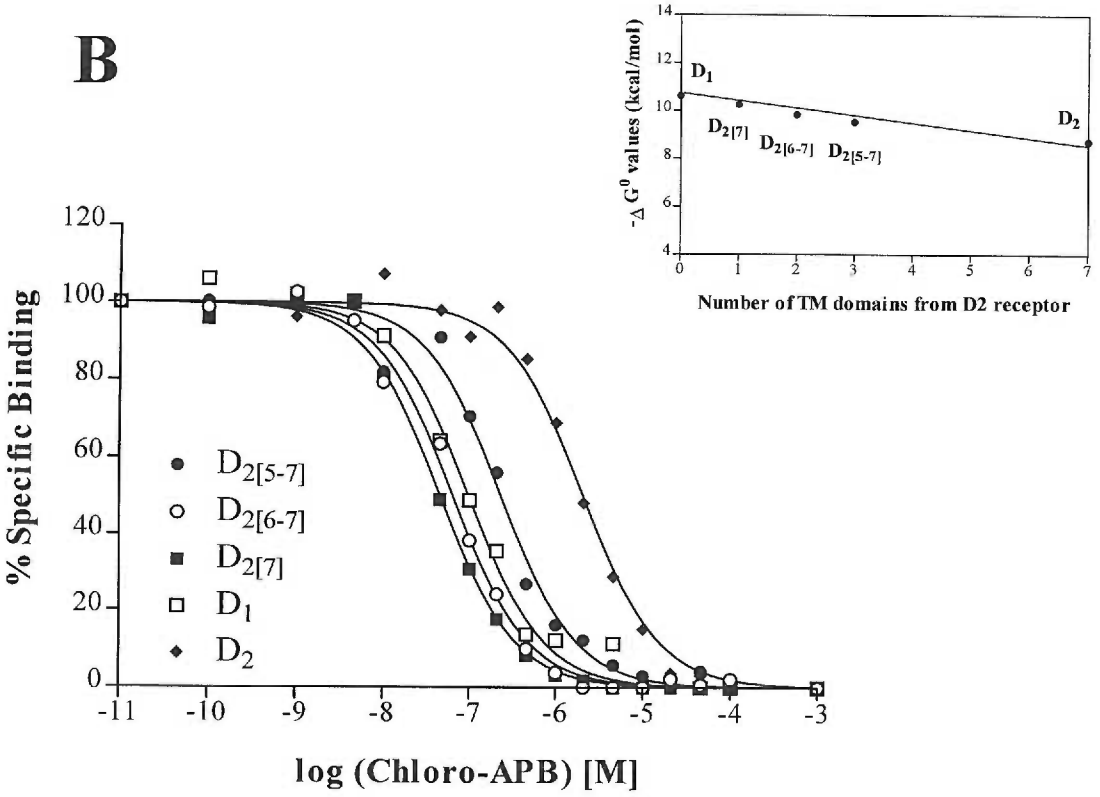
A, B and C, representative data are shown from one of three or more independent experiments in which inhibition of radioligand binding was determined for the indicated drugs. Data are plotted as a percentage of the specific binding in the absence of inhibitor versus *the logarithm of the concentration of (A) clozapine, (B) SCH 23390, or (C) epidepride. In the inset of each panel, the data are expressed as the free energy change of binding, in kilocalories per mole, plotted* versus *the number of TM regions from the D2 receptor. B, the inset includes data for [^{3}H]SCH 23390 (open circles) and unlabeled SCH 23390 (filled circles).*

decrease in affinity. As shown in Fig. 2-2B, the values for the free energy change of binding of SCH 23390 to the chimeras lie close to the line drawn between the values for D_1 and D_2 receptors, suggesting that TM V, VI, and VII each contribute approximately proportionately (1/7) to the selectivity of SCH 23390. That the value for $D_{2[7]}$ is below the line could indicate a greater than 1/7th contribution of TM VII of D_1 receptors. This is consistent with the data obtained in separate experiments by saturation analysis of the binding of [^{3}H]SCH 23390 (Fig 2-2B). The affinity of $D_{2[7]}$ (8 nM) for the radioligand was lower than that of the D_1 receptor (0.4 nM) but similar to $D_{2[6-7]}$ (10 nM).

Interestingly, the benzazepine agonists yielded results very similar to those for SCH 23390 (Table 2-2, Fig. 2-3B). Like the antagonist, the K_i value of SKF 38393 for $D_{2[7]}$ was reduced relative to D_1 receptors, indicating the importance of D_1 TM VII (Table 2-2). Replacement of the D_1 TM V and VI in $D_{2[5-7]}$ and $D_{2[6-7]}$ had little additional effect on the binding of SKF 38393, suggesting that these TM regions contribute proportionately little to the D_1 selectivity of the agonist. On the other hand, the K_i value for chloro-APB roughly doubled as each of TMs VII through V was changed from D_1 receptor sequence to D_2 receptor sequence (Table 2-2). The free energy change of binding of the agonist to each chimera was very close to the calculated line between values for D_1 and D_2 receptors (Fig. 2-3B). This indicates that each of these three TM regions contributes about 1/7th of the selectivity of chloro-APB. The finding that the affinity of $D_{2[5-7]}$ for the drugs in this group is intermediate between the affinity of D_1 and D_2 receptors, and approximately what would be expected assuming that each TM region contributes equally to ligand selectivity, suggests that if nonspecific distortions in the tertiary structure of the chimeric receptors are the basis for the low affinity of the receptors for non-selective and D_2-selective antagonists (see above), the structural distortions are not large.

The affinity of $D_{2[7]}$ for a D_2-selective agonist, quinpirole, was 10-fold higher than the affinity of D_1 receptors for the drug (Table 2-2). Subsequent additions of D_2 receptor sequence in $D_{2[6-7]}$ and $D_{2[5-7]}$ caused no additional increases in affinity. As shown in Figure 2-3C, the value for the free energy change of binding of quinpirole to $D_{2[7]}$ lies above the line drawn between the values for D_1 and D_2 receptors. This gain of


A
% Specific Binding
log (Dopamine) [M]
D2[5-7]
D2[6-7]
D2[7]
D1
D2
-Δ G0 values (kcal/mol)
Number of TM domains from D2 receptor


B
% Specific Binding
log (Chloro-APB) [M]
D2[5-7]
D2[6-7]
D2[7]
D1
D2
-Δ G0 values (kcal/mol)
Number of TM domains from D2 receptor

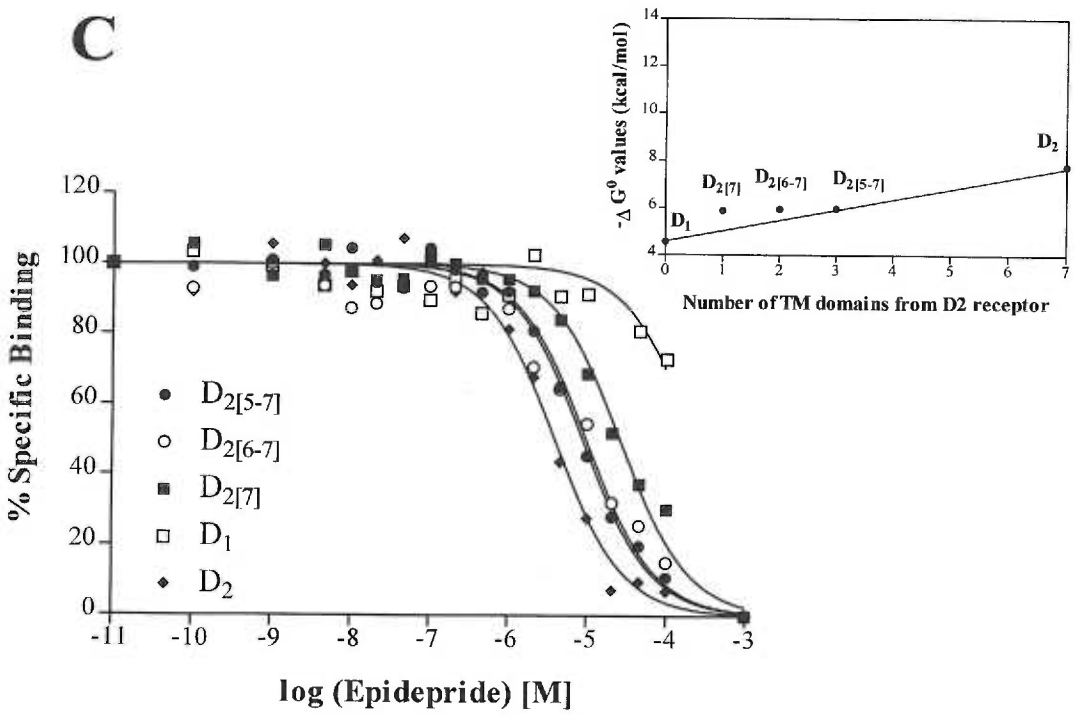


Figure 2-3 Binding of agonists.

A, B and C, representative 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) dopamine, (B) chloro-APB, or (C) quinpirole, in the presence of GTP. In the inset of each panel, the data are expressed as the free energy change of binding, in kilocalories per mole, plotted* versus *the number of TM regions from the D_2 receptor.*

function demonstrates that TM VII from D_2 receptors contributes disproportionately to the selectivity of quinpirole for D_2 receptors. Interestingly, this is complementary to our finding that TM VII of D_1 receptors contributes disproportionately to the D_1 selectivity of two benzazepines, SCH 23390 and SKF 38393, and consistent with the importance of this region in the selectivity of drugs for α_2 and β_2 adrenergic receptors (80).

A substantial body of work has demonstrated the critical role of the 3rd cytoplasmic loop of G protein-coupled receptors in signal transduction (108,109). If the third cytoplasmic loop and the adjoining TM regions were sufficient to select the G

proteins which are activated by dopamine receptors, then we would expect $D_{2[3-7]}$ and $D_{2[5-7]}$ to inhibit and $D_{2[6-7]}$ and $D_{2[7]}$ to stimulate adenylate cyclase activity.

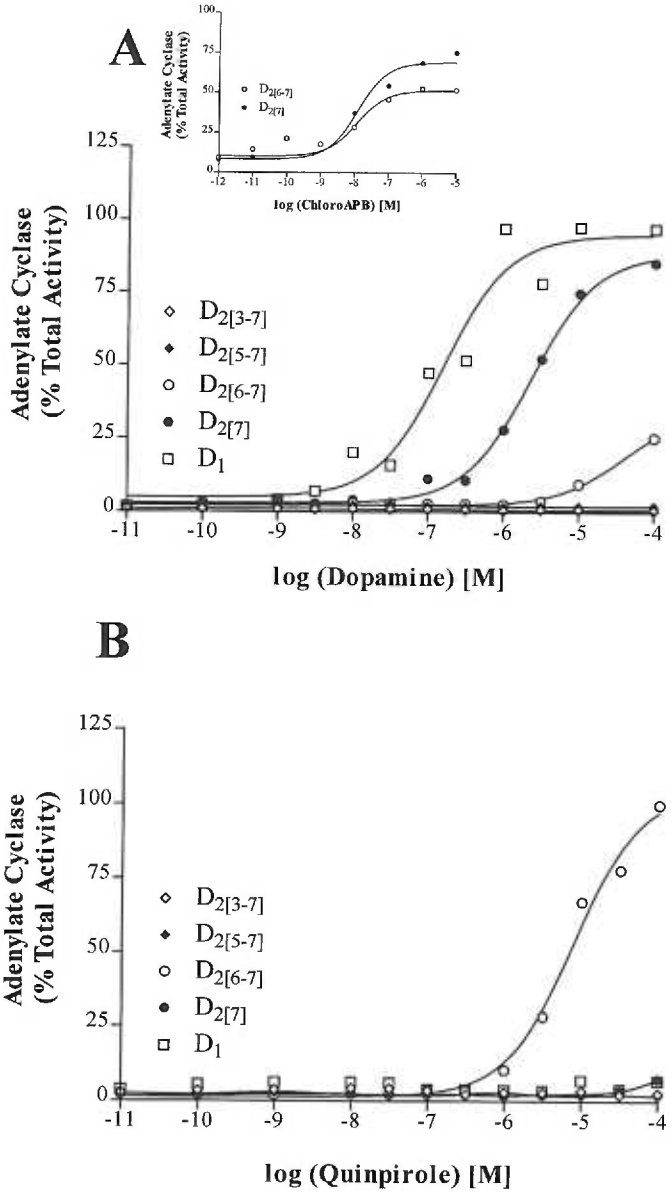


Figure 2-4 Stimulation of adenylate cyclase activity by agonists.
A and B, data are shown from one of three or more independent experiments in which stimulation of adenylate cyclase activity was assessed. A, data are plotted as adenylate cyclase activity, expressed as the percentage of the maximal stimulation at D_1 receptors, versus *the logarithm of the concentration of dopamine. The inset depicts stimulation of adenylate cyclase by chloro-APB at $D_{2[6-7]}$ and $D_{2[7]}$, expressed as a percentage of maximal stimulation at D_1 receptors. B, data are plotted as adenylate cyclase activity, expressed as the percentage of the maximal stimulation at $D_{2[6-7]}$,* versus *the logarithm of the concentration of quinpirole*

In agreement with this hypothesis, $D_{2[6-7]}$ and $D_{2[7]}$, like D_1 receptors, mediated stimulation of adenylate cyclase by dopamine and chloro-APB (Fig. 2-4A), although the reduced maximal stimulation by $D_{2[6-7]}$ and $D_{2[7]}$ relative to D_1 receptors demonstrates that successive additions of D_2 receptor TM VII and VI decreased the efficiency of coupling to G_s. The increased EC_{50} of dopamine at $D_{2[6-7]}$ and $D_{2[7]}$ is consistent with its reduced potency for inhibition of radioligand binding to these receptors. Interestingly, the D_2 agonist quinpirole stimulated adenylate cyclase *via* $D_{2[6-7]}$, but not *via* $D_{2[7]}$ or D_1 receptors (Fig. 2-4B); although the addition of TM VII of D_2 receptors ($D_{2[7]}$) substantially increased affinity for quinpirole, the addition of D_2 receptor TM VI ($D_{2[6-7]}$) was necessary for quinpirole to induce coupling to G_s.

Inhibition of adenylate cyclase activity was assessed for chloro-APB, dopamine, and quinpirole (Fig. 3-5A and 5B). All three agonists inhibited enzyme activity *via* D_2 receptors. However, none of them inhibited adenylate cyclase *via* $D_{2[5-7]}$, $D_{2[6-7]}$, or $D_{2[7]}$ (Fig. 2-5A and 5B) or stimulated adenylate cyclase activity *via* $D_{2[5-7]}$ (Fig. 2-4A and 4B; data for chloro-APB not shown). Although $D_{2[5-7]}$ includes the third cytoplasmic loop of the D_2 dopamine receptor, its inability to stimulate or inhibit adenylate cyclase suggests that other receptor regions must be necessary for signal transduction by D_2 dopamine receptors. This is consistent with the report by McAllister *et al.* (110) that D_3 receptors containing the D_2 receptor third cytoplasmic loop do not couple to adenylate cyclase or arachidonic acid release, two D_2 signaling pathways. In agreement with this conclusion, $D_{2[3-7]}$ was able to mediate modest inhibition of

adenylate cyclase by dopamine (Fig. 2-5A). It appears that the 2nd cytoplasmic loop of D_2 receptors (present in $D_{2[3-7]}$ but not $D_{2[5-7]}$) is necessary for coupling to G_i.

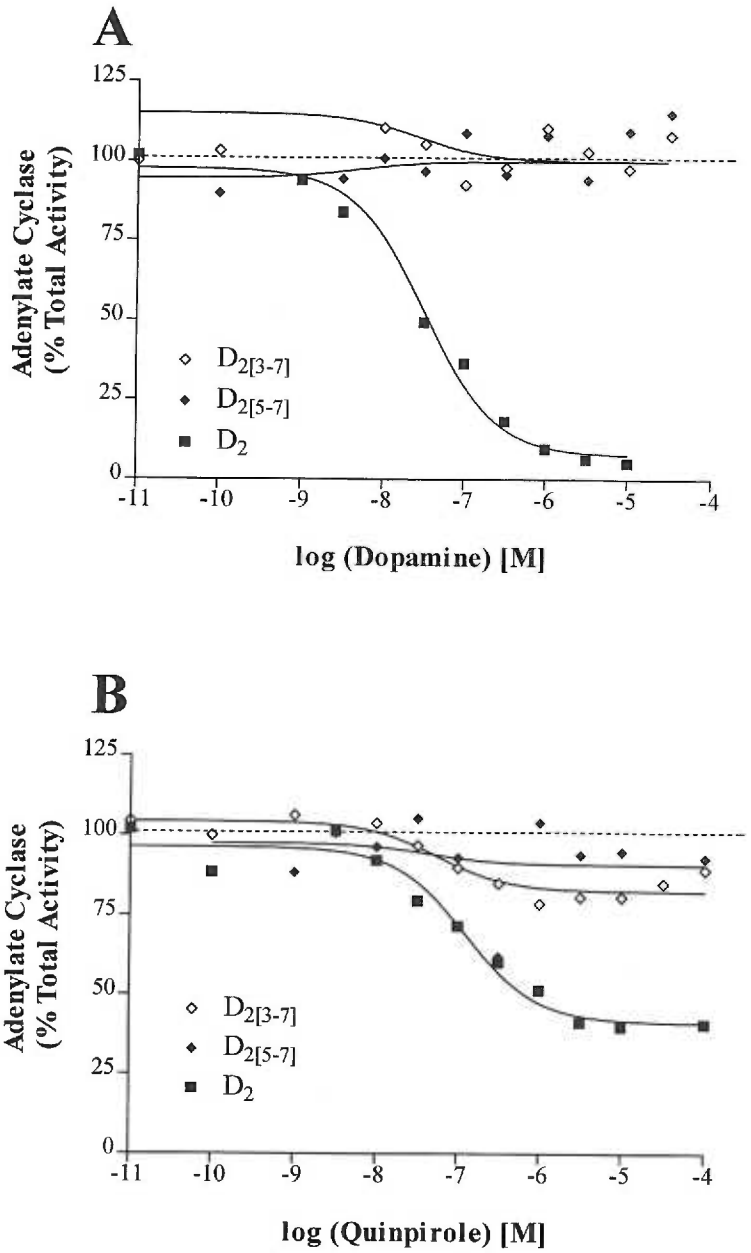


Figure 2-5 Inhibition of adenylate cyclase activity by agonists.
A and B, data are shown from one of three or more independent experiments in which inhibition of adenylate cyclase activity was assessed. Data are plotted as adenylate cyclase activity, expressed as the percentage of total activity stimulated by 1 μM isoproterenol, versus *the logarithm of the concentration of (A) dopamine or (B) quinpirole.*

$D_{2[6\text{-}7]}$ in the present report is virtually identical in structure to a chimeric D_1/D_2 receptor characterized previously (111). MacKenzie *et al.* reported that this chimera has a pronounced loss of affinity for dopamine, relative to D_1 receptors, and decreased affinity for SCH 23390 and SKF 38393. In addition, the affinity of the chimera for quinpirole is increased relative to D_1 receptors, and quinpirole is able to stimulate adenylate cyclase *via* the D_1/D_2 chimera (111). The data presented above confirm these findings and, by extending the results to include two novel D_1/D_2 chimeric receptors, demonstrate that TM VI and VII of the D_2 receptor include determinants of selective efficacy and potency, respectively.

CONCLUSIONS

The pharmacological profiles of the characterized chimeric receptors resemble D_1 more than D_2 receptors. This is consistent with the fact that even in $D_{2[5\text{-}7]}$, the chimera with the most D_2 sequence, only 3 of the 7 membrane-spanning domains are from the D_2 receptor. Still, $D_{2[3\text{-}7]}$, with 5 of the 7 of the transmembrane regions from the D_2 receptor, did not appear to bind the D_2 ligands [^{125}I]epidepride or [^{3}H]spiperone with detectable affinity.

The selectivity profiles of the ligands for the chimeric receptors can be fit into two groups. The non-selective antagonists, the D_2-selective antagonists, and the non-selective agonist dopamine were all less potent at $D_{2[5\text{-}7]}$, $D_{2[6\text{-}7]}$, and $D_{2[7]}$ than at D_1 or D_2 receptors. Our present hypothesis is that this is due to a combination of nonspecific structural perturbations and an important role for TM regions I-IV in the binding of these

ligands to D_2 receptors. To test this hypothesis it will be important to analyze chimeras with D_2 receptor sequence in the amino terminal TM regions. Although the chimeras reciprocal to those described above were not functional, due to a hypothesized incompatibility between TM I or II of the D_2 receptor and TM VII of the D_1 receptor, it should be possible to identify an important role for TM regions I-IV by characterizing chimeras with D_2 sequence at both ends, and internal substitutions of TM regions from the D_1 receptor.

The second type of selectivity profile was shown by the three D_1-selective benzazepines and the D_2-selective agonist quinpirole. All four of the drugs in this group had potencies for the chimeric receptors that were intermediate between their potencies at D_1 and D_2 receptors, so that the calculated values for the free energy change of binding were close to a line drawn between the values for D_1 and D_2 receptors. Furthermore, 3 out of 4 of these drugs had results suggesting an important role for TM VII in the selectivity of drugs for D_1 and D_2 receptors.

Our results support the hypothesis that the third cytoplasmic loop of D_1 dopamine receptors is crucial for the coupling of the receptors to G_s. Coupling to G_i and inhibition of adenylate cyclase, however, required structural features, including the 2nd cytoplasmic loop, in addition to the 3rd cytoplasmic loop of the D_2 receptor. Finally, comparison of the interactions of quinpirole with $D_{2[6\text{-}7]}$ and $D_{2[7]}$ demonstrated that the amino acids that contribute to the selectivity of binding of the drug differ from those responsible for the selective efficacy of the drug. Thus, $D_{2[6\text{-}7]}$ and $D_{2[7]}$ had similar affinity for quinpirole, and whereas both chimeric receptors were able to mediate stimulation of

adenylate cyclase by D_1 agonists, only $D_{2[6\text{-}7]}$ was able to mediate stimulation of adenylate cyclase by the D_2 receptor agonist.

III CONSTITUTIVE ACTIVITY OF A CHIMERIC D_2/D_1 DOPAMINE RECEPTOR

as revised and resubmitted to

Molecular Pharmacology

ABSTRACT

Chimeric D_1/D_2 receptors were constructed to identify structural determinants of drug affinity and efficacy. We previously reported that chimeras that had D_1 receptor transmembrane region VII together with amino-terminal sequence from the D_2 receptor were non-functional (Kozell *et al.*, J. Biol. Chem. 269:30299-30306, 1994). D_2/D_1 chimeras were constructed that contained D_2 receptor sequence at the amino- and carboxyl-terminal ends, and D_1 receptor sequence in the intervening region. Chimeric receptors with D_2 sequence from transmembrane domain 7 to the carboxyl terminus together with D_2 receptor sequence from the amino terminus through transmembrane helix 4 ($D_{2[1-4,7]}$) and 5 ($D_{2[1-5,7]}$) bound [^{3}H]spiperone with high affinity, consistent with the hypothesis that D_2 receptor transmembrane region I or II is incompatible with D_1 receptor transmembrane region VII. $D_{2[1-4,7]}$ and $D_{2[1-5,7]}$ had affinities similar to D_1 and D_2 receptors for most non-selective dopamine antagonists, and had affinities for most of the selective antagonists that were intermediate between the parent receptors.

$D_{2[1-4,7]}$ and $D_{2[1-5,7]}$ mediated dopamine receptor agonist-induced stimulation and inhibition, respectively, of cyclic AMP accumulation. The more efficient coupling of $D_{2[1-5,7]}$ to inhibition of cyclic AMP accumulation, compared to the coupling of $D_{2[5-7]}$ and $D_{2[3-7]}$, supports the view that multiple D_2 receptor cytoplasmic domains acting in concert are necessary for receptor activation of G_i. In contrast, $D_{2[1-4,7]}$, which contains only one cytoplasmic loop (the third) from the D_1 receptor, is capable of activating G_s.

$D_{2[1-4,7]}$ exhibited several characteristics of a constitutively active receptor, including enhanced basal (unliganded) stimulation of cyclic AMP accumulation, high

affinity for agonists even in the presence of GTP, and blunted agonist-stimulated cyclic AMP accumulation. A number of dopamine receptor antagonists were inverse agonists at $D_{2[1\text{-}4,7]}$, inhibiting basal cyclic AMP accumulation. Some of these drugs were also inverse agonists at the D_1 receptor. Interestingly, several antagonists potentiated forskolin-stimulated cyclic AMP accumulation *via* $D_{2[1\text{-}5,7]}$, and also *via* the D_2 receptor, which could reflect inverse agonist inhibition of native constitutive activity of this receptor.

INTRODUCTION

The dopamine receptor family is comprised of D_1-like (D_1 and D_5) and D_2-like (D_{2L}, D_{2S}, D_3, and D_4) receptors (112). The D_1-like dopamine receptors have a shorter third intracellular loop (IC3) and a longer carboxyl terminus than the D_2-like receptors. D_1-like receptors have high affinity for benzazepine ligands, such as SCH 23390, whereas D_2-like receptors have high affinity for benzamide and butyrophenone ligands, such as sulpiride and spiperone. D_1-like dopamine receptors couple to the G protein G_s, stimulating adenylate cyclase, whereas the D_2-like receptors couple to G_i or G_o, inhibiting adenylate cyclase. D_1- and D_2-like receptors both regulate phosphoinositide turnover, and D_2-like dopamine receptors also modulate arachidonic acid release, Na^+/H^+ exchange, K^+ currents, and Ca^{2+} currents (56).

One approach to identifying 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_1 and D_2 receptors (17,73,75,76,113,114). Whereas point mutations can often 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_1/D_2 receptors has suggested that structural determinants of selective potency and efficacy can differ, with TMVI of the D_2 dopamine receptor implicated in selective efficacy of some agonists, and TMVII of D_1 and D_2

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_1 receptors, although inhibition of adenylate cyclase *via* D_2 receptors appears to require both the second intracellular loop (IC2) and IC3 (64,115).

It is often observed that the function of certain chimeric receptors is dramatically impaired due to incompatibilities between adjacent transmembrane regions (80). Such chimeras can be used to identify intramolecular interactions in the parent receptors (66,67). When we constructed chimeric receptors containing the D_2 receptor TMI and TMII, and D_1 receptor TMVII, the chimeras were non-functional (115). 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 (66-69), we hypothesized that incompatibilities between D_1 TMVII and D_2 TMI and TMII produced the non-functional state of these chimeric receptors. We now report that replacement of the D_1 TMVII in the non-functional chimeras with D_2 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 (116).

MATERIALS AND METHODS

Materials. [^{3}H]spiperone (80 Ci/mmol) was purchased from Amersham (Arlington Heights, IL), and [^{3}H]SCH 23390 (70 Ci/mmol) and [^{3}H]cyclic AMP (30 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). SCH 23390, SKF 38393, spiperone, chloro-APB (SKF 82958), 6-chloro-PB, quinpirole (LY17155), apomorphine, bromocriptine, lisuride and forskolin were purchased from Research Biochemicals International (Natick, MA). Dihydrexidine (Dr. Richard Mailman, University of North Carolina), epidepride (NCQ 219; Dr. Tomas de Paulis, Vanderbilt University), fenoldopam (SKF 82526; Dr. Richard Wilcox, University of Texas at Austin), and pergolide (Lilly) were generous gifts. Dopamine (3-hydroxytyramine), IBMX (isobutyl-methylxanthine), and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of chimeric receptor cDNAs. Eight chimeric cDNAs (previously referred to as CH1 to CH8, new nomenclature depicted in Fig. 1A 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_6 glioma cells, and characterization of these receptors was described previously (115). The D_1 TMVII and cytoplasmic tail was removed from $D_{2[1\text{-}2]}$ (CH5), $D_{2[1\text{-}4]}$ (CH6), and $D_{2[1\text{-}5]}$ (CH7) by digestion with *Cla*I and *Eco*RI and replaced with a fragment containing D_2 TMVII and cytoplasmic tail from $D_{2[7]}$ (CH4), creating $D_{2[1\text{-}2,7]}$, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$ (Fig 1C).

Expression of recombinant receptors. D_1, D_{2L}, and chimeric cDNAs ($D_{2[1\text{-}2,7]}$, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$) were stably expressed in HEK293 (human embryonic kidney)

cells by electroporation. HEK293 cells (2.2 x 10^7/ml) were re-suspended with the appropriate cDNA (15 μg) and pBabe Puro (2 μg), to confer resistance to puromycin (17), in DMEM supplemented with 10% calf bovine serum (CBS) 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 DMEM supplemented with 5% fetal bovine serum, 5% CBS, penicillin G (50 U/ml), and streptomycin (50 μg/ml) in a humidified incubator at 37°C in the presence of 10% CO_2. After 48 hr the medium was replaced with growth medium containing puromycin (2 μg/ml). Puromycin-resistant colonies were transferred to duplicate wells and tested for binding of D_1- and D_2-selective radioligands.

Radioligand binding assay. Confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na^+-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 x *g* for 20 min. The crude membrane fraction was resuspended in Tris-buffered saline with a Brinkmann Polytron homogenizer at setting 6 for 10 sec, and used for radioligand binding assays. For determinations of K_i values, the affinity of $D_{2[1\text{-}4,7]}$, $D_{2[1\text{-}5,7]}$, and D_2 receptors for ligands was assessed by inhibition of the binding of [^{3}H]spiperone, whereas the affinity of the D_1 receptor for ligands was quantified by inhibition of the binding of [^{3}H]SCH 23390. 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° C for 1 hour and terminated by filtration through glass fibre filters on a 96-well Tomtec cell harvester. The filters were dried prior to the addition of BetaPlate scintillation fluid, and radioactivity on the filters was determined with a Wallac 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 $MgCl_2$ before addition to assay tubes containing Tris-buffered saline and (final concentrations) 4 mM $MgCl_2$, 1 mM EDTA, 200 μM GTP, 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 μM) was assessed, the crude membrane fraction was added to assay tubes containing 20 mM HEPES pH 7.5, 6 mM $MgCl_2$, 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.

Cyclic AMP accumulation assay. Cells were plated at a density of approximately 100,000 cells/well in 48-well tissue culture clusters. After 2 to 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 to 400,000 cells/well at the time of assay. The cyclic AMP accumulation assay of adenylate

cyclase activity was carried out essentially as described (118). Cells were rinsed on ice two times with 200 µl assay buffer (Earle's balanced salt solution, containing 0.02% ascorbic acid, 2% CBS, and 500 µM IBMX) for 5 min followed by the addition of assay buffer with or without forskolin (10 µM final concentration) and appropriate drugs. Incubations were carried out at 37° C for 10 min, except for agonist stimulation and inverse agonist inhibition of $D_{2[1\text{-}4,7]}$, which was carried out at 40° C, 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 x g for 15 min and stored at 4° C for at least 1 hr before quantification of cyclic AMP.

Quantification of cyclic AMP. Cyclic AMP was quantified using a competitive binding assay (119) as described (118). Samples of the cell lysate from each well (5-20 µl) were added to duplicate assay tubes. [^{3}H]Cyclic AMP (~0.5 pmol) in cyclic AMP assay buffer (100 mM Tris-HCl, pH, 7.4, 100 mM NaCl, 5 mM EDTA) was added to each tube, followed by cyclic AMP-binding protein (100 µg of crude bovine adrenal extract in cyclic AMP assay buffer) for a final volume of 500 µL. 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 cyclic AMP concentration in each sample was estimated from a standard curve ranging from 0.1 to 100 pmol cyclic AMP.

Data analysis. Saturation isotherms, radioligand displacement curves, and dose-response curves for cyclic AMP accumulation were analyzed by nonlinear regression using the program GraphPAD Prism. K_i values are geometric means from three or more independent experiments ± the asymmetrical standard error of the mean. For dopamine, the goodness of fit for one- and two-site analyses was compared using an F test. When $P<0.05$ for the 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 (2-tailed) as indicated. The change in free energy of binding of a drug was calculated using the equation $\Delta G^o = -RT\ln(1/K_i)$ where ΔG^o is the change in free energy, R is the gas constant, T is temperature in degrees Kelvin, and K_i is the apparent affinity of the drug.

RESULTS AND DISCUSSION

To test the hypothesis that incompatible interactions between D_1 TMVII and D_2 TMI and TMII resulted in non-functional chimeric receptors, we modified $D_{2[1-2]}$, $D_{2[1-4]}$, and $D_{2[1-5]}$, (Fig. 3-1) so that 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 HEK293 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}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 ± 70 fmol/mg of membrane protein, with a K_D for [^{3}H]spiperone of 0.60 nM ± 0.04 nM (*n*

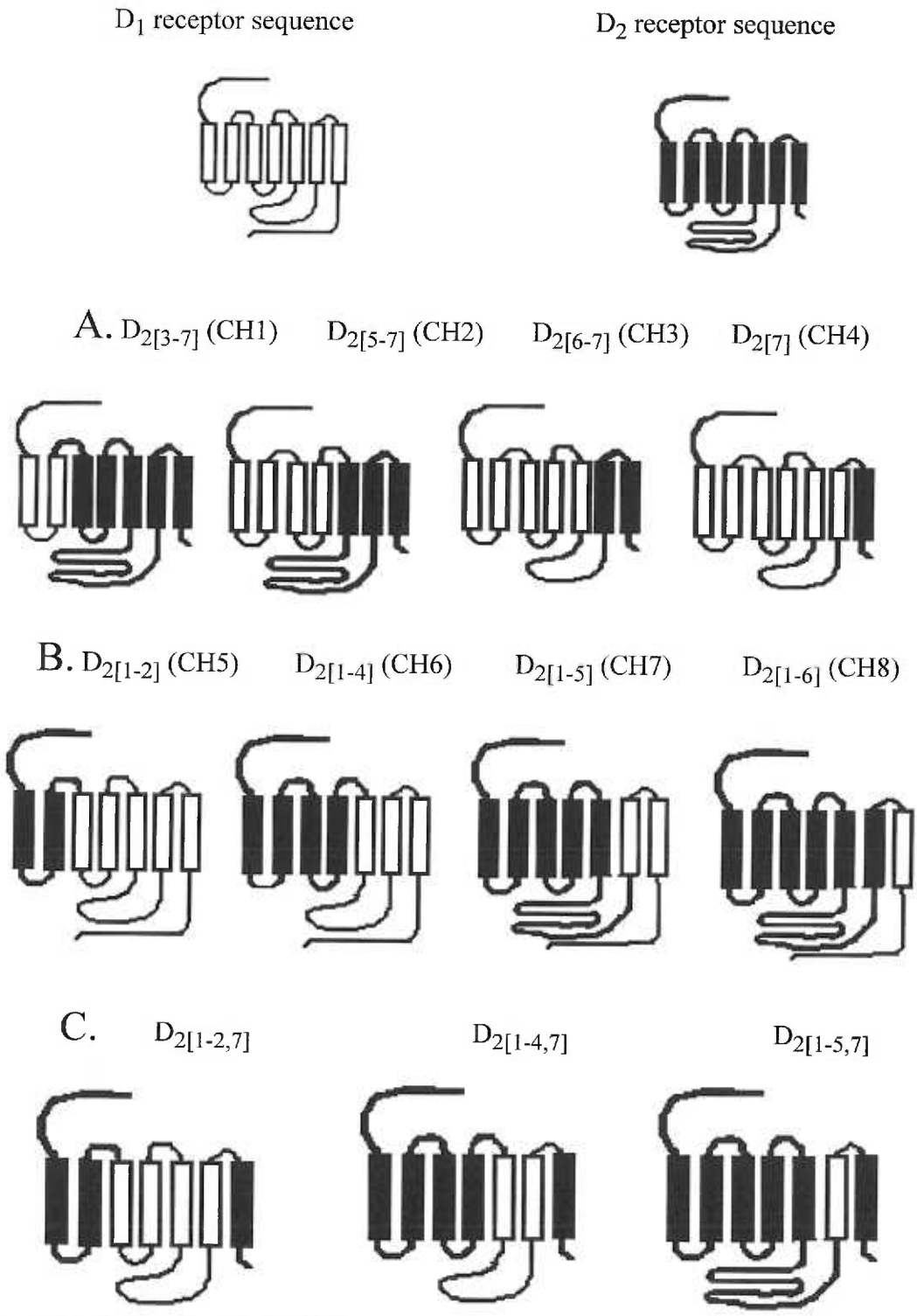


Figure 3-1 D_2/D_1 chimeric receptors.
Schematic representation of structure of the chimeric dopamine receptors. D_1 sequence is denoted by open rectangles for putative TM helices and thin lines for intracellular and extracellular hydrophilic regions. D_2 sequence is denoted by filled rectangles for putative TM helices and thick lines for hydrophilic regions.

= 11), the density of $D_{2[1\text{-}5,7]}$ was 300 ± 30 fmol/mg, with a K_D of 2.00 ± 0.33 nM (n = 13), and the density of D_2 receptors was 480 ± 30 fmol/mg, with a K_D of 0.071 ± 0.018 nM (n = 9). Although we were able to detect specific binding of [^{3}H]spiperone and [^{3}H]clozapine to $D_{2[1\text{-}2,7]}$, and specific binding of [^{3}H]SCH 23390 to $D_{2[1\text{-}2,7]}$, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$, the binding was of such low affinity that accurate estimates of K_D and B_{max} could not be obtained.

Three clonal lines of D_1 receptor-expressing HEK293 cells were used for these experiments. The density of D_1 receptors on the clones used to assess stimulation of adenylate cyclase was 2700 ± 490 fmol/mg (n = 9), and 1100 ± 90 fmol/mg (n = 4) of membrane protein.

Characterization of antagonist and agonist binding.

For catecholamine receptors, structural determinants of ligand binding appear to be primarily in the conserved α helical regions (120). Therefore, we analyzed the chimeras in terms of the number of transmembrane helices that were contributed by D_1 or D_2 receptors. As has been done previously (82,115) we calculated the change in the free energy of binding of a ligand mean ΔG^o 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_1 and D_2 receptors, then a plot of ΔG^o at each chimeric and wild-type receptor *versus* the number of transmembrane regions that are from the D_2 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

Table 3-1 Affinity of D_1, D_2, and chimeric dopamine receptors for antagonists

	K_i (nM)			
Drug	**D_1**	**$D_{2[1\text{-}4,7]}$**	**$D_{2[1\text{-}5,7]}$**	**D_2**
(+)-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)
SCH 23390	1.1 (0.9 - 1.3)	570 (340 - 930)	3,100 (2,900 - 3,400)	2,600 (2,000-3,400)

K_i values for inhibition by the indicated antagonists of the binding of [^{3}H]spiperone to chimeric and D_2 dopamine receptors, and binding of [^{3}H]SCH 23390 to D_1 receptors, determined as described under "Materials and Methods". Affinity values (in nM) represent geometric means from three or more independent experiments with the limits defined by the asymmetrical standard error of the mean given in parentheses.

was calculated for each drug. If the line drawn between ΔG^o for the drug at the wild-type receptors did not intersect the 95% confidence interval for the ΔG^o 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 ΔG^o of binding. A value of ΔG^o 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 non-selective for D_1 and D_2 dopamine receptors. (+)-Butaclamol had similar affinities for all four receptors (D_1, D_2, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$) (Table 3-1; Fig. 3-2A), but the affinity of *cis*-flupenthixol for $D_{2[1\text{-}5,7]}$ was significantly below the line drawn between wild-type receptors. The affinity of $D_{2[1\text{-}4,7]}$ for clozapine however, was significantly greater than the affinity of either wild-type receptor for the drug (Table 3-1).

Although the mean ΔG^o values for the binding of the D_2-selective antagonists epidepride, haloperidol, spiperone, and sulpiride were slightly below the line drawn between values for each drug at D_1 and D_2 dopamine receptors, only for the binding of spiperone and epidepride to $D_{2[1\text{-}5,7]}$ did the line not intersect the 95% confidence interval of the mean (Table 3-1; Fig. 3-2B). There was no difference in the affinity of $D_{2[1\text{-}4,7]}$ and $D_{2[1\text{-}5,7]}$ for these D_2 selective ligands.

The affinity of D_1-selective benzazepines, SCH 23390, SKF 38393 and chloro-PB, for $D_{2[1\text{-}4,7]}$ and $D_{2[1\text{-}5,7]}$ was assessed. The mean ΔG^o values were not significantly different from the line drawn between D_1 and D_2 receptors (Table 3-2; Fig. 3-3A). Also, the affinity of SCH 23390 and SKF 38393 for $D_{2[1\text{-}5,7]}$ and D_2 receptors was not significantly different. For another D_1-selective benzazepine, chloro-APB, the affinity of $D_{2[1\text{-}5,7]}$ was significantly less than predicted, whereas the potency of chloro-APB for

Table 3-2 Affinity of D_1, D_2, and chimeric dopamine receptors for agonists K_i (nM except where indicated)				
Drug	**D_1**	**$D_{2[1\text{-}4,7]}$**	**$D_{2[1\text{-}5,7]}$**	**D_2**
6,7 ADTN	15,000	41	500	600
	(12,000 - 20,000)	(38 - 46)	(310 - 820)	(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)
DHX (μM)	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 (μM)	31 (21 - 44)	0.52 (0.39-0.70)	20 (14 - 29)	13 (11-15)
Bromocriptine	1,400	6.1	9.5	2.2
	(900 - 1,900)	(4.8 - 7.8)	(9.3 - 9.7)	(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	25	29	85
	(1,500-2,800)	(19 - 34)	(27 - 31)	(67 - 110)
Fenoldopam	85	320	520	1,300
	(59 - 120)	(210 - 460)	(400 - 690)	(900 - 1,800)
Quinpirole(μM)	100	13	20	10
	(70 - 120)	(9 - 19)	(17 - 24)	(10 - 14)
6-Chloro-PB	58	800	850	4,800
	43 - 79)	(680 - 950)	(400 - 1400)	(3,600 - 6,500)
Chloro-APB	36	110	1.,900	1,700
	(33 - 38)	(110 - 120)	(1,700 - 2,000)	(1,600 - 1,800)
SKF 38393	0.53	10	20	21
(μM)	(0.34 - 0.84)	(0.8 - 1.4)	(11 - 36)	(18 - 24)

K_i values for inhibition by the indicated agonists of the binding of [^{3}H]spiperone to chimeric and D_2 receptors, and the binding of [^{3}H]SCH 23390 to D_1 receptors, determined as described under "Materials and Methods". All experiments were carried out in the presence of 200 μM GTP. Affinity values (nM except where indicated) represent geometric means from three or more independent experiments with the limits defined by the asymmetrical standard error of the mean given in parentheses.

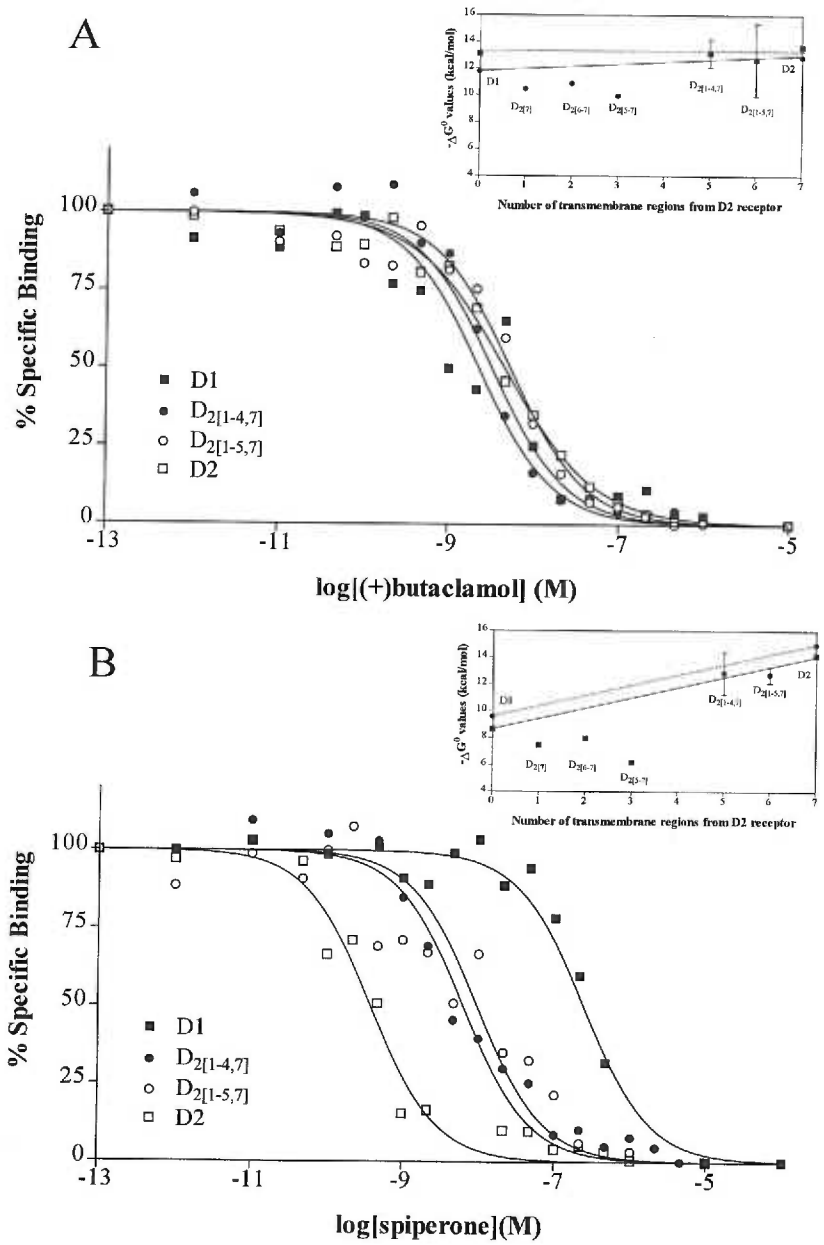


Figure 3-2 Binding of antagonists.

Representative 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. Inset: Averaged data from all experiments are expressed as the free energy change of binding, in kilocalories per mole, plotted* versus *the number of transmembrane regions from the D_2 receptor. The 95% confidence intervals are indicated by the error bars. Data points represented as* squares *are from our previous paper using chimeric and wildtype receptors expressed in C_6 glioma cells (115), and the* solid line *represents the line drawn between D_1 and D_2 receptor ΔG^o values from the earlier work. Data points represented as* circles, *and the* dotted line *drawn between D_1 and D_2 ΔG^o values, are from the current study using receptors expressed in HEK293 cells.*

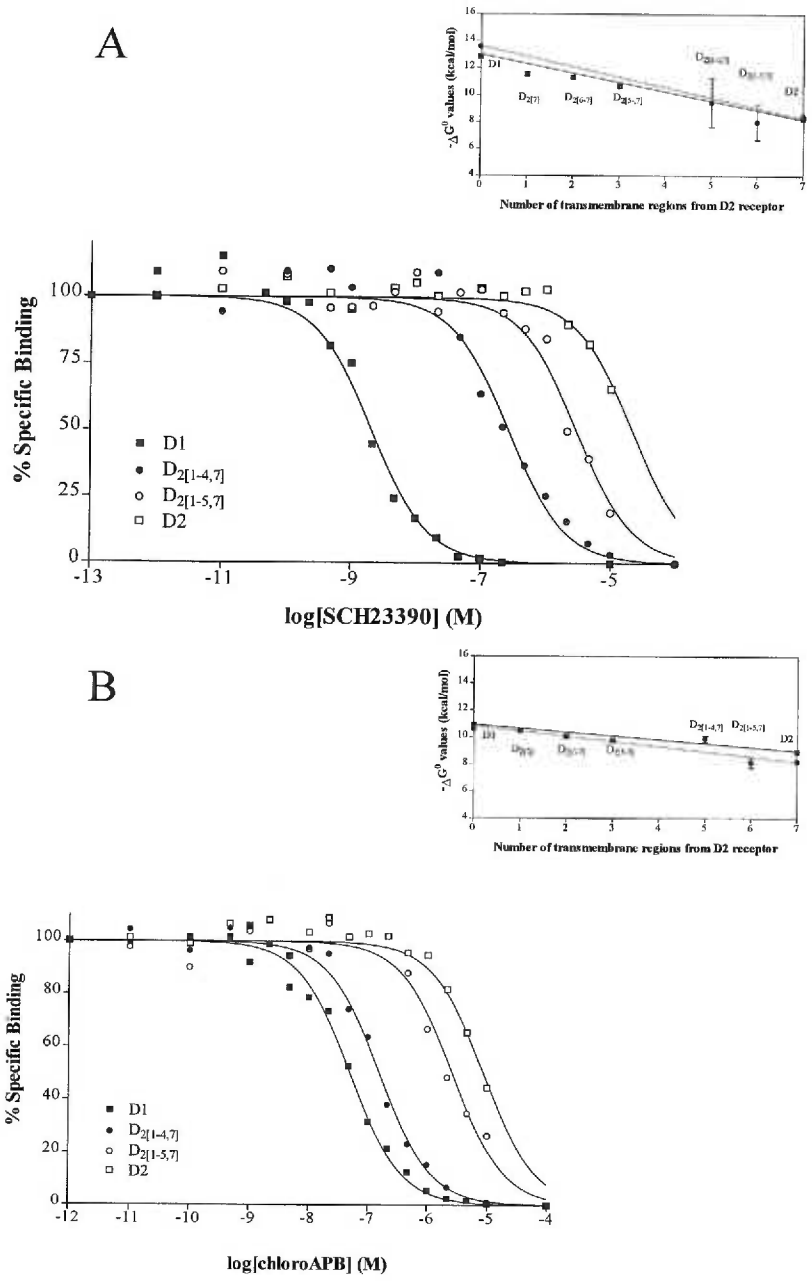


Figure 3-3 Binding of benzazepines.

Representative 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) SCH 23390, or (B) chloro-APB, in the presence of GTP. Inset: Averaged data from all experiments are expressed as free energy change of binding, in kilocalories per mole, plotted* versus *the number of transmembrane regions from the D_2 receptor. The 95% confidence intervals are indicated by the error bars. Data points represented as* squares *are from our previous paper using chimeric and wildtype receptors expressed in C_6 glioma cells (115), and the* solid line *represents the line drawn between D_1 and D_2 receptor ΔG^o values from the earlier work. Data points represented as* circles, *and the* dotted line *drawn between D_1 and D_2 ΔG^o values, are from the current study.*

Table 3-3 Affinity of D_1, D_2, and chimeric dopamine receptors for dopamine in the presence and absence of GTP K_i (μM)

	D_1	% *n*	$D_{2[1\text{-}4,7]}$	% *n*	$D_{2[1\text{-}5,7]}$	% *n*	D_2	% *n*
No GTP								
High	0.08	30	0.05	76	0.06	33	0.3	43
	(0.05-0.13)	*4*	(0.03-0.07)	*3*	(0.04-0.09)	*6*	(0.2 - 0.4)	*5*
Low	4.9	70	11	24	11	67	21	57
	(3.9-6.1)		(0.5 - 2.2)		(7.3-15)		(17-25)	
1 site	3.8	100	0.08	100				
w/o GTP	(3.1-4.7)	*4*	(0.07-0.09)	*3*				
200 μM GTP	5.3		0.12		9.9		7.3	
added	(4.0-7.0)	*6*	(0.09-0.14)	*4*	(7.6-13)	*6*	(5.7 - 9.4)	*6*

K_i values for the binding of [^{3}H]spiperone to chimeric and D_2 dopamine receptors, and binding of [^{3}H]SCH 23390 to D_1 receptors, determined as described under "Materials and Methods". Affinity values (μM) represent geometric means for the number of independent experiments indicated (n) with the limits defined by the asymmetrical standard error of the mean given in parentheses. The proportion of sites in the high and low affinity classes is also indicated (%).

$D_{2[1\text{-}4,7]}$ was higher than would be predicted by the line drawn between D_1 and D_2 receptors (Fig. 3-3B).

For most agonists, ΔG^o values for $D_{2[1\text{-}5,7]}$ were not significantly different from those predicted by the line drawn between D_1 and D_2 receptors. However, $D_{2[1\text{-}4,7]}$ receptors had significantly higher affinity for many dopamine agonists than did D_1 and D_2 receptors (Table 3-2). The affinity of $D_{2[1\text{-}4,7]}$ receptors was higher than either wild-type dopamine receptor for 6,7-ADTN, apomorphine, propylnorapomorphine (NPA), dihydrexidine (DHX), dopamine, lisuride, and pergolide. For every agonist tested, except the partial agonist SKF 38393, ΔG^o values for $D_{2[1\text{-}4,7]}$ were above the line drawn

between D_1 and D_2 receptors (Table 3-2; Fig. 3-4A and 3-B), indicating a higher than expected affinity of $D_{2[1\text{-}4,7]}$ for most agonists. Since one characteristic of constitutively active receptors is high affinity for agonists (116), these results suggested that $D_{2[1\text{-}4,7]}$ is constitutively active.

As an initial test of the hypothesis that $D_{2[1\text{-}4,7]}$ 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 towards the active conformation, resulting in high affinity binding of agonists even in the presence of GTP (116). We were able to detect both high (K_H) and low (K_L) affinity states in the absence of GTP for D_2, D_1, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$ receptors (Table 3-3; Fig. 3-5). However, for $D_{2[1\text{-}4,7]}$ more than 75% of the receptors were in a high affinity state, whereas for D_2, D_1, and $D_{2[1\text{-}5,7]}$ less than 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_1 and $D_{2[1\text{-}4,7]}$ receptors was not significantly improved by assuming the presence of two classes of binding sites. For the D_1 receptor, the K_i for dopamine when only one class of binding sites was detected was close to K_L 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 $D_{2[1\text{-}4,7]}$, the K_i was similar to the value for K_H (Table 3-3). Furthermore, the K_i for dopamine at $D_{2[1\text{-}4,7]}$ was unchanged by the addition of GTP, whereas for D_1, D_2, and

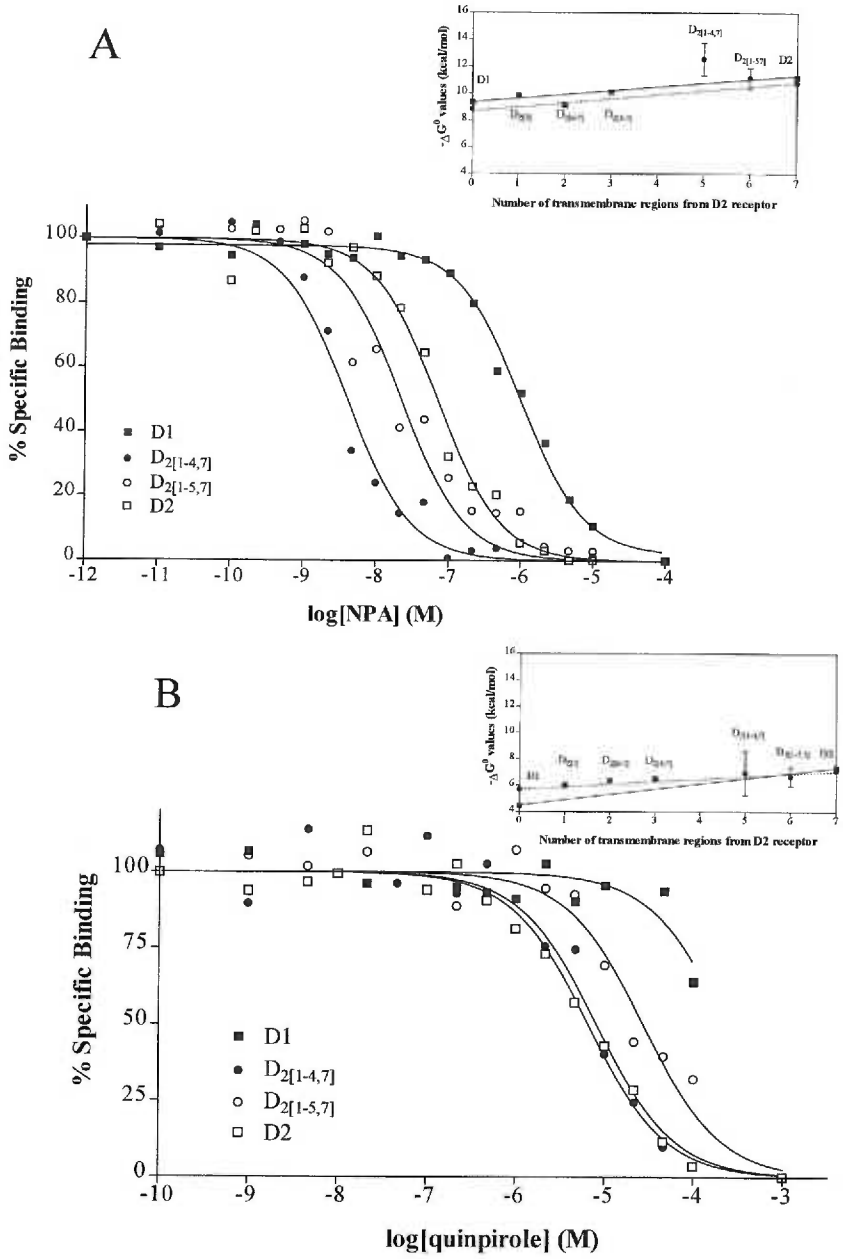


Figure 3-4 Binding of agonists.

Representative 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. Inset: Averaged data from all experiments are expressed as the free energy change of binding, in kilocalories per mole, plotted* versus *the number of transmembrane regions from the D_2 receptor. The 95% confidence intervals are indicated by the error bars. Data points represented as* squares *are from our previous paper using chimeric and wildtype receptors expressed in C_6 glioma cells (115), and the* solid line *represents the line drawn between D_1 and D_2 receptor ΔG^o values from the earlier work. Data points represented as* circles, *and the* dotted line *drawn between D_1 and D_2 ΔG^o values, are from the current study.*

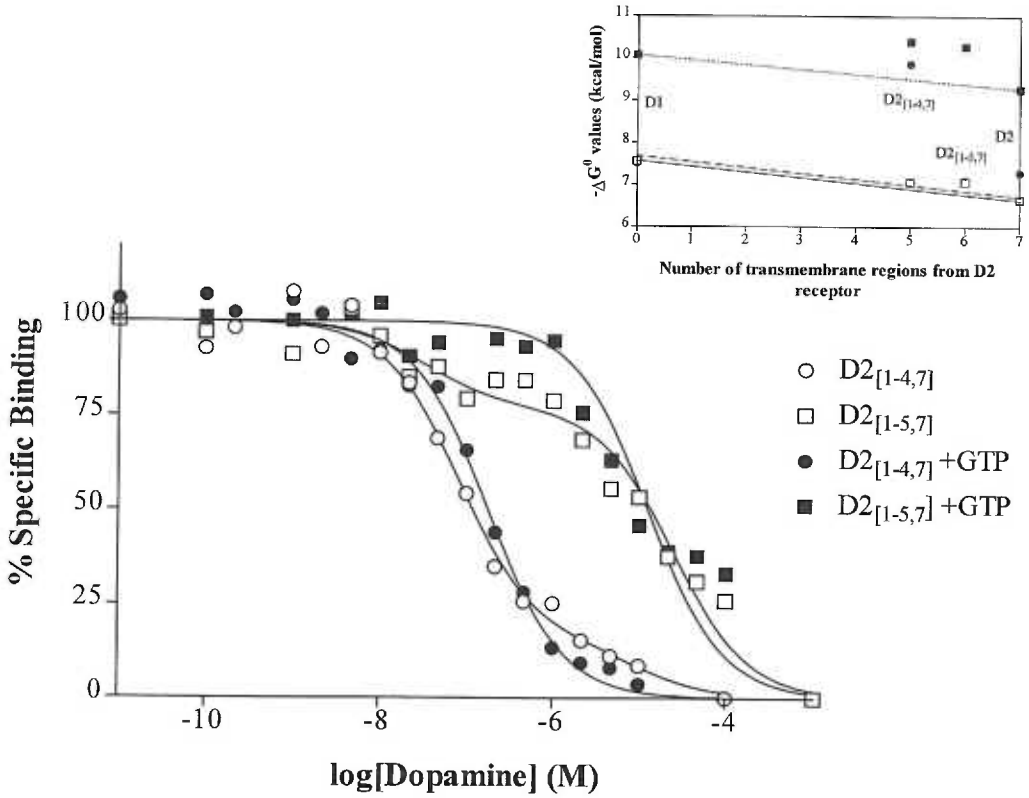


Figure 3-5 GTP-sensitivity of the binding of dopamine.
Representative data are shown from one of three or more independent experiments, in which inhibition of radioligand binding to $D_{2[1-4,7]}$ and $D_{2[1-5,7]}$ was determined for dopamine. Data are plotted as the percentage of the specific binding versus *the logarithm of the concentration of dopamine. Inset: Averaged data from all experiments are expressed as the free energy change of binding, in kilocalories per mole, plotted* versus *the number of transmembrane regions from the D_2 receptor. The filled squares represent ΔG^o values for K_H, and the open squares represent ΔG^o values for K_L, in the absence of GTP. The filled circles represent ΔG^o values in the presence of GTP. The solid line represents the line drawn between D_1 and D_2 receptor ΔG^o values in the presence of GTP. The dotted line represents the line drawn between high affinity states, while the dashed line represents the line drawn between low affinity states, of the D_1 and D_2 receptor ΔG^o values in the absence of GTP.*

$D_{2[1-5,7]}$ receptors, only the low affinity state K_L, could be detected in the presence of GTP.

Stimulation of adenylate cyclase *via* D_1 and $D_{2[1-4,7]}$ receptors.

Consistent with the hypothesis that $D_{2[1-4,7]}$ was constitutively active, cells that expressed $D_{2[1-4,7]}$ receptors had basal cyclic AMP levels that were significantly elevated

compared to non-transfected HEK293 cells, and also compared to cells expressing D_1, $D_{2[1\text{-}5,7]}$, and D_2 receptors (Table 3-4).

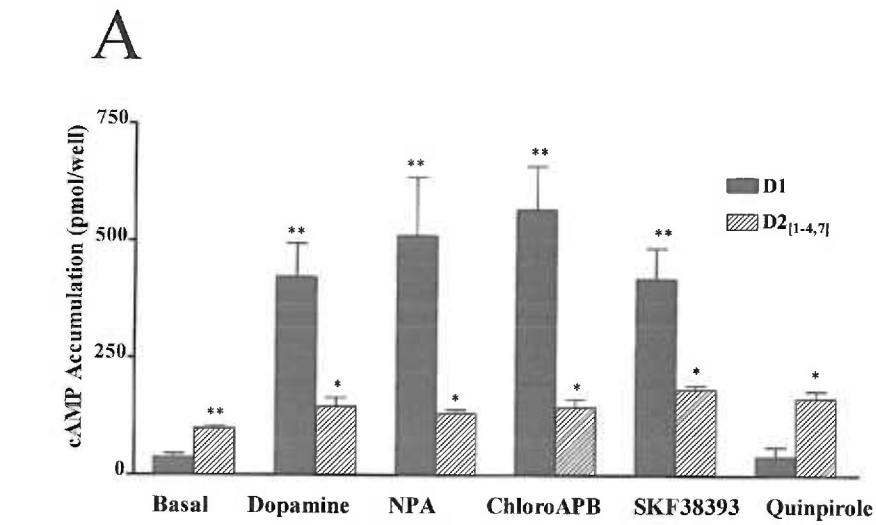
Interestingly, basal cyclic AMP accumulation in non-transfected HEK293 cells was significantly lower than in cells expressing D_1 receptors (Table 3-4), suggestive of a low level of unliganded activity by D_1 receptors expressed in HEK293 cells. Assays

Table 3-4 Basal levels of cAMP in cells not transfected or cells expressing D_1, D_2 and chimeric dopamine receptors

	cyclic AMP (pmoles per well)			
HEK293 cells *Assay at 37°C*	**D_1**	**$D_{2[1\text{-}4,7]}$**	**$D_{2[1\text{-}5,7]}$**	**D_2**
$4.3 \pm 1.1^{b,c}$	$28.6 \pm 3.2^{a,b}$	$101.5 \pm 8.8^{a,c}$	$6.4 \pm 1.0^{b,c}$	$6.3 \pm 1.0^{b,c}$
	Assay at 40°C			
	34.3 ± 6.2^{b}	100 ± 6.2^{c}		

Basal cAMP levels were determined as described under "Materials and Methods". Results shown are means from 10 or more independent experiments ± the standard error of the mean. [a]significantly different from non transfected HEK293 cells, $P<0.01$, [b]significantly different from $D_{2[1\text{-}4,7]}$, $P<0.01$, [c]significantly different from D_1, $P<0.02$

incubated at either 37° or 40° C resulted in similar levels of basal cyclic AMP accumulation (Table 3-4). Because agonist-stimulated accumulation of cyclic AMP was more consistent at 40° C for cells expressing $D_{2[1\text{-}4,7]}$ receptors, those assays were carried out at 40° C.



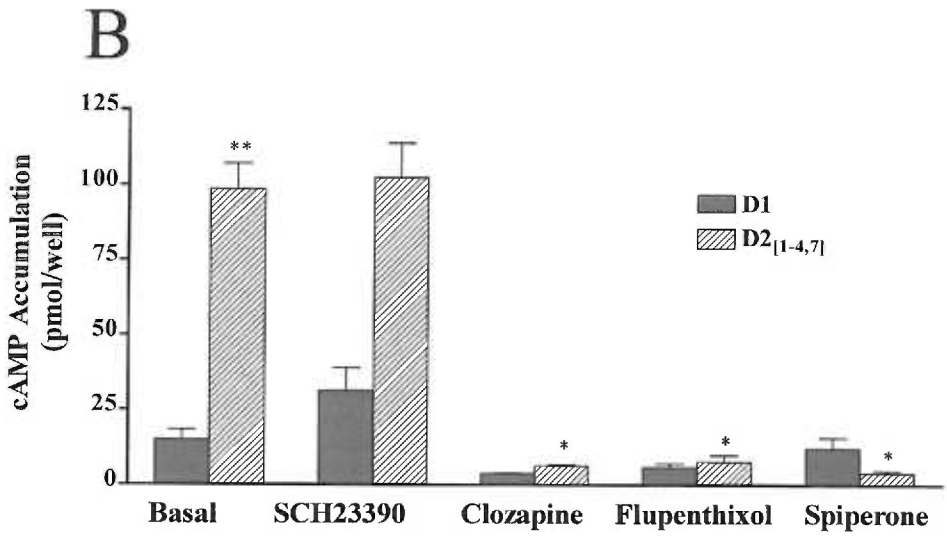


Figure 3-6 Modulation of cyclic AMP accumulation *via* D_1 and $D_{2[1-4,7]}$ receptors.
Data shown are the average ± the standard error of the mean from three or more independent experiments in which (A) stimulation by agonists (1 μM, except 10 μM quinpirole) or (B) inhibition by antagonists (1 μM), of cyclic AMP accumulation via *D_1 and $D_{2[1-4,7]}$ receptors was assessed. Data are expressed as pmoles per well. Assays were performed at 37° C, except for agonist stimulation and antagonist inhibition of $D_{2[1-4,7]}$ which was at 40° C. In other experiments, no stimulation of cyclic AMP accumulation* via *the D_1 receptor was observed in the presence of 100 μM quinpirole (data not shown).*

**P<0.05 compared to basal, Student's t-test for paired means.*

***P<0.05 compared to D_1 basal, Student's t-test for paired means.*

Most dopamine receptor agonists stimulated cyclic AMP accumulation *via* $D_{2[1-4,7]}$ and D_1 receptors (Fig. 3-6A). Apomorphine, NPA, chloro-APB, SKF 38393, dihydrexidine, lisuride, pergolide, and bromocriptine were as efficacious as dopamine via $D_{2[1-4,7]}$. Fenoldopam and chloro-PB did not stimulate cyclic AMP accumulation *via* $D_{2[1-4,7]}$, although both drugs are agonists at D_1 receptors. Interestingly, the D_2 receptor agonist quinpirole stimulated adenylate cyclase *via* $D_{2[1-4,7]}$, although quinpirole lacks efficacy at D_1 receptors. We were unable to detect stimulation of cyclic AMP accumulation by dopamine *via* $D_{2[1-2,7]}$.

Stimulation of adenylate cyclase *via* D_1 receptors resulted in cyclic AMP levels over 450 pmoles per well for many of the agonists tested, whereas agonist-mediated stimulation of adenylate cyclase *via* $D_{2[1-4,7]}$ receptors was blunted, resulting in maximal cyclic AMP levels that were only ~150 pmoles per well (Fig. 3-6A). To determine if the reduced ability to stimulate cyclic AMP accumulation was due to coupling of the D_2 cytoplasmic domains of $D_{2[1-4,7]}$ to $G_{i/o}$, we treated cells with pertussis toxin (25 ng/ml for 18-22 hr). Treatment with pertussis toxin elevated cyclic AMP accumulation stimulated by 1 μM dopamine from 137 ± 10 pmoles/well to 327 ± 43 pmoles/well (n = 3). The cyclic AMP accumulation stimulated by 10 μM forskolin in these cells was not altered by treatment with pertussis toxin (data not shown), indicating that the treatment did not nonspecifically 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 (121). A number of non-selective

antagonists and D_2-selective antagonists appeared to be inverse agonists at $D_{2[1\text{-}4,7]}$ receptors. (+)-Butaclamol, clozapine, epidepride, *cis*-flupenthixol, haloperidol, and spiperone decreased basal cyclic AMP levels in the absence of agonist by approximately 70% (Fig. 3-6B; data for (+)-butaclamol, epidepride, and *cis*-flupenthixol not shown). Inhibition of basal cyclic AMP accumulation by clozapine was not altered by pretreatment with pertussis toxin (data not shown). Although not statistically significant, there was a trend for non-selective antagonists, such as clozapine and haloperidol, to decrease basal cyclic AMP formation in cells expressing D_1 receptors. The D_1-selective antagonist, SCH 23390, did not inhibit basal levels of cyclic AMP formation at either D_1 or $D_{2[1\text{-}4,7]}$ receptors. There was a tendency for SCH 23390 to increase D1 receptor-mediated cyclic AMP formation, consistent with its reported weak partial agonist activity at D_1 receptors (122).

In other experiments, inhibition of forskolin-stimulated cyclic AMP accumulation by antagonists was assessed. Spiperone and clozapine both inhibited forskolin-stimulated cyclic AMP accumulation via $D_{2[1\text{-}4,7]}$ in a dose-dependent manner, with EC_{50} values of 1.9 ± 0.2 nM and 24 nM ± 10 nM, respectively (Fig. 3-7A). To confirm that some antagonists are inverse agonists at the wildtype 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 ± 24 nM, 61 ± 5 nM, and 540 ± 200 nM, respectively (Fig. 3-7B).

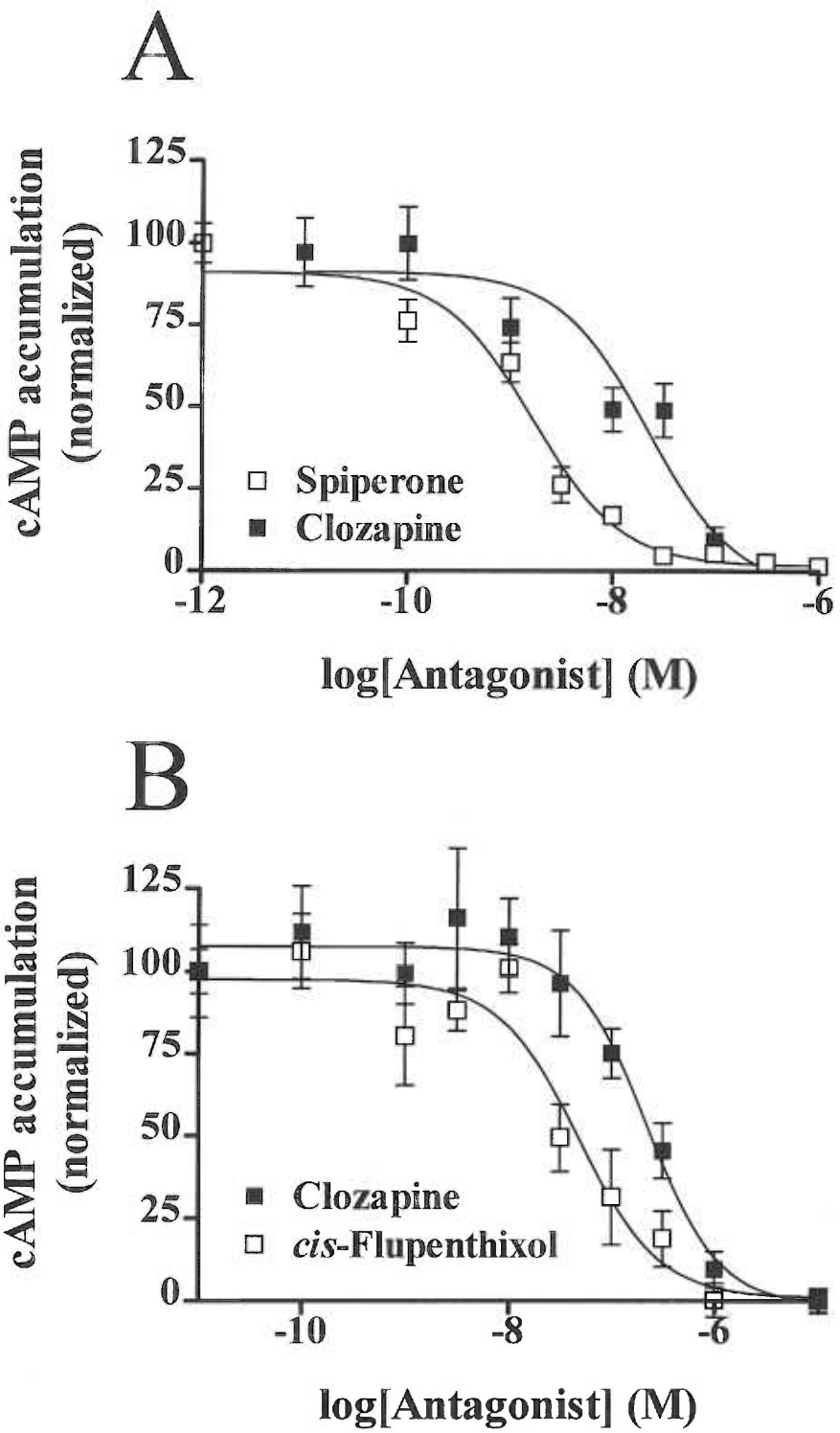


Figure 3-7 Inhibition of forskolin-stimulated cyclic AMP accumulation by inverse agonists. *Data shown are representative of four independent experiments in which inverse agonist inhibition cyclic AMP accumulation in the presence of 100 nM forskolin in cells expressing (A)* $D_{2[1\text{-}4,7]}$ *or (B)* D_1 *receptors was assessed. Data are plotted as a percentage of control cyclic AMP accumulation* versus *the logarithm of the concentration of antagonist.*

Agonist modulation of D_2 and $D_{2[1\text{-}5,7]}$ receptor-mediated cyclic AMP accumulation.

Inhibition of adenylate cyclase activity was assessed in cells expressing wild-type and chimeric receptors. The non-selective agonists dopamine, NPA, and apomorphine inhibited ~80% of forskolin-stimulated adenylate cyclase activity *via* the D_2 receptor, and 50-60% of activity *via* $D_{2[1\text{-}5,7]}$ (Fig. 3-8A; data for apomorphine not shown). Similarly, the D_2-selective agonists bromocriptine, lisuride, pergolide, and quinpirole inhibited adenylate cyclase activity *via* the D_2 receptor by 70-75%, and *via* $D_{2[1\text{-}5,7]}$ by 40 - 50% (Fig. 3-8A; data for pergolide and lisuride not shown). However, only one of the D_1-selective agonists tested, chloro-APB, inhibited adenylate cyclase activity *via* $D_{2[1\text{-}5,7]}$ (60%; Fig. 3-8A). At this chimera, other D_1-selective agonists (SKF 38393, 6-chloro-PB dihydrexidine, and fenoldopam) either had no effect or potentiated forskolin stimulation of cyclic AMP (Fig. 3-8B). SKF 38393, dihydrexidine, and fenoldopam inhibited adenylate cyclase *via* D_2 receptors by about 50%, whereas 6-chloro-PB did not inhibit adenylate cyclase. While 6-chloro-PB was an agonist at D_1 receptors, it appeared to be an antagonist or an inverse agonist at D_2, $D_{2[1\text{-}4,7]}$, and $D_{2[1\text{-}5,7]}$ receptors.

Inverse agonism at $D_{2[1\text{-}5,7]}$ and D_2 receptors.

We also evaluated the ability of antagonists to act as inverse agonists at D_2 receptors. The presence of (+)-butaclamol, epidepride, *cis*-flupenthixol, or haloperidol more than doubled forskolin-stimulated cyclic AMP accumulation in cells expressing D_2 receptors (Fig. 3-8; data for (+)-butaclamol not shown). Clozapine, spiperone, and SCH 23390 produced a more modest potentiation of forskolin-stimulated cAMP accumulation,

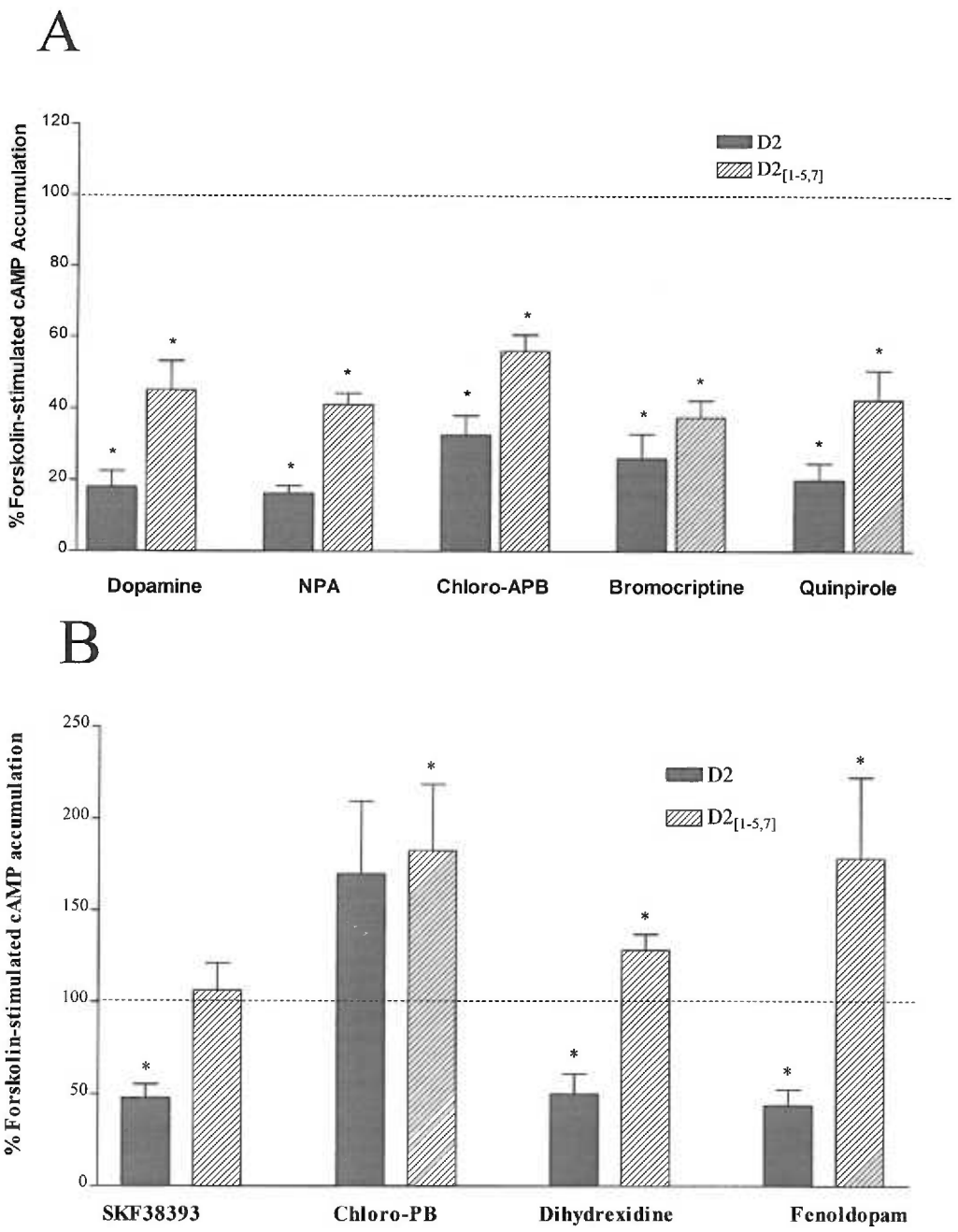


Figure 3-8 Modulation of cyclic AMP accumulation *via* D_2 and $D_{2[1\text{-}5,7]}$ receptors.

The data shown are the average ± the standard error of the mean of four or more independent experiments in which (A) inhibition or (B) potentiation of forskolin-stimulated (10 μM) adenylate cyclase activity by agonists (1 μM) was assessed. Data are expressed as a percentage of forskolin-stimulated cyclic AMP accumulation in the absence of receptor agonist.

**P<0.05 compared to control, Student's t-test for paired means.*

about 40% over control values. Because the binding profile of $D_{2[1\text{-}5,7]}$ was similar to D_2 receptors for most antagonists, we also evaluated the effect of antagonists on forskolin-stimulated cyclic AMP accumulation in cells expressing $D_{2[1\text{-}5,7]}$ receptors. Epidepride, *cis*-flupenthixol, and haloperidol significantly increased forskolin-stimulated cyclic AMP accumulation in cells expressing $D_{2[1\text{-}5,7]}$ by 40-50% (Fig. 3-9). Antagonists had no effect on forskolin-stimulated cyclic AMP accumulation in untransfected HEK293 cells (data not shown).

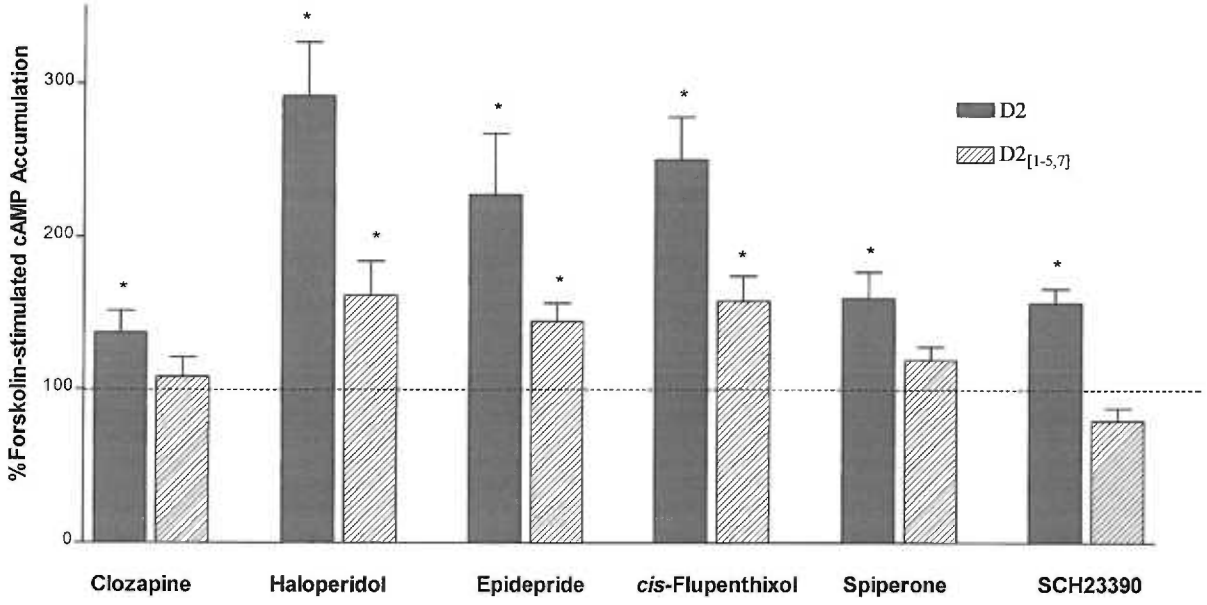


Figure 3-9 Potentiation of forskolin-stimulated adenylate cyclase activity by antagonists.
Data shown are the average ± the standard error of the mean, of four or more independent experiments in which potentiation of forskolin-stimulated (10 μM) adenylate cyclase activity by antagonists (1 μM) was assessed. Data are expressed as a percentage of forskolin-stimulated cyclic AMP accumulation in the absence of antagonist.

**P<0.05 compared to control, Student's t-test for paired means.*

DISCUSSION

We described previously the construction of eight chimeric receptors. Four D_1/D_2 chimeras were functional (Ref. 115; Fig. 1A), whereas reciprocal D_2/D_1 chimeras (Fig. 1B) were not, although the D_2/D_1 chimeric receptors did appear to be expressed in the cells, as assessed by the presence of mRNA. We hypothesized that the chimeric receptors were non-functional due to incompatible interactions between D_1 TMVII and D_2 TMI and TMII, regions which other mutagenesis and modeling data have suggested to be adjacent (66-68). Specifically, amino acid residues in TMII of the serotonin 5-HT_{2A}, gonadotropin-releasing hormone (GnRH), and adrenergic receptors, and TMI of the muscarinic m2 and m5 receptors, interact with amino acid residues in TMVII (66,67,69). For example, mutation of Asn^{87} in TMII to Asp in the GnRH receptor resulted in a non-functional receptor with no detectable binding of ligands, whereas addition of the reciprocal mutation of Asp^{318} in TMVII to Asn restored function to the mutant receptor (69). Also, mutation of Asn^{312} in β_2-adrenergic receptors to the amino acid residue found in the homologous domain of the α_2 adrenergic receptor (Phe) resulted in a nonfunctional protein, whereas replacement of TMI and TMII of the β_2- adrenergic receptor mutant with α_2 adrenergic receptor sequence restored function to the mutant receptor (66). These findings suggest that the low affinity of $D_{2[5-7]}$, $D_{2[6-7]}$, and $D_{2[7]}$ for a number of ligands (115), and the lack of detectable antagonist binding to $D_{2[1-2]}$, $D_{2[1-4]}$, $D_{2[1-5]}$, and $D_{2[1-6]}$ is 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 $D_{2[1-4,7]}$ and $D_{2[1-5,7]}$ 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 $D_{2[1-2]}$, $D_{2[1-4]}$, $D_{2[1-5]}$, and $D_{2[1-6]}$, and perhaps also the low affinity of $D_{2[5-7]}$, $D_{2[6-7]}$, and $D_{2[7]}$ for some ligands, but it is difficult to make firm conclusions regarding other effects, such as the high affinity of $D_{2[1-4,7]}$ and $D_{2[1-5,7]}$ for clozapine, or the low affinity of $D_{2[1-2,7]}$ for the radioligands tested. One finding that was consistent for all the chimeras tested in these experiments and previously (115) is that TMVI of the D_1 receptor apparently contributes little to the selective binding of most benzazepine ligands.

These difficulties of interpretation also apply to functional studies, where 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 (115), such as the ability of $D_{2[1-4,7]}$ and $D_{2[6-7]}$ and the inability of $D_{2[1-5,7]}$ and $D_{2[5-7]}$ to stimulate adenylate cyclase, demonstrate consistently that the third cytoplasmic loop of D_1 receptors is necessary and sufficient for coupling to G_s. Furthermore, the lack of inhibition of isoproterenol-stimulated cyclic AMP accumulation by $D_{2[5-7]}$, and the modest inhibition by $D_{2[3-7]}$, indicated that both IC2 and IC3 from the D_2 receptor are required for inhibition of adenylate cyclase (9). Dopamine activation of $D_{2[3-7]}$ receptors only inhibits cyclic AMP accumulation by ~

20%, but dopamine was able to cause ~ 60% inhibition *via* the $D_{2[1\text{-}5,7]}$ receptor. These results suggest that the first cytoplasmic loop (IC1) of the D_2 receptor is also important for inhibition of adenylate cyclase activity *via* the D_2 receptor. If all three cytoplasmic loops are involved in coupling to G proteins that inhibit adenylate cyclase, it might be predicted that $D_{2[1\text{-}4,7]}$ would activate both $G_{i/o}$ and G_s. Consistent with this, pertussis toxin treatment enhanced stimulation of adenylate cyclase by $D_{2[1\text{-}4,7]}$.

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 (116). 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_1 and D_5 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 (122). Constitutive activation of a receptor describes any manipulation that tends to increase the formation of R* in the absence of agonist. In the present study, basal levels of cyclic AMP accumulation were four-fold higher in HEK293 cells expressing $D_{2[1\text{-}4,7]}$ than in HEK293 cells expressing the wildtype D_1 receptor. Basal cyclic AMP accumulation was in turn higher in HEK293 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 $D_{2[1\text{-}4,7]}$ 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 cyclic AMP in cells expressing the $D_{2[1\text{-}4,7]}$ receptor, other characteristics of constitutively active G protein-coupled receptors, including increased affinity for agonists, attenuation of agonist-induced second messenger signalling, and the ability of inverse agonists to block basal second messenger generation (116,121), were observed.

Whereas the affinity of most agonists for $D_{2[1\text{-}5,7]}$ was approximately what would be expected if all transmembrane regions contribute equally to ligand binding, the affinity of most agonists for $D_{2[1\text{-}4,7]}$ 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* $D_{2[1\text{-}4,7]}$ receptors was only about 30% of the maximal stimulation *via* the D_1 receptor. Some of the reduced responsiveness was apparently due to coupling of $D_{2[1\text{-}4,7]}$ to both $G_{i/o}$ and G_s, since pertussis toxin treatment more than doubled the stimulation of cyclic AMP 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 β_2-adrenergic receptor (123) and the α_2-adrenergic

receptor (124). On the other hand, since $D_{2[1\text{-}4,7]}$ 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 cyclic AMP accumulation *via* $D_{2[6\text{-}7]}$ and $D_{2[7]}$ receptors was also attenuated compared to the D_1 receptor (115).

Every D_1 or D_2 receptor antagonist tested at the $D_{2[1\text{-}4,7]}$ receptor, with the exception of SCH 23390, inhibited basal cyclic AMP accumulation. In addition, clozapine and spiperone both caused dose-dependent decreases in forskolin-stimulated cyclic AMP accumulation, with EC_{50} values similar to their affinities at $D_{2[1\text{-}4,7]}$. 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 inhibit basal cyclic AMP accumulation in HEK293 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 (122).

A mutant D_1 receptor (L286A) with enhanced unliganded stimulation of adenylate cyclase activity was recently described (114), but the characteristics of this receptor differ in several respects from the constitutively active chimeric receptor, $D_{2[1\text{-}4,7]}$. In particular, the affinity of L286A (1.2 μM) for dopamine was similar to the affinity of the wild-type D_1 receptor (1.9 μM). The sensitivity to GTP of high-affinity agonist binding, and the ability of antagonists to inhibit basal cyclic AMP accumulation *via* L268A, was not assessed.

It has been proposed that the structural instability of a mutant β_2–adrenergic receptor confers upon the receptor its constitutive activity, since it is able to isomerize more readily between the R and R* conformations (27). The constitutive activity of $D_{2[1-4,7]}$ is consistent with this interpretation, and could be due to a global effect on helix packing, as proposed for a chimeric m2/m5 muscarinic receptor that constitutively activates G_q (125). Since the IC3 (109) and peptides derived from the IC3 of G protein-coupled receptors (126-129) 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 (130).

We also observed evidence for unliganded activity of the wild-type D_2 receptor. All the antagonists that we tested enhanced forskolin-stimulated cyclic AMP accumulation in HEK293 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 cyclic AMP 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 present study (480 fmol/mg) is similar to the density of D_2 receptors in brain regions where the receptors are expressed most abundantly (131).

In summary, restoration of function in D_2/D_1 chimeric receptors that have D_2 receptor sequence in TMI, II, and VII 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 containing 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 cyclic AMP accumulation.

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IV. DETERMINANTS OF DESENSITIZATION AND DOWN REGULATION OF THE D_1 DOPAMINE RECEPTOR

ABSTRACT

Agonist-mediated regulation of receptor response, or desensitization, occurs rapidly and is characterized by phosphorylation of the receptor, and a loss in high affinity (K_H) binding sites, but no change in receptor number. When the agonist treatment is more prolonged reductions in receptor density, or down regulation, occurs. Phosphorylation of D_1 receptors is potentially mediated by two different kinase families, cyclic AMP dependent protein kinase (PKA), and G protein-coupled receptor kinases (GRKs). To assess the role of phosphorylation *via* PKA in desensitization and down regulation of the D_1 dopamine receptor, we mutated potential PKA phosphorylation sites to alanine.

The potency of dopamine for stimulating adenylate cyclase *via* wildtype D_1 receptors was decreased following two hour treatment with 100 nM dopamine, but this treatment did not decrease the potency of dopamine at two mutant D_1 receptors, T136A and S380A, suggesting the inability to phosphorylate the D_1 receptor at threonine 136 and serine 380 results in the inability of agonist treatment to effect agonist potency. Mutation of five potential PKA phosphorylation sites did not diminish down regulation of the D_1 receptor. However, one mutant D_1 receptor, S127A, was down regulated more quickly than the wildtype D_1 receptor by 1 μM dopamine. Interestingly, both S127A and S380A did not appear to uncouple from G proteins following agonist pretreatment, thus implicating phosphorylation of serine 127 and serine 380 in the uncoupling of G proteins from the D_1 dopamine receptor.

INTRODUCTION

A decreased responsiveness of cells to continuous external stimuli appears to be a mechanism for maintaining cellular homeostasis. Among the G protein-coupled receptors, this decrease in responsiveness following prolonged agonist application is most prominent for receptors that couple to G_s, such as the D_1 dopamine and β_2-adrenergic receptors, mediating stimulation of adenylate cyclase (133). The mechanisms involved in agonist-induced changes in responsiveness of β-adrenergic receptors (β-adrenergic receptors refers only to β_1- and β_2-adrenergic receptors) have been studied in detail, and the β-adrenergic receptors have been used as a prototype for examining common mechanisms for agonist-induced changes in other receptor types.

Continuous agonist treatment of cultured cells expressing β-adrenergic receptors attenuates stimulation of adenylate cyclase. This desensitization can be either homologous, where agonist stimulation of β_2-adrenergic receptors decreases responsiveness only at β_2-adrenergic receptors, or heterologous, where agonist stimulation at another receptor type results in decreased responsiveness of β_2-adrenergic receptors. Homologous and heterologous desensitization both occur following phosphorylation of the β-adrenergic receptor. Phosphorylation of the receptor is mediated by two receptor kinase families, cyclic AMP-dependent protein kinase (PKA) and G protein-coupled receptor kinases (GRKs) (88-90), which include both β-adrenergic receptor kinases, βARK1 and βARK2. Rapid desensitization of β-adrenergic receptors (occurring within a few minutes) is thought to involve phosphorylation by GRKs. The phosphorylation appears to increase the affinity of β-arrestin for the receptor. The

binding of β-arrestin to the phosphorylated receptor inhibits receptor-G_s interactions and functionally uncouples the receptor. A slower (minutes to hours) component to desensitization of β-adrenergic receptors is thought to be mediated by PKA phosphorylation of the receptor. This slower component of desensitization also results in functional uncoupling of β-adrenergic receptor, although β-arrestin does not appear to be involved (133).

Phosphorylation by PKA and GRKs diminishes β-adrenergic receptor activation of adenylate cyclase by decreasing the potency of agonists for stimulation of adenylate cyclase and by decreasing the maximum response generated with agonist treatment. Low agonist concentrations (nM) stimulate phosphorylation by PKA (88,134,135) reducing the potency of the agonist, whereas high agonist concentrations (μM) stimulate phosphorylation by both PKA and GRKs (90,134), reducing both the potency and efficacy of the agonist.

Agonist pretreatment of endogenous or recombinant D_1 dopamine receptors also results in desensitization (57,85-87). Desensitization is associated with phosphorylation (92) of the D_1 receptor , and a loss in the percentage of high affinity agonist binding sites (57), without a change in the receptor density (57,136). Some studies suggest that PKA phosphorylation is involved in desensitization (57,94) whereas other studies suggest that desensitization of the receptor is independent of PKA activation (86,87), therefore the role of phosphorylation by PKA has not been elucidated for the D_1 dopamine receptor.

More prolonged treatment of cells or tissue expressing β-adrenergic or D_1 dopamine receptors (hours to days) leads to a decrease in receptor number, or down

regulation (137). Down regulation of the β-adrenergic receptor is at least partially mediated by PKA phosphorylation (46,95). Both β_1 and β_2-adrenergic receptors have potential PKA phosphorylation sites (R-R/K-X-S/T), in the carboxyl-terminal region of the third cytoplasmic loop and in the cytoplasmic tail (90,138). Phosphorylation by PKA has also been implicated in down regulation of the D_1 dopamine receptor (87). Both rhesus macaque and human D_1 dopamine receptors have two good potential PKA phosphorylation sites, T136 in the second cytoplasmic loop (IC2), and T268 in the third cytoplasmic loop (IC3). There are also several sites which have lower potential as PKA phosphorylation sites (R-$X_{(1-2)}$ S/T), S127 in IC2, S263 in IC3, and S380 in the carboxyl tail. At least one of these sites, S380, is phosphorylated in vitro by PKA, as assessed by the phosphorylation of a fusion protein containing the amino acids 372 - 442 of the D_1 receptor cytoplasmic tail (139).

The density of D_1 receptors expressed in C_6 cells is maximally down regulated following four hour agonist treatment (60). For D_2 receptors expressed in the same cell line, agonist treatment increases the density of receptors but it takes at least 14 hours for the up regulation to be maximal (64; See Appendix A). Also, the modulation of the density of the D_2 receptors requires higher concentrations (100 μM *versus* 10 μM) of dopamine than modulation of D_1 receptor density (64). Because of these differences in the regulation of D_1 and D_2 receptors, the assessment of the regulation of three D_1/D_2 chimeric receptors was potentially very informative. The chimeric receptors ($D_{2[5-7]}$, $D_{2[6-7]}$, and $D_{2[7]}$) have the first four, five and six transmembrane domains (TMs) from the D_1 receptor, with D_2 sequence in the carboxyl tail region. The affinity of dopamine

ligands and the ability of these receptors to couple to adenylate cyclase has been described previously (115, Chapter 2).

To assess the role of phosphorylation *via* PKA in desensitization and down regulation of the D_1 receptor, we constructed five mutant D_1 dopamine receptors in which single potential PKA phosphorylation sites (serine or threonine), were mutated to alanine. We also assessed the role of D_1 and D_2 TM regions in subtype specific modulation of receptor density using D_1/D_2 chimeric receptors.

MATERIALS AND METHODS

Materials. [^{3}H]SCH 23390 (80 Ci/mmol), [^{3}H]spiperone (80 Ci/mmol), and [^{3}H]cyclic AMP (30 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). Dopamine (3-hydroxytyramine), IBMX (3-isobutyl-methylxanthine), quinpirole, NPA, and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction, expression and characterization of mutant and chimeric receptor cDNAs. Three chimeric cDNAs (previously referred to as CH2 to CH4, new nomenclature $D_{2[1\text{-}2,7]}$, $D_{2[1\text{-}4,7]}$, $D_{2[1\text{-}5,7]}$) were constructed as described previously (115). I mutated four single potential protein kinase A (PKA) phophorylation sites using trans-polymerase chain reaction. The mutant cDNA were cloned into *Pst*I and *Sph*I sites of pcDNA-1 (S127A, T136A, S263A, T268A). Mutation of the last mutant D_1 receptor was performed by Minh Vu. S380A cDNA was cloned into pcDNA-3. The expression

and characterization of the mutant and chimeric cDNAs in C_6 glioma cells was done as described previously (115).

Radioligand binding assay. Confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na^+-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 x g for 20 min. The crude membrane fraction was resuspended in Tris-buffered saline with a Brinkmann Polytron homogenizer at setting 6 for 10 sec, and used for radioligand binding assays. For assays in which down regulation of receptor density by dopamine was assessed, the medium was replaced with DMEM containing 3% fetal bovine serum (FBS), 2% fetal calf serum (FCS), and 0.025% ascorbic acid with or without 1 μM dopamine. The plates were incubated in a 37° C incubator for one, two, or four hours before rinsing them two times with 10 mL of DMEM containing 3% FBS and 2% FCS for 5 minutes each rinse. Following the rinse, the cells were lysed by replacing the medium with ice-cold hypotonic buffer and then frozen at -70° C until the radioligand binding assay was performed. The cells were thawed, scraped into centrifuge tubes, and centrifuged, and the crude membrane fraction was resuspended and added to tubes as indicated above.

For competition binding studies in which dopamine displacement of binding in the absence of GTP was assessed, the plates were pretreated and rinsed in the same manner as the plates assessed for down regulation. Following the rinse, the cells were lysed by replacing the medium with ice-cold hypotonic buffer and then frozen at -70° C until the competition binding assay was performed. The plates were thawed, scraped off

the plate and centrifuged at 24,000 x g for 20 min. The crude membrane fraction was resuspended, incubated at 37° C for 15 to 20 min, re-centrifuged and resuspended a second time. The resulting crude membrane fraction was added to assay tubes containing 20 mM HEPES pH 7.5, with 6 mM $MgCl_2$, 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.

Cyclic AMP accumulation assay. Cells were plated at a density which resulted in confluency of the cells in 48 well clusters in three to four days. When the cells were confluent, the plates were used for assays in which desensitization of agonist-mediated adenylate cyclase stimulation was assessed. The medium was replaced with DMEM containing 0.02% ascorbic acid in the absence or presence of various concentrations of dopamine and the cells were placed in a 37° C incubator for one, two, or four hours. The cells were rinsed two times with 200 µl Earle's balanced salt solution (EBSS) for a total of less than 6 minutes, followed by the addition of assay buffer and appropriate concentrations of dopamine. Incubations were carried out at 37° C for 10 min. 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 x g for 15 min and stored at 4° C for at least 1 hr before quantification of cyclic AMP.

Quantification of cyclic AMP. Cyclic AMP was quantified using a competitive binding assay (119) as described (118). Samples of the cell lysate from each well (10 µl) were added to duplicate assay tubes. [^{3}H]Cyclic AMP (~0.5 pmol) in cyclic AMP assay

buffer (100 mM Tris-HCl, pH, 7.4, 100 mM NaCl, 5 mM EDTA) was added to each tube, followed by cyclic AMP-binding protein (100 μg of crude bovine adrenal extract in cyclic AMP assay buffer) for a final volume of 500 μL. The reaction tubes were incubated on ice for 2 to 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 cyclic AMP concentration in each sample was estimated from a standard curve ranging from 0.1 to 100 pmol cyclic AMP.

Data analysis. Saturation isotherms, radioligand displacement curves, and dose-response curves for cyclic AMP accumulation were analyzed by nonlinear regression using the program GraphPAD Prism. K_i values are geometric means from three or more independent experiments ± the asymmetrical standard error of the mean. For displacement of radioligand binding by dopamine, the goodness of fit for one- and two-site analyses was compared using an F test. When $P<0.05$ for the improvement of the fit assuming two classes of binding sites, data were analyzed in terms of two classes of binding sites. One way analysis of variance along with Dunnett's test was used to analyze whether the response, to dopamine stimulation of adenylate cyclase activity, of the mutant receptors differed from wildtype D_1 receptors, following agonist treatment. Student's t-tests for paired means were also used to analyze the effects of agonist treatment on the efficacy and potency of dopamine for stimulating cAMP accumulation in cells expressing wildtype and mutant D_1 receptors.

RESULTS

Following the construction of five D_1 dopamine receptors, S127A, T136A, S263A, T268A, and S380A, in which single potential phosphorylation sites (serine or threonine) were mutated to alanine, we assessed whether the affinity of the mutant receptors for the prototypic D_1 antagonist, SCH 23390 was changed. The K_D for the radioligand [^{3}H]SCH 23390 was not altered by any of the mutations, and the density of mutant and D_1 receptors expressed in C_6 glioma cells ranged from 140 to 700 fmol/mg protein (Table 4-1).

Table 4-1 D_1 and mutant D_1 receptor density and K_D values

Receptor	B_{max} (fmol/mg protein)	average K_D (nM)
D1	611 ± 71	0.33
S127A	264 ± 42	0.29
T136A	704 ± 89	0.31
S263A	140 ± 51	0.32
T268A	407 ± 59	0.30
S380A	231 ± 24	0.32

Comparison of D_1 wild-type and mutant K_D values and receptor densities from radioligand binding isotherms. There was no significant difference between the K_D value for [^{3}H]SCH 23390 binding to the D_1 dopamine receptor and K_D values for binding to any of the mutant receptors, average K_D value of three to six independent experiments. The densities of receptors are mean ± the standard error of the mean from six to twelve independent experiments.

Uncoupling of the receptor from G protein

Prior to examining the function of the mutant and wildtype D_1 receptors, we examined physical uncoupling of the receptor from G proteins (presumably G_s) following dopamine treatment. Four of the mutant D_1 receptors had higher affinity for dopamine (values for

K_H were lower) than wildtype D_1 receptors, but following dopamine pretreatment values for K_H were not significantly different. After 1 μM dopamine treatment for four hours, cells expressing the D_1 or mutant receptors were assessed for changes in the percentage of high affinity agonist binding sites (K_H) (Table 4-2).

Table 4-2 Mutant and D_1 receptor K_i values for dopamine and % K_H

	Control			4 HR DA pretreatment		
	K_H (nM)	K_L (μM)	%K_H	K_H (nM)	K_L (μM)	%K_H
D1	21	1.3	**39**	3.3	1.1	**17****
	(17-28)	(1.2-1.5)		(1.3-8.3)	(1.0-1.1)	
S127A	8.0*	1.1	**41**	6.7	1.5	**43***
	(6.4-10)	(0.9-1.4)		(4-11)	(1.3-1.6)	
T136A	4.6*	1.6	**26**	3.3***	1.2	**9**
	(3.7-5.8)	(1.1-2.4)			(0.9-1.6)	
S263A	6.1*	1.5	**34**	4.2	1.2	**17**
	(4.1-9.4)	(0.9-2.3)		(3.4-5.2)	(0.9-1.7)	
T268A	34	1.5	**59**	13	0.8	**27****
	(30-40)	(1.2-1.8)		(11-16)	(0.6-0.9)	
S380A	6.4*	0.9	**44**	9.4	1.1	**33**
	(4.1-9.9)	(0.6-1.2)		(7.7-12)	(0.8-1.6)	

K_i values for dopamine inhibition of the binding of [^{3}H]SCH 23390 to mutant and D_1 receptors in the absence of GTP were determined as described under "Materials and Methods". Affinity values for K_H and K_L are the geometric means of affinity values, in nM or μM, with the limits defined by the asymmetrical standard error of the mean given in parentheses. The values for percentage of receptors in the high affinity binding state (K_H) were determined using the goodness of fit for one- and two-site analyses compared using an F test, as described in data analysis. The proportion of sites in the high affinity class is also indicated (%K_H). Values are from three to five independent experiments.
* significantly different from D_1, Student's t-test, $P<0.05$, ** significantly different from control, Student's t-test for paired means, $P<0.05$, *** cannot determine average K_H.

In untreated cells expressing wildtype D_1 receptors, 39% of the receptors were in the high affinity state. Agonist treatment resulted in a 50% decrease in the percentage of receptors in a high affinity state (17%). Similar decreases in the percentage of receptors in the high affinity binding state were also seen with the mutant receptors, T136A, S263A and T268A. In contrast, only a 25% decrease (from 44% to 33%) in the percentage of high affinity binding was seen with the mutant S380A, and there was no change in the percentage of high affinity binding sites of the mutant S127A (41% *versus* 44%).

Functional desensitization of receptors following agonist exposure

The functional coupling of the wildtype and mutant D_1 receptors was assessed by the ability of dopamine to stimulate adenylate cyclase. The wildtype and mutant D_1 receptors stimulated adenylate cyclase activity to similar extents in assays performed together. Additionally, dopamine had significantly reduced potency for stimulation of adenylate cyclase at three of the mutant receptors (See Table 4-3B Control EC_{50} values).

To assess the effects of mutating potential PKA phosphorylation sites, cells expressing D_1 and mutant receptors were treated for one, two, or four hours with either 10 or 100 nM dopamine prior to measuring dopamine-stimulated cyclic AMP accumulation. Both the potency and the efficacy of the stimulation of adenylate cyclase by dopamine were determined. A decreased efficacy of an agonist cannot be overcome by increasing the agonist concentration, and is reflected in a decreased maximal response. Reduced potency means that higher concentrations of the agonist are necessary to elicit an equivalent response, which is reflected in a parallel shift to the right of the dose response curve.

Treatment with 10 nM dopamine for one hour resulted in a 20% decrease in the efficacy of dopamine at D_1 receptors (Table 4-3A). By two hours the decrease was maximal (30%), with no further decrease following four hour treatment. One hour treatment of the mutant D_1 receptors decreased the efficacy of dopamine only at the T136A mutant receptor (Table 4-3A). The efficacy of dopamine at the other mutants was

Table 4-3A Efficacy of dopamine for stimulating cyclic AMP accumulation following 10 nM dopamine treatment

	1 HR % control stimulation	2 HR % control stimulation	4 HR % control stimulation
D_1	82 ± 4.1	71 ± 12*	73 ± 5.1*
S127A	81 ± 18	82 ± 10	137 ± 15**
T136A	82 ± 2.6	64 ± 9.0*	44 ± 30
S263A	96 ± 9.2	57 ± 8.4*	63 ± 6.4*
T268A	89 ± 14	92 ± 7.7	57 ± 14
S380A	98 ± 15	97 ± 18	60 ± 13

Data shown are expressed as the percentage of control values ± the standard error of the mean from three or more independent experiments in which the efficacy of dopamine for stimulating cAMP accumulation following 10 nM dopamine treatment was assessed as described in "Materials and Methods". Maximal stimulation is defined as cAMP accumulation in the presence of 1 μM dopamine. * significantly different from control, Student's t-test for paired means, $P<0.05$; ** significantly different from D_1, one way analysis of variance, with Dunnett's Multiple Comparison Test as post test, $P<0.005$;

not significantly different from control values, or from one hour pretreated D_1 receptor values due in part to high standard error of the means. Two hour pretreatment of the mutant receptors with 10 nM dopamine decreased the efficacy of dopamine at T136A, S127A, and S263A to an extent similar to the reduction in the maximal responsiveness of the wildtype receptor. The efficacy of dopamine at T268A and S380A, however, did not

Table 4-3B Potency of dopamine for stimulating adenylate cyclase following 10 nM dopamine treatment

EC_{50} (nM)

	Average Control	1 HR	2 HR	4 HR
D_1	2.2 ± 0.03	3.8 ± 0.5	3.2 ± 0.3**	2.2 ± 1.0
S127A	8.7 ± 1.7*	4.9 ± 0.1	33 ± 17	5.4 ± 0.3
T136A	27 ± 7.3*	36 ± 24	40 ±	24 ± 11
S263A	16 ± 3.5*	16 ± 8.8	40 ± 15	11 ± 3.6
T268A	2.0 ± 0.1	---	1.5 ± 0.5	1.8 ± 0.9
S380A	2.2 ± 0.04	2.5 ± 0.5	3.7 ± 0.7	2.2 ± 1.1

Data shown are expressed as the concentration of dopamine that produces 50% of the maximal stimulation of cAMP accumulation (EC_{50}) ± the standard error of the mean from two or more independent experiments. Cells were treated with 10 nM dopamine for 1, 2, or 4 hours prior to determining dose response curves for dopamine stimulation of cAMP accumulation as described in "Materials and Methods". * significantly different from D_1 control, Student's t-test, $P<0.05$; ** significantly different from control, Student's t-test for paired means, $P<0.05$.

change from control values. Following 10 nM dopamine treatment for four hours the efficacy of dopamine at four of the mutants was decreased to an extent similar to the decreased responsiveness of the wildtype D_1 receptor. However the efficacy of dopamine at S127A did not decrease, but increased following 10 nM dopamine treatment for four hours.

The potency of dopamine for stimulation of adenylate cyclase *via* wildtype D_1 receptors decreased following treatment with 10 nM dopamine for two hours, but returned to control D_1 values following four hour treatment (Table 4-3B). The potency of dopamine for stimulation of adenylate cyclase *via* the mutant D_1 receptors did not change following 10 nM dopamine pretreatment. Although we were unable to generate EC_{50}

Table 4-4A Efficacy of dopamine for stimulating cyclic AMP accumulation following 100 nM DA pretreatment			
	1 HR % control stimulation	**2 HR** % control stimulation	**4 HR** % control stimulation
D_1	72 ± 5.8*	55 ± 6.4*	62 ± 5.1*
S127A	83 ± 13	61 ± 13*	93 ± 16
T136A	64 ± 5.4*	41 ± 9.2*	47 ± 30
S263A	74 ± 12	57 ± 8.4*	57 ± 8.0*
T268A	83 ±16	57 ± 8.9*	49 ± 9.5*
S380A	74 ± 15	57 ± 12	51 ± 14

Data shown are expressed as the percentage of control values ± the standard error of the mean from three or more independent experiments in which the efficacy of dopamine for stimulating cAMP accumulation following 100 nM dopamine treatment was assessed as described in "Materials and Methods". Maximal stimulation is defined as cAMP accumulation in the presence of 1 μM dopamine. * significantly different from control, Student's t-test for paired means, $P<0.05$;

values following 1 hour dopamine treatment of T268A, we were able to determine maximal stimulation of cyclic AMP accumulation.

The efficacy of dopamine at the wildtype D_1 receptors and all the D_1 mutant receptors was reduced to a similar extent following one hour (20-30%), and two hour (40-60%) 100 nM dopamine treatment. There was no further reduction in efficacy following 100 nM dopamine treatment for four hours (Table 4-4A). At S127A, however, the efficacy of dopamine returned to control (pretreated levels) following four hours of dopamine treatment. This result was similar to the increased efficacy of this mutant following 10 nM dopamine treatment for four hours.

The potency of dopamine for stimulating adenylate cyclase *via* the wildtype D_1 receptor was reduced following 100 nM dopamine treatment for two and four hours

Table 4-4B Potency of dopamine for stimulating adenylate cyclase following 100 nM dopamine treatment

	EC_{50} (nM)			
	Average Control	**1 HR**	**2 HR**	**4 HR**
D_1	2.2 ± 0.03	4.4 ± 1.1	4.7 ± 1.0**	7.0 ± 0.2**
S127A	8.7 ± 1.7*	9.4 ± 1.9	26 ± 6.2**	10 ± 1.8
T136A	27 ± 7.3*	95 ± 51	37 ± 33	30 ± 13
S263A	16 ± 3.5*	27 ± 11	49 ± 7.7**	33 ± 8.9**
T268A	2.0 ± 0.1	---	23 ± 15	9.4 ± 5.7
S380A	2.2 ± 0.04	2.9 ± 0.5	4.1 ± 1.6	1.3 ± 1.0

Data shown are expressed as the concentration of dopamine that produces 50% of the maximal stimulation of cAMP accumulation ± the standard error of the mean from two or more independent experiments. Cells were treated with 100 nM dopamine for 1, 2, or 4 hours prior to determining dose response curves for dopamine stimulation of cAMP accumulation as described in "Materials and Methods". * significantly different from D_1 control, Student's t-test, $P<0.05$; ** significantly different from control, Student's t-test for paired means, $P<0.05$.

(Table 4-4B). Dopamine also had decreased potency for stimulating adenylate cyclase activity *via* S263A and T268A following 100 nM dopamine treatment for two and four hours, although the differences were not always statistically significant. Two hour dopamine treatment significantly reduced the potency of dopamine at mutant S127A, but after four hours the potency of dopamine was the same as control values, consistent with the observation that maximal stimulation by dopamine also returned to control values. The potency of dopamine for stimulation of adenylate cyclase activity *via* T136A and S380A was unchanged following 100 nM dopamine pretreatment.

GRKs phosphorylate the β-adrenergic receptors on serine and threonine residues that are clustered in the cytoplasmic tail of the receptor in close proximity to acidic

residues (aspartate or glutamate). The mutant and D_1 receptors contain serine and threonine residues in this region which are close to acidic amino acid residues. The human D_1 receptor has been shown to be phosphorylated by several GRKs (GRK2, GRK3 and GRK5) in transfected HEK293 cells (140), therefore it is very likely that the rhesus macaque D_1 receptor (99.5% identical with human) is phosphorylated by GRKs. As a comparison to 10 nM and 100 nM dopamine pretreatment, and to assess desensitization of mutant and D_1 dopamine receptors mediated by both PKA and GRKs, cells expressing the receptors were pretreated with 1 μM dopamine for one, two, or four hours and stimulation of adenylate cyclase by dopamine was assessed.

Table 4-5A Efficacy of dopamine for stimulating cyclic AMP accumulation following 1 μM dopamine treatment

	1 HR % control stimulation	2 HR % control stimulation	4 HR % control stimulation
D_1	61 ± 5.5*	40 ± 7.2*	40 ± 2.2*
S127A	69 ± 21	38 ± 11*	59 ± 25
T136A	53 ± 8.6*	38 ± 11*	50 ± 28
S263A	64 ± 1.5*	29 ± 11*	22 ± 1.4*
T268A	68 ± 25	66 ± 16	34 ± 1.8*
S380A	72 ± 9.4	51 ± 5.1*	22 ± 1.3*

Data shown are expressed as the percentage of control values ± the standard error of the mean from three or more independent experiments in which the efficacy of dopamine for stimulating cAMP accumulation following 1 μM dopamine treatment was assessed as described in "Materials and Methods". Maximal stimulation is defined as cAMP accumulation in the presence of 1 μM dopamine. * significantly different from control, Student's t-test for paired means, $P<0.05$;

Treatment with 1 μM dopamine of cells expressing wild-type D_1 receptors for 30 minutes resulted in no significant changes in the efficacy of dopamine (N=3, data not

shown), however pretreatment for 1 hour resulted in a 40% reduction in the efficacy of dopamine (Table 4-5A). There was a 60% reduction in the efficacy of dopamine at wildtype D_1 receptors following 1 μM dopamine treatment for two hours, with no further decreases in efficacy after four hours. The reductions in the efficacy of dopamine at the mutant receptors were similar to wildtype D_1 receptors following 1 μM dopamine treatment. However, the maximum reductions in efficacy at S263A, and S380A was greater following 1 μM dopamine treatment for four hours than at the wildtype receptor.

There was a tendency for the potency of dopamine for stimulating adenylate cyclase to be reduced following 1 μM dopamine pretreatment (Table 4-5B), but this difference was not statistically significant for the wildtype D_1 receptor. The potency of

Table 4-5B Potency of dopamine for stimulating adenylate cyclase following 1 μM dopamine treatment

EC_{50} (nM)

	Average Control	1 HR	2 HR	4 HR
D_1	2.2 ± 0.03	6.3 ± 5.4	13 ± 4.6	7.6 ± 1.3
S127A	8.7 ± 1.7*	17 ± 3.1**	41 ± 23	32 ± 1.3**
T136A	27 ± 7.3*	20 ± 12	135 ± 130	200 ± 170
S263A	16 ± 3.5*	83 ± 34	243 ± 100*	84 ± 14**
T268A	2.0 ± 0.1		24 ± 9.0	16 ± 4.5**
S380A	2.2 ± 0.04	32 ± 21	20 ± 19	11 ± 8.4

Data shown are expressed as the concentration of dopamine that produces 50% of the maximal stimulation of cAMP accumulation ± the standard error of the mean from two or more independent experiments. Cells were treated with 1 μM dopamine for 1, 2, or 4 hours prior to determining dose response curves for dopamine stimulation of cAMP accumulation as described in "Materials and Methods". * significantly different from D_1 control, Student's t-test, $P<0.05$; ** significantly different from control, Student's t-test for paired means, $P<0.05$.

dopamine-mediated stimulation of adenylate cyclase *via* the mutant receptors S127A, S263A, and T268A was significantly reduced following 1 mM dopamine treatment for four hours. The very large standard error of the means for T136A and S380A made it difficult to determine whether there were decreases in the potency of dopamine at these mutant receptors.

D_1 receptor down regulation

The role of phosphorylation by PKA in down regulation of the D_1 receptor was assessed using D_1 receptors with single potential PKA phosphorylation sites mutated to alanine. Treatment with 1 μM dopamine for one or two hours did not result in a significant change in the density of wildtype D_1 dopamine receptors. There was a 25%

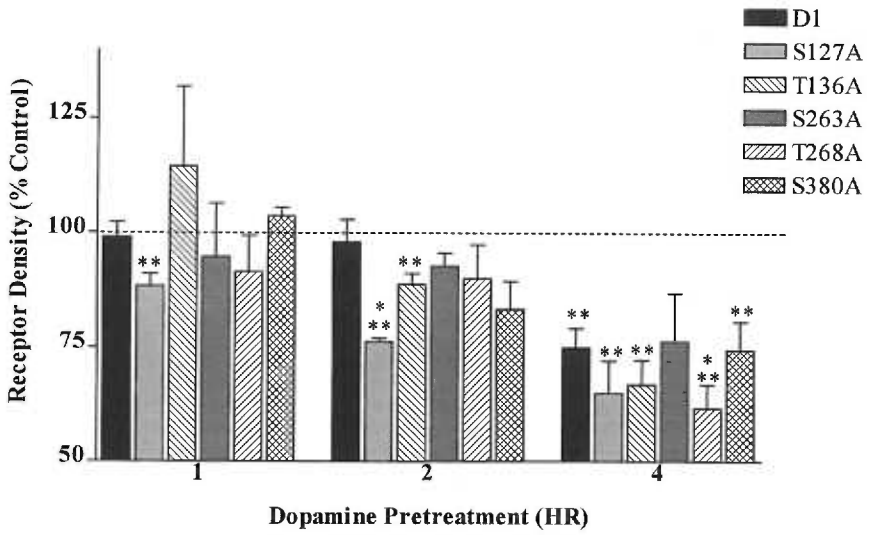

Figure 4-1 Reduction in receptor density following agonist treatment

*Cells expressing D_1 and mutant D_1 receptors were treated for one, two, or four hours with 1 μM dopamine prior to assessment of receptor density as described in 'Materials and Methods'. Data are expressed as the mean ± the standard error of the mean (bars) from two to five independent experiments. Results are expressed as a percentage of the density of receptors on untreated cells. * P<0.05, significantly different from the D_1 control, **P<0.05, significantly different from control, 1-tailed Student's t-test.*

decrease in receptor density following 1 μM dopamine treatment for four hours. (Figure 4-1). Although all the mutant receptors had decreases in receptor density following four hour pretreatment (25-40%), mutant S127A had increased sensitivity to down regulation. Specifically, 1 μM dopamine pretreatment of S127A for one and two hours resulted in a significant (12% and 24%, respectively) decrease in receptor density, whereas the densities of the other mutant receptors were not significantly reduced, indicating that mutation of S127 enhanced the rate, but not the extent of receptor down regulation.

The role of the D_1 receptor third cytoplasmic loop and carboxyl tail in down regulation of the receptor was assessed using chimeric receptors. One of the chimeric receptors, $D_{2[5-7]}$, had D_2 receptor sequence from TMV through the carboxyl tail, whereas the other chimeric receptors had D_2 receptor sequence from TMVI ($D_{2[6-7]}$) or TMVII ($D_{2[7]}$) through the carboxyl tail of the D_2 receptor, respectively.

Four hour pretreatment with 10 μM dopamine decreased the density of wildtype D_1 receptors, $D_{2[6-7]}$ and $D_{2[7]}$ (Figure 4-2). Following 10 μM dopamine pretreatment of $D_{2[5-7]}$ for four hours, the density of receptors was increased. Four hour (not shown, see (64)) 10 μM dopamine treatment of the D_2 receptor did not result in significant changes in receptor density.

Overnight pretreatment with 10 μM dopamine, 10 μM NPA or 100 μM quinpirole decreased the density of $D_{2[6-7]}$ receptors, whereas the density of $D_{2[5-7]}$ receptors was increased (Figure 4-1). D_1 and $D_{2[7]}$ receptors were down regulated by both dopamine and NPA, whereas quinpirole pretreatment produced no significant reduction in the

density of these receptors. Quinpirole and NPA pretreatment increased the density of D_2 receptors. Treatment with 10 μM dopamine treatment did not effect D_2 receptor density,

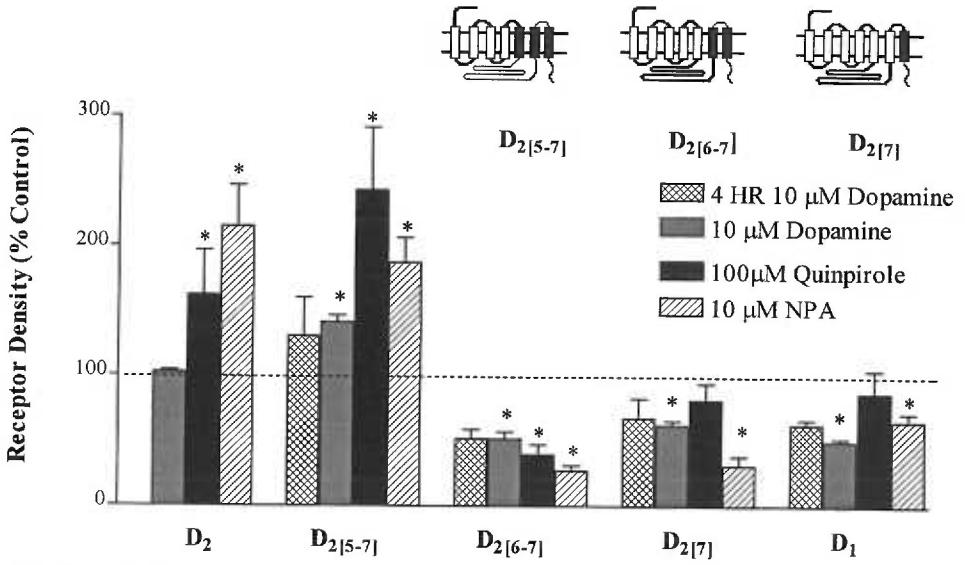

Figure 4-2 Regulation of receptor density at wildtype and D_1/D_2 dopamine receptors.
*The D_1 receptor and the chimeras, $D_{2[6-7]}$ and $D_{2[7]}$, were down regulated by 4 hour treatment with dopamine or overnight dopamine or NPA pretreatment. Quinpirole reduced the receptor density of $D_{2[6-7]}$, but did not decrease the density of either D_1 or $D_{2[7]}$ receptors. The density of D_2 or $D_{2[5-7]}$ receptors was significantly increased by overnight pretreatment with either NPA or quinpirole, but only the density of $D_{2[5-7]}$ was increased following 10 μM dopamine pretreatment. * $P<0.05$, significantly different from control.*

although overnight treatment with 100 μM dopamine will up regulate receptor levels (64). The affinity of quinpirole for the chimeric receptors $D_{2[5-7]}$ and $D_{2[6-7]}$ is similar, but quinpirole did not mediate significant changes in the density of $D_{2[7]}$, whereas it significantly reduced the density of $D_{2[6-7]}$.

DISCUSSION

D_1 receptor coupling to G proteins

One of the consequences of chronic agonist occupancy of the D_1 receptor is a decrease (50% in this study) in the percentage of high affinity sites for agonist, reflecting physical uncoupling of the receptors from the G protein. Whereas mutation of serine 380 to alanine appeared to partially inhibit this effect, mutation of serine 127 to alanine abolished this receptor response.

If phosphorylation of a particular residue is crucial for regulation of receptor function, then we would expect to see that mutation of that residue alters regulation of the receptor in response to agonist. Our data suggest that phosphorylation of S127 and S380 may be involved in regulating G_s uncoupling from the D_1 receptor.

D_1 receptor desensitization

Desensitization is a mechanism that is evoked in cells continuously exposed to agonist, resulting in a reduction of the cellular response. Continuous exposure of cells expressing β-adrenergic receptors to agonist results in an attenuation of agonist-mediated adenylate cyclase activity. Hausdorff et al. (134) demonstrated that exposure to low (nM) concentrations of agonist preferentially induces PKA phosphorylation of β_2-adrenergic receptors. Phosphorylation of either β_1 or β_2-adrenergic receptors by PKA decreases the potency of agonists (134,135), with no change in maximal response. The catalytic subunit of PKA mimics the attenuation of agonist potency in membranes expressing β_1-adrenergic (135) or human D_1 dopaminergic receptors (94), and this reduction in agonist

potency can be blocked by a protein kinase inhibitor added to permeabilized cells. In contrast, in transient transfections of Cos 7 cells, human D_1 dopamine receptors in which the distal part of the carboxyl tail (site of potential GRK phosphorylation) was truncated were unable to desensitize (141). However this truncation removes one potential PKA phosphorylation site, serine 380, in addition to a number of potential sites of phosphorylation by GRKs. In opossum kidney cells, increases in cyclic AMP levels are required for down regulation but not desensitization of endogenous D_1 receptors (87). However, these studies used relatively high (10 to 100 μM) concentrations of dopamine. Therefore the role of PKA in desensitization of the D_1 receptor is still remains to be elucidated.

The efficacy of dopamine at D_1 receptors

The efficacy of dopamine for stimulating adenylate cyclase activity was reduced following 10 nM and 100 nM dopamine treatment. Two of the mutant receptors, T268A and S380A appeared to desensitize at a slower rate following 10 nM dopamine treatment. For one mutant, S127A, the efficacy of dopamine for stimulating adenylate cyclase activity increased above (pretreated) control levels following 10 nM dopamine, and returned to control levels following 100 nM dopamine treatment for four hours.

A slower time course for agonist-induced desensitization of T268A and S380A may be consistent with a role for threonine 268 and serine 380 in mediating desensitization of the D_1 dopamine receptor. The increased (relative to two hour pretreatment) efficacy of dopamine at S127A following four hours of dopamine pretreatment may be due to increased G_s coupling of this mutant, as suggested by an

inability of this mutant to uncouple from G_s (no reduction in high affinity binding following dopamine treatment).

Agonist potency for stimulation of adenylate cyclase

Low dopamine (10 and 100 nM) concentrations were used to induce preferential phosphorylation by PKA of wildtype and mutant D_1 dopamine receptors. The potency of dopamine for stimulating adenylate cyclase *via* wildtype D_1 receptors was reduced following 10 nM and 100 nM dopamine treatment for two hours. The potency of dopamine for stimulating adenylate cyclase activity was unchanged in cells expressing two of the mutant receptors, T136A and S380A, suggesting that phosphorylation of threonine 136 and serine 380 may be involved in desensitization of the D_1 receptor.

Desensitization following of 1 μM dopamine treatment

Following two hour 1 μM dopamine treatment the efficacy of dopamine for stimulating adenylate cyclase activity *via* wildtype D_1 receptors was maximally reduced (60%). The efficacy of dopamine for stimulating adenylate cyclase activity in two mutant receptor cell lines, S263A and S380A, was decreased to a greater extent than in cells expressing wildtype D_1 receptors (80%).

Treatment with 1 μM dopamine of cells expressing wildtype D_1 receptors resulted in decreases in the potency of dopamine that were non significant. The potency of dopamine for stimulating adenylate cyclase was reduced significantly following four hour dopamine treatment of S127A, S263A and T268A.

The decreased potency of dopamine for stimulating adenylate cyclase activity *via* S127A, S263A and T268A suggests that these mutants may be more sensitive to phosphorylation of other sites of the D_1 receptor mediating desensitization. These mutants may be more sensitive to desensitization mediated by other regions of the D_1 dopamine receptor.

D_1 receptor down regulation

Down regulation of receptor density occurs after chronic agonist treatment for many G_s-coupled receptors, and is mediated in part *via* phosphorylation by protein kinase A (PKA). In our current study, there was a significant reduction in wildtype D_1 receptor density (25%) following four hours of agonist pretreatment. None of the D_1 receptor mutants were resistant to down regulation, and one of the mutant receptors, S127A, actually had increased sensitivity to down regulation. Whereas there was no significant decrease in the density of D_1 or the other mutant receptors prior to the four hour time point, the density of S127A receptors were significantly decreased following one (12%), two (24%), and four (35%) hour pretreatment with dopamine. The inability of mutant S127A to respond to chronic agonist treatment by a decrease in high affinity binding sites would increase the likelihood that a particular receptor would be occupied by agonist, possibly increasing the rate of removal of the agonist-occupied receptor from the membrane, leading to an increased rate of down regulation.

Because agonist-induced changes in receptor density of D_1 and D_2 dopamine receptor are in opposite directions, we wanted to determine whether the use of chimeric receptors would elucidate regions of the receptor involved in receptor-specific regulation

of receptor number. The density of a chimeric receptor containing D_2 TMV through the cytoplasmic tail, $D_{2[5\text{-}7]}$, changed with a rate and sensitivity to lower concentrations of dopamine that was similar to the time course and agonist sensitivity of the D_1 receptor, but, the density of $D_{2[5\text{-}7]}$ increased, similar to agonist-modulation of the D_2 dopamine receptor (64). Agonist treatment of the other chimeric receptors, $D_{2[6\text{-}7]}$ and $D_{2[7]}$ resulted in D_1 like regulation. These results suggest that regions of the D_1 receptor contributing to the faster time course and the ability of lower concentrations of dopamine (1 and 10 μM for D_1 *versus* 100 μM dopamine for D_2) to induce changes in receptor density are located within the amino terminus to the carboxyl end of TMIV, and that determinants of up or down regulation of the D_1 or D_2 receptor are located in TMV and IC3. Although S263A and T268A are in the third cytoplasmic loop of the D_1 receptor, these mutants are down regulated in a manner similar to the wildtype D_1 receptor suggesting that other components of the receptor, possibly multiple phosphorylation sites, regulate agonist-induced down regulation of the D_1 dopamine receptor.

CONCLUSIONS

Desensitization of the D_1 dopamine receptor as observed in this study is not entirely analogous to the desensitization seen with the β_2-adrenergic receptor. The efficacy of dopamine was reduced following 10 nM dopamine treatment of D_1 receptors, whereas for the β_2-adrenergic receptor pretreatment with low concentrations of agonists decreased the potency of agonists without changing the maximal response. However, following pretreatment with 1 μM dopamine there was a reduction in both potency (non-

significant) and efficacy of dopamine at D_1 receptors, which is consistent with the desensitization occurring at the β_2-adrenergic receptor.

Desensitization and down regulation of several of the mutant D_1 receptors differed from that of wildtype D_1 receptors. Mutation of one potential PKA phosphorylation site, serine 127, resulted in an inability of the D_1 receptor to uncouple from G proteins following agonist treatment. This mutant receptor, S127A, was also down regulated at a faster rate than the wildtype D_1 receptor. Another mutant D_1 receptor, S380A, had a reduced ability to uncouple from G proteins following agonist treatment. Furthermore, following agonist pretreatment (100 nM) of C_6 cells expressing S380A there was no loss in the potency, and a slower rate in loss of efficacy, of dopamine for stimulating adenylate cyclase activity compared to wildtype D_1 receptors.

V. SUMMARY AND CONCLUSIONS

LIGAND BINDING AND SIGNAL TRANSDUCTION

The goals of my dissertation were to elucidate regions of the dopamine receptor that are involved in determining the potency of selective ligand binding, the selectivity of signal transduction, and the modulation of receptor function and expression. To this end, chimeric D_1/D_2 dopamine receptors were constructed to analyze the contribution of particular transmembrane domains (TMs) to ligand binding and to selective modulation of adenylate cyclase. There are several advantages to using chimeric receptors for determining TM contributions to ligand binding and to ligand modulation of signal transduction. Although *in vitro* mutagenesis of single amino acids has identified a number of conserved amino acid residues as important for ligand binding and signal transduction, many aspects of receptor function involve the concurrent action of multiple, often contiguous, amino acid residues, that would not be identified by point mutations. Chimeric receptors can also be designed so that a "gain of function" (such as enhanced affinity for agonist or the ability to modulate a signal transduction pathway), can be measured and the particular receptor region determining that function can be revealed. Construction of chimeric receptors is not without its disadvantages. In particular, it can be difficult to construct chimeric receptors that are functional and have high affinity for a radioligand, so that agonist and antagonist affinities can be determined. Furthermore, incompatibilities between transmembrane helices may perturb receptor structure, making it difficult to determine whether a decrease in ligand binding is due to the TM composition of the chimera or the result of nonspecific changes in helical packing.

Adrenergic and dopaminergic receptors have a number of conserved amino acid residues and share the same signaling pathways. Chimeric α_2/β_2 adrenergic receptors (80) were functional, and were useful for identifying specific determinants of ligand binding and signal transduction. We hypothesized that the construction of chimeric D_1/D_2 dopamine receptors would also be informative because the receptors are similar enough that nonspecific structural distortions would not an insurmountable problem, but are different enough pharmacologically and functionally that specific properties of each receptors can be determined.

We constructed two sets (See Fig. 3-1A & 1B set one; Fig 3-1C set two) of chimeric receptors. Chimeras in the first set were constructed by Dr. Rachael Neve, whereas I constructed those in the second set. We hypothesized that a substantial gain of selective ligand binding at one chimeric receptor would result in a substantial loss at the reciprocal chimeric receptor. However, following transfection of the first set of chimeric receptor cDNAs into mammalian cell lines, we were only able to detect specific ligand binding to three of the chimeric receptors. These chimeras, $D_{2[5\text{-}7]}$, $D_{2[6\text{-}7]}$, and $D_{2[7]}$, containing four, five, or six transmembrane domains of the D_1 receptor, respectively, bound the D_1 receptor antagonist [^{3}H]SCH 23390 with high affinity. Reciprocal chimeric receptors, containing D_1 receptor transmembrane region VII together with amino-terminal sequence from the D_2 receptor, were non-functional, and displayed no detectable specific binding of radiolabeled D_1 or D_2 antagonists although abundant mRNA for these chimeric receptors was expressed in the cells.

Three-dimensional models of G protein-coupled receptors suggest that the seven membrane spanning regions form a barrel-like structure in the cell membrane, with TMI and TMII located next to TMVII. Chimeric and mutant receptor studies in which amino acid residues in these membrane spanning domains were mutated in several different G protein-coupled receptors support this model (66-69). Since the four chimeric receptors with D_2 sequence for TMI and TMII along with D_1 sequence for TMVII through the carboxyl tail were non-functional, we hypothesized that replacing TMVII in these chimeric receptors with TMVII from the D_2 receptor would restore function. Following replacement of D_1 TMVII and carboxyl tail with D_2 TMVII and carboxyl tail, we expressed these chimeric receptors in mammalian cell lines, and found that two of the chimeric receptors, $D_{2[1\text{-}4,7]}$ and $D_{2[1\text{-}5,7]}$, displayed high affinity binding of [^{3}H]spiperone. The other chimeric receptor, $D_{2[1\text{-}2,7]}$, displayed low affinity binding of radioligands, precluding analysis of saturation isotherms.

POTENCY OF ANTAGONISTS AND AGONISTS

The structural determinants of the binding of ligands to G protein-coupled receptors are thought to lie within the conserved α-helical regions (10); thus, the chimeras were analyzed in terms of the number of TM regions that were from D_1 or D_2 receptors. If each transmembrane region contributes an equal amount, or one-seventh, of the difference between the affinity of a given ligand for the D_1 and D_2 receptors, then a plot of the change in free energy of binding of a ligand (ΔG°) to each chimeric and wild-type receptor *versus* the number of transmembrane regions that are from the D_2 receptor

should be linear, with the values for the chimeric receptors falling on a line drawn between the wild-type receptors. A ΔG^o value 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. This analysis allowed us to make several specific conclusions concerning the binding of some antagonists or agonists to D_1 or D_2 dopamine receptors, but the binding of some ligands to the receptors did not appear to follow any specific pattern.

The low specific binding to $D_{2[3\text{-}7]}$ and $D_{2[1\text{-}2,7]}$ hindered the analysis of radioligand binding to these chimeras. Therefore, we cannot include $D_{2[3\text{-}7]}$ or $D_{2[1\text{-}2,7]}$ in our analysis of ligand binding, because we cannot predict whether the low affinity of radioligands for these chimeric receptors is due to decreased affinity for the radioligands, or to incompatibilities in the chimeric receptor structure.

Our first hypothesis was that the binding of some ligands involves important interactions with TMVII. Several D_1-selective benzazepines had reduced potency for chimeric receptors when the D_1 TMVII was replaced by D_2 TMVII, and quinpirole, a D_2-selective agonist, had increased potency for these chimeric receptors. However, the affinity of other D_1-selective benzazepines and the agonist NPA for the chimeric receptors did not demonstrate an important role for TMVII in the selective affinity of all benzazepines or agonists.

Our second hypothesis was that TMI through TMIV of the D_2 receptor are important determinants of the binding of non-selective and D_2-selective antagonists to D_2

receptors. The binding of a number of non-selective and D_2-selective antagonists was greatly reduced at chimeric receptors in which D_2 TMI through TMIV were replaced with D_1 TM regions. In chimeric receptors with D_2 TMI through TMIV, the affinity of these antagonists for the chimeric receptors was intermediate between D_1 and D_2 receptors, and did not appear to differ significantly from the line drawn between D_1 and D_2 ΔG° values, suggesting that each TM contributes equally to the selectivity of the ligand. Reduced affinity may reflect conformational changes resulting in nonspecific perturbations in the chimeric receptor structure, but increased affinity of other ligands for these chimeras suggests that any perturbations in the chimeric structure are relatively minor. The serotonin (5HT) receptor, $5HT_2$, also has high affinity for butyrophenone ligands. The shift of affinity following replacement of TMI through TMIII from the serotonin $5HT_2$ receptor with those of the $5HT_{1C}$ receptor (142) is consistent with the involvement of TMI through TMIII in selective binding of haloperidol and spiperone. These results also support a role for D_2 TMI through TMIV in the selective binding of these two antagonists, haloperidol and spiperone, to the D_2 dopamine receptor.

The affinity of most agonists for one chimeric receptor, $D_{2[1\text{-}4,7]}$, were greater than expected based on values predicted from the line drawn between D_1 and D_2 receptors. Since one characteristic of constitutively active receptors is high affinity for agonists (116), we hypothesized that $D_{2[1\text{-}4,7]}$ was constitutively active (See section on constitutive activity below).

MODULATION OF ADENYLATE CYCLASE ACTIVITY

We hypothesized that IC3 would determine the G protein coupling of the chimeric receptors. Therefore, chimeric receptors containing D_1 IC3 would stimulate, whereas chimeras containing D_2 IC3 would inhibit adenylate cyclase activity.

Cells expressing chimeric receptors $D_{2[6\text{-}7]}$, $D_{2[7]}$, and $D_{2[1\text{-}4,7]}$ with the IC3 from the D_1 receptor all stimulated of adenylate cyclase activity in the presence of dopamine agonists. Maximal stimulation of adenylate cyclase cannot be compared for $D_{2[6\text{-}7]}$, $D_{2[7]}$, and $D_{2[1\text{-}4,7]}$, because the receptors were expressed in different cell lines (C_6 and HEK293) and the assays determining adenylate cyclase activity were different. However, we can compare the maximal stimulation of each chimera to wildtype D_1 receptors. When this comparison was done, it appeared that dopamine-mediated stimulation of adenylate cyclase via $D_{2[6\text{-}7]}$ was ~25%, $D_{2[7]}$ was ~80%, and $D_{2[1\text{-}4,7]}$ was ~33% of wildtype D_1 receptor maximum values.

The attenuation of the efficacy of dopamine at $D_{2[1\text{-}4,7]}$ was not the result of decreased potency of dopamine at this chimeric receptor, because it had greater affinity for dopamine than either D_1 or D_2 receptors. Overnight treatment of cells expressing $D_{2[1\text{-}4,7]}$ with pertussis toxin resulted in an increased ability of the chimeric receptor to stimulate cAMP accumulation (~70%), compared to wildtype D_1 receptors. These results suggest that at least part of the explanation for the attenuated efficacy of agonists at $D_{2[1\text{-}4,7]}$ is due to coupling of the chimeric receptor to pertussis toxin sensitive G proteins ($G_{i/o}$). There are other possible explanations for the reduced efficacy of agonists at $D_{2[1\text{-}4,7]}$. Agonist stimulation of adenylate cyclase *via* the $D_{2[1\text{-}4,7]}$ receptor may be decreased

because the receptor is a chimera and is missing structural components which are necessary for full agonist efficacy, or stimulation may be attenuated due to desensitization of the chimeric receptor, even in the absence of agonist. Constitutive activation of the cellular mechanisms involved in desensitization of this receptor would decrease the ability of the chimera to respond to agonist stimulation, as has been suggested for constitutively active mutants of the β_2- (123) and α_2-adrenergic receptor (124).

Further confirmation of the importance of the role of IC3 in G protein coupling came from chimeric receptors containing the third cytoplasmic loop from D_2 receptor. Cells expressing $D_{2[3\text{-}7]}$ or $D_{2[1\text{-}5,7]}$, but not cells expressing $D_{2[5\text{-}7]}$, mediated inhibition of isoproterenol-(C_6 cells) or forskolin-stimulated (HEK293 cells) adenylate cyclase activity in response to agonists. Dopamine only inhibited cyclic AMP accumulation by ~ 20%, in cells expressing $D_{2[3\text{-}7]}$ receptors, but inhibited cyclic AMP accumulation by ~ 60% in cells expressing the $D_{2[1\text{-}5,7]}$ receptor.

In conclusion, the third cytoplasmic loop is necessary, and perhaps sufficient, for D_1 receptor-mediated stimulation of adenylate cyclase. However, a comparison of the efficacy of dopamine at two chimeric receptors, $D_{2[7]}$ and $D_{2[1\text{-}4,7]}$, suggests that the efficiency D_1 receptor coupling to G_s would be improved by the addition of other receptor cytoplasmic domains. Coupling of $D_{2[1\text{-}4,7]}$ to pertussis toxin-sensitive G proteins interfered with coupling to G_s, as demonstrated by the ability of $D_{2[1\text{-}4,7]}$ to stimulate adenylate cyclase with higher efficacy (~70% of wildtype stimulation)

following treatment with pertussis toxin, therefore the addition of D_1 receptor cytoplasmic loops may reduce G_i/G_o coupling to the chimeric receptor.

The inability of $D_{2[5\text{-}7]}$ to mediate inhibition of adenylate cyclase activity suggested that whereas IC3 was crucial for the coupling of receptors to G proteins, other structural features of the D_2 receptor were also necessary for inhibition of adenylate cyclase. Moreover, the greater efficacy of agonists at $D_{2[1\text{-}5,7]}$, compared to efficacy of agonists at $D_{2[5\text{-}7]}$ and $D_{2[3\text{-}7]}$, pointed out that contributions from multiple D_2 receptor cytoplasmic domains, acting together were necessary for D_2 receptor activation of G_i.

To determine which intracellular loops, other than IC3, might improve G_s-coupling to D_1, and G_i-coupling to D_2, and to determine which regions are involved in pertussis toxin-sensitive G protein-coupling to $D_{2[1\text{-}4,7]}$, it will be necessary to construct chimeric receptors in which only cytoplasmic domains from one parent receptor are replaced with cytoplasmic domains from the other parent receptor.

CONSTITUTIVE ACTIVITY OF A CHIMERIC RECEPTOR

The extended ternary complex model of receptor activation of G proteins proposes that receptors spontaneously isomerize between an inactive (R) and an active (R*) conformation, with agonists having higher affinity for and stabilizing or inducing the active conformation (116). Constitutive activation of a receptor describes any modification which increases the formation of R* in the absence of agonist. In addition to unliganded stimulation of second messengers in cells expressing constitutively active G protein-coupled receptors, other characteristics of constitutive activity include

increased affinity for agonists even in the presence of GTP, attenuation of agonist-induced second messenger signaling, and the ability of inverse agonists to block basal second messenger generation (116,121).

The chimeric receptor, $D_{2[1\text{-}4,7]}$, exhibited several characteristics of a constitutively active receptor. First, the basal activity of adenylate cyclase, as measured by cyclic AMP accumulation in the absence of agonist, was three to four times greater for the chimeric receptor, $D_{2[1\text{-}4,7]}$, than for wildtype D_1 receptors (100 pmoles *versus* 30 pmoles per well). Second, the affinity of most agonists for $D_{2[1\text{-}4,7]}$, was greater than the affinity of the agonists for either D_1 or D_2 receptors (See Potency of Antagonists and Agonists), however the efficacy of agonists for stimulating adenylate cyclase activity *via* $D_{2[1\text{-}4,7]}$ was reduced compared to wildtype D_1 receptors. Third, in the absence of GTP, when two affinity states were detected, the percentage of high affinity binding sites ($\%K_H$) of $D_{2[1\text{-}4,7]}$ was greater than the $\%K_H$ for either wildtype receptor. In assays in which only one class of binding site was detected, the affinity for dopamine was similar to the K_H when two sites were detected. The addition of GTP did not change the affinity of dopamine for $D_{2[1\text{-}4,7]}$, indicating that the high affinity did not result from coupling to G proteins.

The extended ternary complex model of G protein activation predicts that most receptors will have some ability to activate G proteins in the absence of agonist. The amount of unliganded receptor activity will depend on several factors, including receptor density and the equilibrium between R and R* states of the receptor. Agonists would bind and stabilize formation of the active (R*) state, whereas inverse agonist would

modulate receptor signaling in an opposite fashion to agonists, stabilizing formation of the inactive (R) state of the receptor, and antagonists would not differentiate between inactive or active receptor states. We found that several dopamine receptor antagonists functioned as inverse agonists and significantly inhibited the elevated basal cyclic AMP accumulation at $D_{2[1\text{-}4,7]}$.

In conclusion, constitutive activity of the chimeric receptor, $D_{2[1\text{-}4,7]}$, was suggested by the elevated basal cyclic AMP levels in cells expressing $D_{2[1\text{-}4,7]}$, the high affinity of agonists for $D_{2[1\text{-}4,7]}$, the inability of GTP to affect the high affinity agonist binding state, and the ability of inverse agonists to block basal cyclic AMP accumulation.

INVERSE AGONISM AT DOPAMINE RECEPTORS

Cells expressing the D_1 receptor exhibited significantly greater basal levels of cyclic AMP than untransfected cells. Also, several antagonists appeared to inhibit basal cyclic AMP accumulation in cells expressing the D_1 receptor. These results suggested that there was some basal activity of D_1 receptors. Further studies determined that antagonists could dose-dependently block forskolin-stimulated adenylate cyclase activity in cells expressing D_1 receptors. The D_1-selective antagonist, SCH 23390, did not inhibit basal levels of cyclic AMP formation at either D_1 or $D_{2[1\text{-}4,7]}$ receptors, but there was a tendency for SCH 23390 to increase D_1 receptor-mediated cyclic AMP formation, consistent with its reported weak partial agonist activity at D_1 receptors (122).

D_2 receptors inhibit adenylate cyclase when stimulated by agonists. In cells expressing wildtype D_2 receptors, the addition of antagonists enhanced, or potentiated,

forskolin stimulation of adenylate cyclase activity. The chimeric receptor $D_{2[1-5,7]}$ also mediated potentiation of forskolin-stimulated adenylate cyclase activity in the presence of several antagonists. In conclusion, D_1 and D_2 dopamine receptors, have the ability to modulate G protein activation of adenylate cyclase, even in the absence of agonist. This low level of constitutive activity is consistent with the extended ternary complex model of G protein-coupled receptor activation.

REGULATION OF THE D_1 DOPAMINE RECEPTOR

Prolonged agonist treatment of cells expressing D_1 dopamine receptors attenuates the ability of the D_1 receptor to stimulate adenylate cyclase. This attenuation of receptor responsiveness, or desensitization, is characterized by decreased ability to activate the second messenger pathway (94), phosphorylation of the receptor (92), and a loss of high affinity (K_H) binding sites for agonists (57). There is no change in receptor density with desensitization of the receptor, but more prolonged agonist treatment results in down regulation of receptor number (87). Phosphorylation of the D_1 receptor appears to be necessary for both desensitization and down regulation of the receptor. This phosphorylation is potentially mediated by two different kinase families, cyclic AMP dependent protein kinase (PKA), and the G protein-coupled receptor kinase (GRK) family, which includes β-ARK1 and β-ARK2.

Potential sites for PKA phosphorylation are found in the second and third cytoplasmic loops and in the carboxyl terminal tail of the D_1 receptor (112). To test the hypothesis that phosphorylation by PKA is involved in desensitization and down

regulation of the D_1 dopamine receptor, mutant D_1 receptors with potential phosphorylation sites mutated to alanine were constructed. The ability of dopamine to desensitize and down regulate mutant and wildtype D_1 receptors was assessed.

Because prolonged agonist treatment results in down regulation of D_1 receptor and up regulation of D_2 receptors, we reasoned that an analysis of the regulation of chimeric receptors could allow us to determine which receptor regions were involved in modulation of receptor density. Therefore, we also assessed the ability of dopamine agonists to up or down regulate three chimeric receptors and wildtype D_1 and D_2 receptors.

Desensitization of the D_1 dopamine receptor

Desensitization of the β-adrenergic receptors by PKA phosphorylation results in reduced agonist potency for stimulation of adenylate cyclase with no change in the efficacy of the agonist. However, desensitization of the D_1 dopamine receptor resulted in decreased potency and efficacy of agonists for stimulating adenylate cyclase activity.

Treatment with 100 nM dopamine for two hours desensitized the wildtype D_1 receptor, decreasing both potency and efficacy of dopamine, but there was no change in the potency of dopamine for stimulating adenylate cyclase *via* T136A or S380A receptors. At two mutant receptors, T268A and S380A, there was a slower rate for the loss of efficacy of dopamine for stimulating adenylate cyclase activity. These results suggested that PKA phosphorylation of serine 380 is involved in desensitization and point out that phosphorylation at more than one site may be involved in PKA-mediated

desensitization. The mutant S380A was also resistant to agonist-induced uncoupling from G proteins which may be a further confirmation of a role for serine 380 in receptor regulation.

Down regulation of D_1 receptors

The agonist-induced down regulation of mutant D_1 receptors suggests that no single PKA phosphorylation site determines down regulation of the D_1 dopamine receptor. One mutant D_1 receptor, S127A, was down regulated more quickly than the wildtype D_1 receptor. There was no change in the percentage of high affinity binding sites following dopamine treatment (four hours) of S127A, but both the efficacy and potency of dopamine-mediated stimulation of adenylate cyclase were reduced. The increased rate of down regulation may be due the inability of this receptor to undergo a loss of G protein coupling.

Treatment with 10 μM dopamine for four hours or overnight maximally down regulated D_1 receptors without changing the density of D_2 receptors. However, following overnight treatment with 100 μM dopamine the density of D_2 receptors was increased (64). The density of a chimeric receptor containing D_2 TMV throughout the cytoplasmic tail, $D_{2[5\text{-}7]}$, was up regulated with a time course and sensitivity to dopamine that was more similar to the D_1 receptor, but the direction of regulation of receptor density was more similar to D_2 receptors (64). Agonist-induced regulation of two other chimeric receptors, $D_{2[6\text{-}7]}$ and $D_{2[7]}$ followed a D_1-like receptor pattern. Therefore, regions of the receptor contributing to the shorter time course and increased agonist sensitivity of D_1 receptor regulation are located within the amino terminus to the carboxyl end of TMIV,

whereas determinants of up or down regulation of the D_1 or D_2 receptor are located in TMV and IC3.

In conclusion, the lack of single potential PKA phosphorylation sites did not abolish or diminish the ability of mutant D_1 receptors to down regulate in response to agonist treatment. However, mutation of one potential phosphorylation site, S127, increased the rate of receptor down regulation. Interestingly, this mutant was also resistant to the ability of agonist treatment to uncouple the receptor from G proteins. Whether of not these two phenomena are related is not known.

A comparison of the time course, sensitivity to dopamine, and direction of regulation of the density of D_1 *versus* D_2 receptors using chimeric receptors, has allowed us to differentiate regions of the receptor that are important in the direction of modulation of receptor density from those important in the time course and sensitivity to dopamine.

CONCLUSIONS

The construction and expression of chimeric and mutant receptors is a valuable tool for structure-function analysis of receptor proteins. One of the advantages of this technique is that reciprocal chimeras can be used to verify the importance of a region in a function. Removal of the region from one parent results in the loss of function, whereas addition of the region to the other parent results in a gain of function. For example, several D_1/D_2 chimeric receptors demonstrated a loss of selective benzazepine binding and a gain of selective quinpirole binding with the replacement of a particular TM region (TMVII). Another example of this is the gain of the ability to stimulate or inhibit

adenylate cyclase with the addition to the chimera of D_1 IC3 or D_2 IC3, respectively. We also demonstrated that incompatibilities between TM I and II of the D_2 receptor with D_1 TMVII resulted in a loss of receptor function, whereas replacement of D_1 TMVII with D_2 TMVII restored the function of the receptors.

Another advantage to using chimeric receptors for structure-function analysis is the ability to differentiate between regions involved in one function, but not another. Therefore, we were able to differentiate regions of the receptor that were involved in selective potency from those regions involved in the selective efficacy of a ligand. We determined that TMV and the IC3 were important for specific modulation of receptor density, whereas the D_1 TMI through TMIV were involved in the faster rate and increased agonist sensitivity of D_1 receptors to modulation of receptor density. We also demonstrated that mutation of one amino acid increased the sensitivity of the receptor to agonist-induced changes in receptor density, and also resulted in the inability of the mutant receptor to uncouple from G proteins.

There are a number of disadvantages to using mutagenesis for structure-function analysis of receptors. It can be difficult to construct chimeric receptors that are functional. Several of the chimeric receptors displayed low affinity for radioligands, precluding analysis of ligand binding. We could not determine whether the low affinity of these chimeric receptors was due to structural perturbations or to the loss of receptor regions important for selective ligand binding.

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APPENDIX A

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Drug-Induced Up-Regulation of Dopamine D2 Receptors on Cultured Cells

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Abstract: Ligand-induced up-regulation of recombinant dopamine D2 receptors was assessed using C_6 glioma cells stably expressing the short (415-amino-acid; $D2_S$) and long (444-amino-acid; $D2_L$) forms of the receptor. Overnight treatment of C6-$D2_L$ cells with *N*-propylnorapomorphine (NPA) caused a time- and concentration-dependent increase in the density of receptors, as assessed by the binding of radioligand to membranes prepared from the cells, with no change in the affinity of the receptors for the radioligand. The effect of 10 μM NPA was maximal after 10 h, at which time the density of $D2_L$ receptors was more than doubled. The agonists dopamine and quinpirole also increased the density of $D2_L$ receptors. The receptor up-regulation was not specific for agonists, because the antagonists epidepride, sulpiride, and domperidone caused smaller (30–60%) increases in receptor density. Prolonged treatment with 10 μM NPA desensitized $D2_L$ receptors, as evidenced by a reduced ability of dopamine to inhibit adenylyl cyclase, whereas treatment with sulpiride was associated with an enhanced responsiveness to dopamine. The magnitude of NPA-induced receptor up-regulation in each of four clonal lines of C6-$D2_L$ cells (mean increase, 80%) was greater than in all four lines of C6-$D2_S$ cells (33%). Inactivation of pertussis toxin-sensitive G proteins had no effect on the basal density of $D2_L$ receptors or on the NPA-induced receptor up-regulation. Treatment with 5 μg/ml of cycloheximide, on the other hand, decreased the basal density of receptors and attenuated, but did not prevent, the NPA-induced increase. Chimeric D1/D2 receptors were used to identify structural determinants of dopamine receptor regulation. Treatment with the D1/D2 agonist NPA decreased the density of D1 and chimeric CH4 and CH3 receptors. The latter two receptors have D1 sequence from the amino-terminus to the amino-terminal end of transmembrane region (TM) VII and VI, respectively. CH2, with D1 sequence up to the amino-terminal end of TM V, and thus the third cytoplasmic loop of the D2 receptor, was up-regulated by NPA or the D2-selective agonist quinpirole. Quinpirole treatment decreased the density of CH3 and had no effect on CH4 or D1 receptors. The different responses of CH2 and CH3 to agonist treatment suggest a role for TM V and the third cytoplasmic loop in the direction of receptor regulation. **Key Words:** Dopamine—Dopamine D2 receptors—cDNA—Chimeric receptors.

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One of the most thoroughly documented phenomena in pharmacology is the inverse relationship between the extent of stimulation of neurohormonal receptors and their responsiveness. When the neural afferent to an excitable tissue is removed or blocked, the tissue becomes supersensitive to the transmitter released by the afferent, a response that has been termed "the law of denervation" (Cannon and Rosenblueth, 1949). One component of supersensitivity is often an up-regulation of receptors for the transmitter (Thesleff, 1974). Similarly, stimulation of a tissue causes desensitization, frequently due in part to a decreased density of receptors, attenuating the response to prolonged or repeated stimulation (Harden, 1983).

The rat mesostriatal dopamine system has been used extensively as a CNS model of super- and subsensitivity. Destruction of the pathway results in behavioral supersensitivity (Ungerstedt, 1971), accompanied by an increase in the rate of synthesis of D2 dopamine receptors (Creese et al., 1977; Neve et al., 1985, 1991), whereas treatment with dopamine receptor agonists reverses supersensitivity (List and Seeman, 1979) and may induce subsensitivity of previously normosensitive dopamine receptors (Quik and Iversen, 1978; Chen et al., 1993). Some characteristics of the regulation of dopamine receptors in vitro also fit a model in which overstimulation of the receptors causes a compensatory decrease in the density or responsiveness of the receptors. Treatment with an agonist decreases the responsiveness and density of D1 dopamine receptors on cultured cells (Balmforth et al., 1990; Barton and

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Abbreviations used: CH2, CH3, and CH4, chimeric receptor-2, -3, and -4, respectively; $D2_S$, short (415-amino-acid) form of D2 receptors; $D2_L$, long (444-amino-acid) form of D2 receptors; epidepride, (*S*)-(−)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,3-dimethoxy-5-iodobenzamide; [^{125}I]epidepride, (*S*)-(−)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,3-dimethoxy-5-[^{125}I]iodobenzamide; NPA, *N*-propylnorapomorphine; TM, transmembrane region.

Sibley, 1990; Machida et al., 1992) and also decreases the responsiveness of D2 receptors (Agui et al., 1988; Barton et al., 1991; Bates et al., 1991). There are, however, aspects of the regulation of dopamine receptors in vivo and in vitro that are apparently at variance with a compensatory model. Treating rats with an indirect agonist such as amphetamine or L-DOPA may increase the density of D2 receptors (Klawans et al., 1979; Wilner et al., 1980). Furthermore, destruction of the mesotelencephalic dopamine system decreases the density of D1 receptors in the neostriatum (Marshall et al., 1989; Gerfen et al., 1990), although not all studies are in agreement on this issue (Buonamici et al., 1986; Porceddu et al., 1987). Recently, there have been several reports that D2 receptors on cultured cells are up-regulated by treatment with agonists (Ivins et al., 1991; Filtz et al., 1993, 1994; Zhang et al., 1994).

The experiments described below address the mechanisms of the paradoxical regulation of recombinant D2 receptors expressed in C_6 glioma cells. We now report that the receptor up-regulation is only partially prevented by cycloheximide and is unaffected by the inactivation of pertussis toxin-sensitive G proteins. Up-regulation by the agonist *N*-propylnorapomorphine (NPA), but not the antagonist sulpiride, is associated with desensitization of D2 receptors. Through the use of chimeric D1/D2 dopamine receptors, we have localized structural features that determine the direction (up or down) of regulation of the receptors to within the third cytoplasmic loop and transmembrane region (TM) V.

MATERIALS AND METHODS

Materials

$Na^{125}I$ and [^{3}H]SCH-23390 were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.), and [^{3}H]spiperone was purchased from Amersham (Arlington Heights, IL, U.S.A.). Spiperone and domperidone (Janssen), quinpirole (Lilly), (*S*)-(−)-*N*-[(1-ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide and (*S*)-(−)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,3-dimethoxy-5-iodobenzamide (epidepride) (Dr. T. de Paulis, Vanderbilt University), and cDNAs for rat short (415-amino-acid) and long (444-amino-acid) forms of D2 receptors ($D2_S$ and $D2_L$, respectively) (Dr. O. Civelli, Oregon Health Sciences University) were generous gifts. Sulpiride and *R*(−)-NPA were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Dopamine (3-hydroxytyramine) and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The D1 receptor used in these studies and in the construction of the chimeric receptors is the rhesus macaque D1 receptor gene described previously (Machida et al., 1992).

Expression of D2 receptors in C_6 cells

C_6 cells used for stable expression of D2 receptor cDNAs were maintained as described previously (Cox et al., 1992). Transfection of C_6 cells was carried out by calcium phosphate precipitation (Chen and Okayama, 1988) exactly as described previously (Cox et al., 1992), combining either $pRSV\text{-}D2_S$ or $pRSV\text{-}D2_L$ cDNA (15 μg) with pBabe Puro (2 μg), to confer resistance to puromycin (Morgenstern and Land, 1990).

Drug-induced up-regulation of D2 receptors

All drug solutions were freshly prepared and sterilized by filtration before use. Indicated drugs were diluted 100- or 1,000-fold by addition to cells grown on 10-cm-diameter plates in Dulbecco's modified Eagle's medium. At the end of the incubation, cells were washed with calcium-, magnesium-free phosphate-buffered saline, refed with Dulbecco's modified Eagle's medium, and returned to the incubator. After 10 min, cells were washed three times with growth medium and then harvested. For harvesting, cells were lysed with ice-cold hypotonic buffer [1 m*M* Na^+-HEPES (pH 7.4) and 2 m*M* EDTA]. After swelling for 10–15 min, the cells were scraped from the plate and spun at 24,000 *g* for 20 min. The resulting crude membrane fraction was resuspended in Tris-buffered saline with a Brinkmann Polytron homogenizer at a setting of 6 for 10 s. This membrane preparation was then spun again at 24,000 *g* and resuspended. When the time course of receptor up-regulation was assessed, cells were frozen in hypotonic buffer at each time point and stored overnight at −20°C before centrifugation and quantification of receptors.

Radioligand binding assays

(*S*)-(−)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,3-dimethoxy-5-[^{125}I]iodobenzamide ([^{125}I]epidepride; NCQ 219) was prepared from its tributyltin precursor using a modification (Neve, 1991) of the method of Clanton et al. (1991). The binding of [^{125}I]epidepride or [^{3}H]spiperone was assessed essentially as described (Bunzow et al., 1988; Cox et al., 1992). Aliquots of the membrane preparation (3–40 μg of protein) were added to duplicate assay tubes containing the following (final concentrations): 50 m*M* Tris-HCl (pH 7.4) with 155 m*M* NaCl (Tris-buffered saline), 0.001% bovine serum albumin, radioligand, and appropriate drugs. Spiperone or (+)-butaclamol (2 μ*M*) was used to define nonspecific binding of [^{125}I]epidepride or [^{3}H]-spiperone, respectively. Incubations were carried out at 30 ([^{125}I]epidepride) or 37°C ([^{3}H]spiperone) for 60 min, in a volume of 0.5 and 1.0 ml, respectively. [^{3}H]SCH-23390 was used to quantify D1 and chimeric receptors and to determine the affinity of agonists for the receptors in the presence of GTP, exactly as described previously (Kozell et al., 1994).

Adenylyl cyclase activity

Cells were seeded in six-well cluster dishes at a density of 18,000/cm^2. On day 3, overnight drug treatment was initiated. The next day the cells were washed as described above, 1.5 ml of HEPES-buffered L15 medium was added to each well, and the cells were incubated in air for 2 h. Adenylyl cyclase activity was assessed by adding [^{3}H]-adenine (1 μCi per well) to the incubation medium 15 min before drug or vehicle. Incubation with drugs was carried out for 7 min and terminated by rinsing two times with ice-cold phosphate-buffered saline. [^{3}H]ATP and [^{3}H]cyclic AMP were extracted in 3% trichloroacetic acid and separated using successive Dowex and alumina columns. Results are expressed as percentages of [^{3}H]ATP converted to [^{3}H]-cyclic AMP.

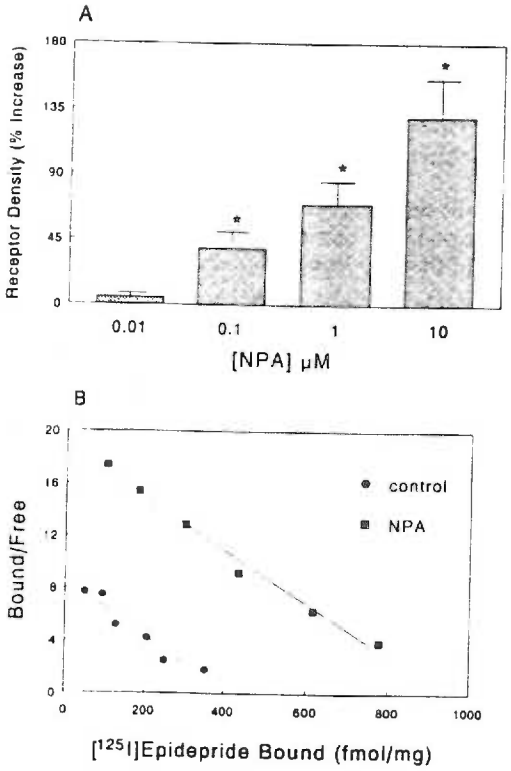

FIG. 1. **A:** Overnight treatment of C6-$D2_L$ cells with NPA caused concentration-dependent increases in receptor density. The density of D2 receptors on membranes prepared from treated cells was determined by saturation analysis of the binding of [^{125}I]epidepride. Data are mean ± SEM (bars) values from four independent experiments. Results are plotted as receptor density, expressed as the percent increase over the control density of receptors, versus the concentration of NPA used for treatment. **B:** The binding of [^{125}I]epidepride to membranes prepared from control C6-$D2_L$ cells or cells treated with 10 μM NPA overnight was characterized. Results shown are from a representative experiment in which the amount of radioligand specifically bound/the free radioligand concentration is plotted versus the amount specifically bound (fmol/mg of protein). *$p < 0.05$ compared with the control density of receptors.

Data analysis

Data were analyzed by nonlinear regression using the programs GraphPAD and Prizm. The free concentration of radioligand was calculated as the concentration added minus the concentration bound. Averages for K_D values are the geometric means [the antilogarithm of mean logarithms (Fleming et al., 1972)].

RESULTS

Agonist-induced up-regulation of $D2_L$ receptors

Overnight (14-h) treatment of C6-$D2_L$ cells with the D2 receptor agonist NPA caused a concentration-dependent increase in receptor density, as determined by saturation analysis of the binding of [^{125}I]-epidepride (Fig. 1A). A 39% increase was apparent after treatment with 0.1 μM NPA, and 10 μM NPA increased the density of $D2_L$ receptors by 130%. Higher concentrations of NPA were not tested because they appeared to be toxic to the cells and because of the difficulty of removing residual drug from the membranes before quantification of the density of receptors. As depicted in Fig. 1B, NPA treatment increased the density of binding sites without altering the affinity of the receptors for [^{125}I]epidepride.

The time course of NPA-induced receptor up-regulation was determined by harvesting C6-$D2_L$ cells after various periods of treatment with 10 μM NPA. The effect of NPA on the binding of [^{125}I]epidepride was maximal after 10–14 h (Fig. 2).

Like NPA, the agonists dopamine and quinpirole increased the density of $D2_L$ receptors (Table 1). Higher concentrations of these drugs were required, so that the density of receptors was only slightly increased by 1 μM quinpirole and not significantly increased by 1 μM dopamine. Robust increases were observed after treatment with either agonist at 100 μM.

Antagonist-induced up-regulation of $D2_L$ receptors

To ascertain if the drug-induced up-regulation of receptors depended on occupation of the receptors by an agonist, C6-$D2_L$ cells were treated for 14 h with the antagonists epidepride, sulpiride, or domperidone before quantification of the density of receptors. Each of the antagonists significantly increased the density of $D2_L$ receptors (Table 1).

Responsiveness of $D2_L$ receptors after drug treatment

C6-$D2_L$ cells were treated for 14 h with 10 μM NPA or 10 μM sulpiride, then rinsed three times with phosphate-buffered saline before quantification of dopamine-inhibited adenylyl cyclase activity (Fig. 3). Treatment with NPA caused an apparent desensitization of adenylyl cyclase. Although curves from two of

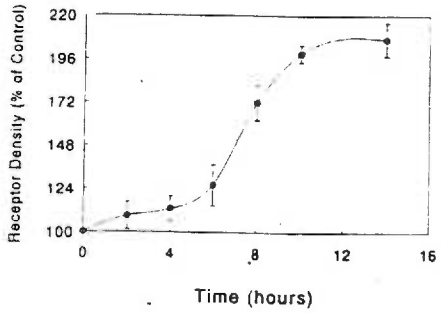

FIG. 2. The time course of NPA-induced receptor up-regulation was determined by saturation analysis of the binding of [^{125}I]-epidepride to membranes prepared after various periods of treatment with 10 μM NPA. Results are plotted as the percentage of the control density of receptors versus the duration of treatment. Data are mean ± SEM (bars) values from three independent experiments.

TABLE 1. *Drug-induced up-regulation of $D2_L$ receptors*

Drug	Density (% of control)	Affinity (pM)
Dopamine (4)		
1 μM	102 ± 1	37 (35–40)
10 μM	101 ± 4	40 (30–52)
100 μM	147 ± 6[a]	42 (39–46)
Quinpirole (4)		
1 μM	106 ± 2	41 (40–42)
10 μM	131 ± 12[a]	41 (34–49)
100 μM	162 ± 6[a]	46 (42–54)
Epidepride (5)		
10 nM	111 ± 6.99	62 (55–70)
100 nM	118 ± 12.63	74 (66–83)
1 μM	126 ± 6.07[a]	87 (77–98)
Sulpiride (3)		
100 nM	126 ± 14.75	39 (34–45)
1 μM	132 ± 8.49	40 (35–46)
10 μM	160 ± 3.52[a]	46 (34–61)
Domperidone (3), 100 nM	149 ± 15.49[a]	59 (39–87)

$C6\text{-}D2_L$ cells were treated with the indicated drugs for 14 h (overnight), and then membranes were prepared and the density of D2 receptors was determined as described in Materials and Methods. The number of independent experiments is in parentheses after each drug. K_D values for [^{125}I]epidepride, in pM, are the geometric means, followed by the 95% confidence limits of the mean in parentheses. In the experiments in which dopamine and quinpirole were used, the B_{max} and K_D of [^{125}I]epidepride in untreated cells were 622 ± 90 fmol/mg of protein and 44 pM (42–48 pM), respectively. In the experiments using sulpiride, epidepride, and domperidone, the B_{max} and K_D of [^{125}I]epidepride in untreated cells were 986 ± 91 fmol/mg of protein and 49 pM (40–60 pM), respectively. The densities of receptors are mean ± SEM values, expressed as percentages of the control density of receptors.

[a] $p < 0.05$, significant increase relative to the control value.

four independent experiments with NPA-treated cells could not be fit to the data because of a failure to reach a plateau at 10 μM dopamine, the combined data from all four experiments were best fit by a curve in which the maximal inhibition of isoproterenol-stimulated adenylyl cyclase activity was 28% of total stimulated activity ($EC_{50} = 0.5\ \mu M$), whereas the maximal inhibition in control (untreated) cells was 60% of the total ($EC_{50} = 0.03\ \mu M$). In contrast, treatment with sulpiride increased the apparent efficacy of dopamine, as reflected in an increase in the maximal inhibition of adenylyl cyclase from 61 ± 3% in control cells to 77 ± 2% in sulpiride-treated cells ($n = 4$, $p < 0.01$).

Comparison of $D2_L$ and $D2_S$

We compared the response of the alternatively spliced forms of D2 receptors by treating $C6\text{-}D2_L$ and $C6\text{-}D2_S$ cells for 14 h with 10 μM NPA. To be sure that variations in the magnitude of the response were not due to unique characteristics of one clone, or due to differences in the initial density of receptors on a cell line, we tested several clones expressing each form of the receptor. In four clones of $C6\text{-}D2_L$ cells with control receptor densities of 186 ± 52 ($n = 3$), 187 ± 18 ($n = 13$), 295 ± 37 ($n = 15$), and 977 ± 165 ($n = 4$) fmol/mg of protein, treatment with 10 μM NPA increased the density of receptors by 156, 110, 79, and 66%, for a combined mean ± SEM increase of 82 ± 10%. In four clones of $C6\text{-}D2_S$ cells with control receptor densities of 128 ± 19 ($n = 6$), 325 ± 30 ($n = 5$), 436 ± 72 ($n = 4$), and 605 ± 45 ($n = 16$) fmol/mg of protein, treatment with 10 μM NPA increased the density of receptors by 54, 33, 40, and 24%, respectively, for a combined mean ± SEM increase of 33 ± 7%. Consistent with the reduced magnitude of the NPA-induced up-regulation of $D2_S$ receptors expressed in C_6 glioma cells, NPA (10 μM) had a small but significant effect on the density of D2 receptors on LZR1 cells, mouse L cells that express $D2_S$ (Bunzow et al., 1988; Neve et al., 1989), increasing the density of receptors 26% from 435 ± 51 to 549 ± 75 fmol/mg of protein.

Mechanism of up-regulation of $D2_L$

The role of pertussis toxin-sensitive G proteins in the up-regulation of D2 receptors was assessed by addition of pertussis toxin to the culture medium at a final concentration of 100 ng/ml 16 h before treatment with 10 μM NPA. Exposure to the toxin continued throughout overnight treatment with NPA. This concentration of pertussis toxin was previously determined to abolish coupling of D2 receptors to G proteins in intact C_6 cells (Neve et al., 1992); the efficacy of the batch of toxin used in the present experiments was verified by assessing D2 receptor-mediated inhibition of adenylyl cyclase (data not shown). Treatment with pertussis toxin had no effect on the NPA-induced up-regulation of $D2_L$ receptors (Fig. 4). The basal density of receptors was 614 ± 47 (control) and 655 ± 31 (pertussis toxin) fmol/mg of protein, as assessed by the binding of [^{125}I]epidepride. Treatment with 10 μM

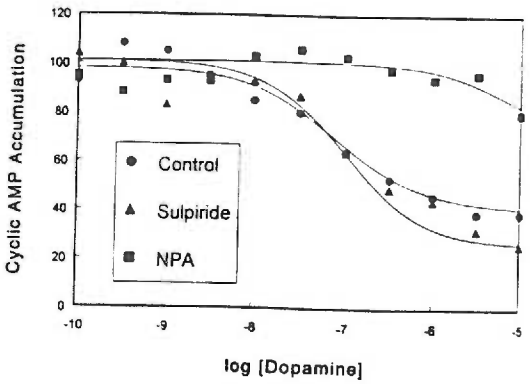

FIG. 3. Treatment of $C6\text{-}D2_L$ cells with NPA decreased and sulpiride treatment increased subsequent inhibition of adenylyl cyclase by dopamine. A representative experiment is shown in which cells were treated with 10 μM NPA, 10 μM sulpiride, or vehicle (control), followed by determination of the ability of dopamine to inhibit adenylyl cyclase. Results are expressed as a percentage of the total enzyme activity stimulated by 1 μM isoproterenol, which was 5.5% (control), 5.5% (NPA), and 5.9% (sulpiride) conversion of [^{3}H]ATP to [^{3}H]cyclic AMP.

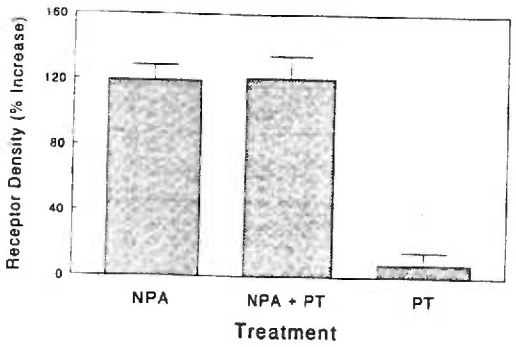

FIG. 4. Pertussis toxin (PT) had no effect on NPA-induced up-regulation of $D2_L$ receptors. C6-$D2_L$ cells were treated with 100 ng/ml of PT for 16 h before treatment with 10 μM NPA. The density of receptors on membranes prepared from PT-treated cells, cells treated with only NPA, and cells treated with NPA and PT was determined by saturation analysis of the binding of [^{125}I]epidepride. Data are mean ± SEM (bars) values from four independent experiments, expressed as the percent increase over the control density of receptors.

NPA increased the density of receptors to 1,346 ± 109 and 1,345 ± 58 fmol/mg of protein in the absence or presence, respectively, of pertussis toxin.

In separate experiments, the effect of the protein synthesis inhibitor cycloheximide on NPA-induced receptor up-regulation was assessed (Fig. 5). Cycloheximide [5 μg/ml, a concentration that inhibits protein synthesis by >95% in C_6 cells (Neve and Molinoff, 1986)] was added to the cells simultaneously with the addition of 10 μM NPA, and the density of receptors on membranes prepared from the cells was determined by analysis of the binding of [^{3}H]spiperone. After treatment for 14 h, cycloheximide decreased the density of D2 receptors by >50%, from 926 to 394 fmol/mg of protein, whereas NPA almost doubled the density of receptors, to 1,796 fmol/mg of protein. Cycloheximide treatment reduced, but did not prevent, the NPA-induced receptor up-regulation, as indicated by the 36% greater density of receptors in the NPA plus cycloheximide-treated cells (531 fmol/mg of protein) compared with cells treated with cycloheximide alone.

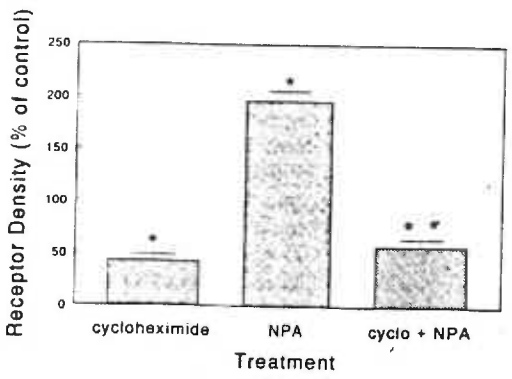

FIG. 5. The effect of cycloheximide on NPA-induced receptor up-regulation was determined by saturation analysis of the binding of [^{3}H]spiperone. C6-$D2_L$ cells were treated with 5 μg/ml of cycloheximide, 10 μM NPA, or both (cyclo + NPA). Data are mean ± SEM (bars) values from five independent experiments, expressed as the percentage of the control density of receptors (926 fmol/mg of protein). $^*p < 0.05$, significantly different from the control values; $^{**}p < 0.05$, significantly different from the cycloheximide value.

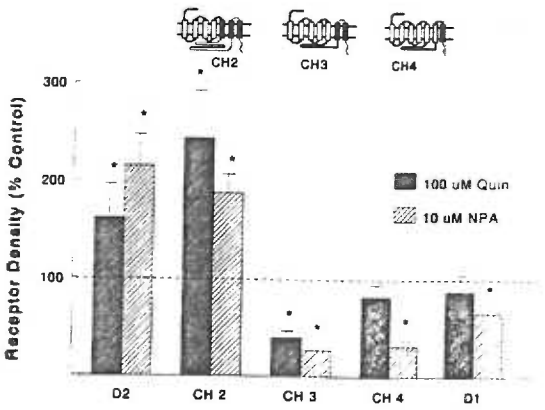

FIG. 6. C_6 cells expressing D1, $D2_L$, or chimeric D1/D2 receptors were treated overnight with 10 μM NPA or 100 μM quinpirole (Quin). The density of $D2_L$ receptors was determined by saturation analysis of the binding of [^{125}I]epidepride (NPA-treated and control cells) or [^{3}H]spiperone (Quin-treated and control cells). The density of CH2, CH3, CH4, and D1 receptors was determined by saturation analysis of the binding of [^{3}H]SCH-23390. Data are mean ± SEM (bars) values from three to five independent experiments. Results are expressed as a percentage of the density of receptors on untreated cells. **Inset:** Schematic representation of the structure of the chimeric receptors. D1 sequence is denoted by open rectangles for putative TM domains and thick lines for intracellular and extracellular hydrophilic regions. D2 sequence is denoted by solid rectangles for the putative TM domains and thin lines for hydrophilic regions. $^*p < 0.05$, significantly different from the control value.

Structural determinants of dopamine receptor up- and down-regulation

The effect of 10 μM NPA and 100 μM quinpirole on the density of chimeric D1/D2 receptors was assessed to permit the initial localization of receptor domains that determine the direction of receptor regulation. Figure 6 illustrates that both the D1/D2 agonist NPA and the D2 agonist quinpirole increased the density of D2 receptors, whereas NPA, but not quinpirole, decreased the density of D1 receptors by 35 ± 6%. It is interesting that chimeric receptor-2 (CH2), which has D2 sequence from the amino-terminal end of TM V to the carboxy-terminus (Fig. 6, inset), was up-regulated by both agonists even more than the wild-type D2 receptors. Treatment with 100 μM quinpirole more than doubled the density of CH2, from 158 to 383 fmol/mg of protein. On the other hand, both agonists decreased the density of chimeric receptor-3 (CH3), which has D2 sequence from the amino-terminal end of TM VI to the carboxy-terminus, from 167 to 66

TABLE 2. *Agonist affinity for D1, D2, and chimeric dopamine receptors*

Agonist	Apparent affinity				
	D2	CH2	CH3	CH4	D1
Quinpirole[a]	2.6 (1.5–4.5)	51 (40–64)	53 (32–86)	59 (31–110)	530 (370–740)
NPA	0.014 (0.001–0.020)	0.1 (0.06–0.1)	0.38 (0.27–0.54)	0.12 (0.03–0.4)	0.3 (0.2–0.4)

The values shown are the geometric means of K_i values, in μM, with the asymmetrical SEM in parentheses.
[a] Values from Kozell et al. (1994).

fmol/mg (quinpirole) and from 442 to 120 fmol/mg of protein (NPA). Only NPA decreased the density of chimeric receptor-4 (CH4), which has D2 receptor sequence from the amino-terminal end of TM VII to the carboxy-terminus, from 268 to 94 fmol/mg. The concentration of NPA (10 μM) is at least 20 times the apparent affinity of NPA for each of the receptors in Fig. 6. The concentration of quinpirole with which the cells were treated (100 μM) was considerably lower relative to the affinity of the chimeric and D1 receptors for quinpirole (Table 2). Nevertheless, it is noteworthy that CH2, CH3, and CH4 displayed three qualitatively different responses to quinpirole treatment despite having similar affinities for this agonist. Because the difference in sequence between CH2 (up-regulated) and CH3 (down-regulated by both agonists) is in TM V and the third cytoplasmic loop, this region is implicated in the direction of receptor regulation.

DISCUSSION

Although the classical response of receptors to stimulation by an agonist is down-regulation, our results confirm the observation that the density of D2 dopamine receptors on cells in culture is increased by agonist treatment. NPA was the most potent of the three drugs tested, causing up-regulation of receptors at a concentration at least 10-fold lower than the lowest effective concentration of dopamine or quinpirole. This is consistent with the greater potency of NPA at D2 receptors as assessed by inhibition of the binding of radioligands to D2 receptors (Cox et al., 1992). The effect of 10 μM NPA was maximal after treatment for ~10 h. Agonist-induced receptor up-regulation has now been described for a cell line that expresses endogenous D2 receptors (Ivins et al., 1991), as well as recombinant D2 receptors expressed in HEK-293 cells (Filtz et al., 1993), Chinese hamster ovary cells (Zhang et al., 1994), C_6 glioma cells, and Ltk^- cells (present study). As detailed below, however, there are both qualitative and quantitative differences in results that may reflect differences in the cell lines or expression systems.

The up-regulation of $D2_L$ receptors was induced not only by agonists, but also by three antagonists, epidepride, sulpiride, and domperidone. The magnitude of the increase was less for the antagonists than for the agonists, implying some selectivity for agonists in inducing receptor up-regulation. These results are similar to those reported by Filtz et al. (1993, 1994).

Agonist-induced up-regulation of $D2_L$ receptors was accompanied by desensitization, or a reduced ability of D2 receptors to inhibit adenylyl cyclase. This resulted in a decrease in maximal inhibition as well as a decrease in the potency of dopamine, in confirmation of the results of Zhang et al. (1994). It is interesting that the receptor up-regulation induced by the antagonist sulpiride was accompanied by an increased responsiveness to dopamine, so that maximal inhibition of adenylyl cyclase was increased. We speculate that the apparent reduction in the potency of dopamine after sulpiride treatment was due to incomplete removal of sulpiride from the cells.

Comparison of several independent clones of C6-$D2_L$ and -$D2_S$ indicated that the alternatively spliced forms of D2 receptors are both up-regulated by treatment with NPA, as reported by Filtz et al. (1993), but in contrast to the results of Zhang et al. (1994), who found that the density of $D2_S$ expressed in Chinese hamster ovary cells is decreased by treatment with agonists whereas the density of $D2_L$ is increased. The present results indicated that in C_6 glioma cells, $D2_S$ was up-regulated less than $D2_L$ after treatment with 10 μM NPA.

Several manipulations were carried out to assess the mechanism of ligand-induced D2 receptor up-regulation. D2 dopamine receptors couple to the pertussis toxin-sensitive G proteins, G_i and G_o, and inactivation of these G proteins by treatment with pertussis toxin prevents most D2 receptor modulation of second messengers (Vallar and Meldolesi, 1989). Pretreatment of C6-$D2_L$ cells with 100 ng/ml of pertussis toxin had no effect on NPA-induced D2 receptor up-regulation, indicating that an interaction with pertussis toxin-sensitive G proteins is not necessary for receptor up-regulation to occur. The lack of effect of pertussis toxin on receptor up-regulation does not exclude the possibility that an interaction with pertussis toxin-insensitive G proteins is necessary for receptor up-regulation. Because several responses to activation of D2 receptors are not inhibited by pertussis toxin (Ganz et al., 1990; Neve et al., 1992; Senogles, 1994), modulation of one of these signaling pathways could be involved in pertussis toxin-insensitive receptor up-regulation. Never-

theless, the insensitivity to pertussis toxin is consistent with the finding that antagonists can induce receptor up-regulation, because antagonists would not be expected to induce coupling of receptors to G proteins. Together these data suggest that modulation of second messengers is not necessary for ligand-induced receptor up-regulation. Our results are in agreement with those of Filtz et al. (1994), but at variance with those of Zhang et al. (1994), who reported that treatment with 1 μg/ml of pertussis toxin prevents NPA-induced up-regulation of $D2_L$ receptors. The discrepancy could be due either to the 10-fold higher concentrations of pertussis toxin used by Zhang et al. (1994) or to differences between C_6 and Chinese hamster ovary cells.

To assess if protein synthesis was necessary for NPA-induced up-regulation of $D2_L$ receptors, we treated cells with cycloheximide. We observed that cycloheximide treatment by itself decreased the density of receptors. Comparison of the density of receptors in cycloheximide-treated cells with the density in cells treated with both 10 μM NPA and cycloheximide indicated that inhibition of protein synthesis reduced, but did not prevent, NPA-induced receptor up-regulation. These results are in close agreement with those of Filtz et al. (1994) but differ slightly from those of Zhang et al. (1994), who observed complete inhibition of dopamine-induced $D2_L$ up-regulation by cycloheximide. All other experiments in this report used the substituted benzamide derivative [^{125}I]epidepride to quantify the density of D2 receptors, but the effect of NPA and cycloheximide was quantified using the butyrophenone radioligand [^{3}H]spiperone. Although there is one report that the effect of agonist treatment may differ according to whether D2 receptor density is assessed using a butyrophenone or a substituted benzamide radioligand (Barton et al., 1991), in the present experiments the effect of treatment with NPA was the same regardless of the radioligand used.

We recently characterized chimeric D1/D2 receptors, determining that CH2, with the first and second cytoplasmic loops from the D1 receptor and the third cytoplasmic loop and C-terminus from the D2 receptor, is not able to stimulate or inhibit adenylyl cyclase (Kozell et al., 1994). In contrast, CH3 and CH4, with all three cytoplasmic loops from the D1 receptor and the C-terminus from the D2 receptor, stimulate but do not inhibit adenylyl cyclase. Furthermore, quinpirole, which has virtually no ability to stimulate adenylyl cyclase via D1 receptors or CH4, gains the ability to stimulate adenylyl cyclase with the addition of TM VI from the D2 receptor (CH3) but then loses coupling to adenylyl cyclase with the further addition of the third cytoplasmic loop and TM V from the D2 receptor (CH2). Analysis of chimeric receptor regulation by agonists should suggest important domains for receptor regulation.

As we reported previously (Machida et al., 1992), treatment with a D1 receptor agonist decreases the density of recombinant D1 receptors. Quinpirole had no effect on the density of D1 receptors, which is consistent with its low affinity and efficacy for this subtype (Kozell et al., 1994). The mixed D1/D2 agonist NPA increased the density of CH2 but decreased the density of CH3. Because the sequence difference between these two chimeric receptors is in TM V and the third cytoplasmic loop, this suggests that somewhere within this region are key structural determinants of receptor regulation. These data do not differentiate between two possibilities. First, the D2 receptor TM V and third cytoplasmic loop could be important for up-regulation. Second, the D1 receptor TM V and third cytoplasmic loop, and the ability to couple to G_S and to stimulate adenylyl cyclase activity that is conferred by this domain, could be important for agonist-induced down-regulation. In support of the second possibility, quinpirole (Kozell et al., 1994) and NPA (authors' unpublished data) stimulate adenylyl cyclase activity at CH3 receptors, and both agonists decreased the density of CH3. In particular, quinpirole gains the ability to stimulate adenylyl cyclase via CH3 and also down-regulated this chimera, but not CH4 or D1 receptors. Quinpirole does not affect adenylyl cyclase via CH4 or D1 receptors (Kozell et al., 1994). The first possibility mentioned above could also be correct, as the two are not mutually exclusive, but it is at least equally likely that it is the *absence* of the D1 third cytoplasmic loop rather than the *presence* of the D2 third cytoplasmic loop that leads to up-regulation of CH2 and D2 receptors. Neither agonist modulates adenylyl cyclase via CH2, and both up-regulated this chimera.

Like D2 receptors, the density of recombinant dopamine D3 receptors on C_6 cells is increased by treatment with agonists (Cox et al., 1995). Although the characteristics of the up-regulation of these two D2-like receptors are similar in many respects, there are also some differences. For example, epidepride and sulpiride increase the density of D2 receptors (Filtz et al., 1993; present study) but do not increase the density of D3 receptors (Cox et al., 1995). Also, cycloheximide completely prevents NPA-induced up-regulation of D3 receptors, although it only partially prevents the proliferation of D2 receptors (Filtz et al., 1994; present study).

The physiological relevance of agonist-induced up-regulation of D2-like receptors is not clear, considering the high concentrations of agonist required to induce the response in C_6 cells. Still, receptor up-regulation in culture could be related to the observation that either acute or chronic treatment with compounds that stimulate the release of dopamine increases the density of D2 receptors in vivo (Klawans et al., 1979; Saelens et al., 1980; Wilner et al., 1980; Vassout et al., 1993; Seeman and Van Tol, 1994). Agonist-induced up-regulation of receptors is unusual but not unprecedented and seems to involve multiple mechanisms that may vary depending on the tissue or receptor. The density of serotonin 5-HT_2 receptors on cultured cerebellar

neurons and β_3-adrenergic receptors on 3T3 cells is increased by persistent stimulation with agonists, owing to an increased abundance of receptor mRNA (Thomas et al., 1992; Akiyoshi et al., 1993). The density of gonadotropin-releasing hormone receptors is increased by low concentrations of agonist, apparently as a result of increased efficiency of translation of an unchanged pool of mRNA (Tsutsumi et al., 1993). The density of vitamin D_3 receptors is also increased by agonists, and at least two mechanisms appear to be involved. The receptors appear to be more stable when in an agonist-binding conformation, i.e., the rate of degradation of receptors that are occupied by agonist is decreased (Costa and Feldman, 1987; Wiese et al., 1992). In addition to a large decrease in the rate of receptor degradation, agonist binding causes a smaller increase in the rate of synthesis of the receptors (Costa and Feldman, 1987), probably owing to increased gene expression (Mangelsdorf et al., 1987; Kamei et al., 1993). Agonist-induced increases in $D2_L$ receptor mRNA levels were observed by Zhang et al. (1994) but not by Filtz et al. (1994).

Our data do not unequivocally identify a mechanism for ligand-induced up-regulation of D2 receptors. Several lines of evidence, however, including the antagonist-induced up-regulation, the lack of effect of pertussis toxin, and the failure of cycloheximide to prevent completely the NPA-induced increase, suggest that stabilization of the receptors by ligands is one likely mechanism. The ability of cycloheximide to reduce the response and the results of Filtz et al. (1994), assessing changes in the rate of receptor synthesis and degradation, indicate that increased receptor synthesis also contributes to D2 receptor up-regulation.

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