CHARACTERIZATION OF DOPAMINE D2 AND D3 RECEPTORS

by

Barbara Alden Cox

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This thesis is dedicated, in loving memory, to Bob Hartshorne, a very good friend.

Abstract

Four dopamine (DA) D2 receptor mutants were constructed, in each of which an alanine residue was substituted for one of four conserved serine residues, Ser-193, Ser-194, Ser-197, and Ser-391. Wild-type and mutant receptors were expressed transiently in COS-7 cells and stably in C6 glioma cells for analysis of ligand-receptor interactions. In radioligand binding assays, the affinity of D2 receptors for DA was decreased 50-fold by substitution of alanine for Ser-193, implicating this residue in the binding of dopamine. Each mutant had smaller decreases in affinity for one or more of the ligands tested, with no apparent relationship between the class of ligand and the pattern of mutation-induced changes in affinity, except that the potency of agonists was decreased by substitution for Ser-193. The potency of DA for inhibition of adenylyl cyclase was substantially reduced by substitution of alanine for Ser-193 or Ser-197. Mutation of Ser-194 led to a complete loss of efficacy for DA and *para*-tyramine, which would be consistent with an interaction between Ser-194 and the *para*-hydroxyl substituent of DA that is necessary for activation of the receptors to occur.

Since mutation of the corresponding residues of β_2-adrenergic receptors has very different consequences, we conclude that although the position of these serine residues is highly conserved among catecholamine receptors, and the residues as a group are important in ligand binding and activation of receptors by agonists, the function of each of the residues considered separately varies among catecholamine receptors.

Additionally, we have stably expressed a rat D3 receptor cDNA in C6 glioma cells (C6-D3) and characterized the binding of the D2 radioligand [^{125}I]epidepride, which also has a high affinity for D3 receptors (Kd=60-120 pM). The binding of [^{125}]epidepride to recombinant D3 and D2 receptors was compared to binding to membranes prepared from

two regions of rat brain, the neostriatum and the ventral forebrain from which the neostriatum had been removed (extrastraiatal basal forebrain). Two-site analysis of the data indicated that for the agonist quinpirole and for the antagonists spiperone and domperidone a small population (approximately 20%) of binding sites in the rat extrastriatal basal forebrain had a pharmacological profile similar to that of C6-D3 receptors. DA and quinpirole inhibited isoproterenol-stimulated adenylyl cyclase activity by 37% and 47% respectively. Quinpirole also induced a 9-16% increase in the rate of extracellular acidification by C6-D3 cells. Epidepride inhibited this quinpirole-induced increase in rate, but pertussis toxin treatment had no effect on quinpirole-induced extracellular acidification. Overnight treatment of C6-D3 cells with agonists DA, quinpirole, and N-propylnorapomorphine (NPA) resulted in large concentration-dependent increases (over 500%) in the density of D3 receptors on membranes prepared from cells. Antagonists had no effect on the density of D3 receptors in C6-D3 cells, except for domperidone, treatment with which caused a small increase in the density of D3 receptors. Treatment with pertussis toxin had no effect on the increase in receptor density caused by the agonist treatments. Analysis by densitometry of northern blots of RNA showed no significant NPA-induced increase in D3 receptor message over that of control. Pretreatment of C6-D2 cells with 10 *u*M DA substantially increased isoproterenol-stimulated adenylyl cyclase activity, and this was inhibited when 1*u*M spiperone was added to the treatment. Treatment with 10 *u*M DA had little effect on wild type C6 cells, but isoproterenol-stimulated enzyme activity was inhibited by greater than 50% in C6-D3 cells that had been pretreated with DA.

Introduction

The catecholamine dopamine (DA) is a neurotransmitter involved in many major functions such as motor control, cognition, emotion, neuroendocrine and cardiovascular regulation. Dopaminergic systems are implicated in various pathologies such as schizophrenia, tardive dyskinesia, Parkinson's disease, Tourette's syndrome, and drug addiction. At the cellular and biochemical level, dopamine also has a wide range of effects, the mediation of which is accomplished through interactions with specific receptor proteins called DA receptors.

DA receptors are members of the family of guanine nucleotide protein (G protein)-coupled receptors. Receptors of this super family have many common features. For example, hydropathy plots of the amino acid sequences of G protein-coupled receptors yield profiles that suggest receptors in which hydrophobic regions traverse the membrane seven times (Fig. 1). These regions are each at least 20 amino acids in length, are thought to sit within the membrane as alpha helices, and are referred to as transmembrane domains or regions (TD). These TDs are interconnected by intracellular and extracellular loops. The third intracellular loop is believed to be a region which interacts with G proteins (Strader et al., 1989b). A comparison of the primary sequence of amino acids of G protein-coupled receptors yields subsets of this large super family (Mountjoy et al., 1992). Specific amino acid residues are highly conserved among these subsets. These conserved residues are largely confined to the TDs and define receptor subsets in that amino acid structure determines receptor function. Studies of β–adrenergic receptors have determined that aspartic acid residues in TDII and III and serine residues in TDV are important for ligand binding and receptor conformation (Emorine et al., 1989). The aspartic acid 79 in TDII is important in maintaining or permitting the induction by agonists of a receptor conformation capable of interacting with G proteins (Emorine et al., 1989). Aspartic acid 113 in TDIII of β_2-adrenergic receptors has an electrostatic

interaction with the cationic amines (Strader et al., 1988). Serines 204 and 207 in TDV are thought to form hydrogen bonds with the *meta*- and *para*-hydroxyl groups of catecholamines (Strader et al., 1989a).

DA receptors have classically been defined as being either DA D1 or DA D2 receptors based on their pharmacology and their respective ability to stimulate or inhibit adenylyl cyclase (Onali et al., 1985; De Camilli et al., 1979). In fact, both endogenous and recombinant D2 receptors are known to modulate a number of signaling pathways such as potassium channels (Lacey et al., 1987; Freedman and Weight, 1988; Lledo et al., 1990), calcium mobilization and polyphosphoinositide hydrolysis (Malgaroli et al., 1987; Nussinovitch and Kleinhaus, 1992; Lledo et al., 1992; Vallar et al., 1990), and Na+/H+ exchange (Ganz et al., 1990). Recombinant D2 receptors also potentiate arachidonic acid release (Felder et al., 1984; Piomelli et al., 1991) and stimulate NA+/H+ exchange (Neve et al., 1992).

DA receptors are now classified as being D2-like (D2, D3, D4) or D1-like (D1A, D1B) based on their sequence identity, pharmacology and function. The cloning of a cDNA for the DA D2 receptor by Bunzow et al. (1988) led the way for the cloning of cDNAs for D1(Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1991; Zhou et al., 1990), D1B, also referred to as D5 (Grandy et al., 1991; Tiberi et al., 1991; Weinshank et al., 1991), D3 (Sokoloff et al., 1990), and D4 (Van Tol et al., 1991) receptors. Within the TDs, sequence homology is high for all dopamine receptors. The sequence of the D1B receptor is about 50% identical to the sequence of D1A and is 78% identical within the TDs (Tiberi et al., 1991; Grandy et al., 1991; Weinshank et al., 1991). D2 and D3 receptors have approximately 50% overall homology at the amino acid level. The homology increases to 75% within the TDs (Sokoloff et al., 1990). The D4 receptor has less homology with only about 40% overall homology to D2 receptors, increasing to

53% within the TDs (Van Tol et al., 1991). Within the TDs there is only 42% homology between the human D2 and human D1 receptor (Zhou et al., 1990) which further argues for the role of amino acid structure in determination of specific receptor function.

The D1A and D1B receptors share very similar ligand binding properties, but there are some differences among the D2-like receptors. In general, D1-like receptors have high affinity for benzazepines and low affinity for butyrophenone neuroleptics and substituted benzamides, with the reverse being true for the D2-like receptors (Table 1). Among the D2-like receptors, the general pharmacological profile holds with some interesting exceptions. Several agonists are more potent at D3 receptors than at D2 receptors, and a number of drugs that bind with equal potency to D3 and D2 receptors have been described as 'atypical' neuroleptics, whose use is associated with fewer extrapyramidal side-effects (Sokoloff et al., 1990; Sokoloff et al., 1992). Most antagonists have a lower affinity for D4 receptors than for D2 receptors except for spiperone, which has equal potency for both D2 and D4 receptors, and clozapine, which has higher potency at D4 receptors than at D2 receptors (Van Tol et al., 1991).

D2 receptors have introns in the coding region, but the D1A and D1B receptor genes do not, which is typical for G protein-coupled receptor genes. The D2 receptor coding region is interrupted by five to six introns (Dal Toso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989a; Selbie et al., 1989), the D3 receptor by five (Sokoloff et al., 1990), and the D4 receptor by four (Van Tol et al., 1991). Introns shared among all three D2-like receptors are found in the second transmembrane domain, in the third cytoplasmic loop, and at the beginning of the sixth transmembrane domain. Having introns within the coding regions of D2-like receptors allows for alternate splicing of the exons. The D2 receptor has two molecular forms due to alternate splicing of the 87 base pair exon between introns four and five, resulting in a

difference of twenty nine amino acids in the third intracellular loop (Selbie et al., 1989; Monsma et al., 1989a; Giros et al., 1989; Dal Toso et al., 1989). In rat the longer form is 444 amino acids in length and is referred to as $D2_{444}$ with the shorter form being 415 amino acids in length, called $D2_{415}$. As the coding regions for the third intracellular loops of D3 and D4 receptors are interrupted by only single introns, no splice variants occur in this region of these receptors. However, the human D3 receptor lacks a forty six amino acid segment of the third intracellular loop that is present in the rat D3 receptor (Sokoloff et al., 1992). The human D3 receptor may, therefore, have an additional intron not found in the rat D3 receptor (Gingrich and Caron, 1993). Splice variants of the rat D3 receptor have been detected by using reverse transcription-polymerase chain reaction (Giros et al., 1991). Two of the splice variants appear to be formed by the use of alternate splice acceptor sites, leading to frameshifts in the normal D3 open reading frame resulting in truncated proteins, the function of which is still being examined. The exact placement of the splice site for the third intron of D4 receptors is imprecise as it is an unusual intron/exon junction site that lacks the normal donor-acceptor sequences (Van Tol et al., 1991). There are at least seven allelic variants of the D4 receptor, that vary in the number of direct repeats of a 48 base pair motif located in the third intracellular loop (Van Tol et al., 1992).

D1A receptors have a wide distribution of message whereas D1B receptors are largely limited to limbic structures. D1 receptor mRNA is abundant in the DA target regions such as the caudate-putamen, the nucleus accumbens, and the olfactory tubercles. D1A mRNA is also found in the cerebral cortex, limbic system, hypothalamus, and thalamus (Sunahara et al., 1990). D1B message is low in the classical DA target regions in the forebrain. In fact, D1B receptor message is confined largely to the lateral mammillary nuclei, the parafascicular nuclei of the thalamus, and several layers of the hippocampus (Tiberi et al., 1991).

For D2-like receptors, overlap of message is seen in some regions, but there are discrete differences in density and overall regional specificity. The distribution of D2 receptor mRNA has been characterized by northern blot analysis (Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989a), PCR amplification of mRNA (Giros et al., 1989), or *in situ* hybridization (Mansour et al., 1992; Meador-Woodruff et al., 1989; Monsma et al., 1989b; Najlerahim et al., 1989; Weiner and Brann, 1989; Weiner et al., 1991). D2 receptor message is most prominant in the caudate-putamen, olfactory tubercle, and the nucleus accumbens. The mRNA for the D2 receptor is also found in the substantia nigra and in the ventral tegmental area, the respective A9 and A10 nuclei which give rise to the major dopaminergic tracts of the brain. Additionally, D2 receptor message is found in the anterior and intermediate lobes of the pituitary gland.

The long form of the D2 receptor, $D2_{444}$, is more abundantly expressed and there is little tissue specificity between the two forms. In fact, the expression of message for each form is maintained at the same ratio for most tissues with the exception of brainstem (Autelitano et al., 1989; Montmayeur and Borrelli, 1991). Also, in the low expressing tissues, the ratio of $D2_{444}$ and $D2_{415}$ message tends to be lower (Neve et al., 1991b).

D3 receptor message is most abundant in the ventral forebrain, with olfactory tubercle having the highest density, and is also present in other areas such as the hippocampus, septum, and substantia nigra. D3 receptor message is generally present at much lower levels than D2 receptor message with the only exception being the islands of Calleja where the density of D3 receptors is quite high (Sokoloff et al., 1990; Bouthenet et al., 1991).

D4 receptor mRNA is found in medulla, amygdala, midbrain, frontal cortex and striatum, and lower levels have been detected in olfactory tubercle and hippocampus (Van Tol et al., 1991).

The molecular cloning of the many subtypes of dopamine receptors has made it possible to compare the characteristics of defined populations of receptors on identical cell backgrounds. By stably expressing recombinant receptors in cells, one can examine the ligand binding properties, function, and regulation of these receptors in isolated sytems. Furthermore, the primary structure of a receptor can be altered in a site-specific manner through the use of *in vitro* mutagenesis. Thus, the functional consequences of a single amino acid substitution can be evaluated. This approach has been used to examine the conserved amino acid residues found in the TDs of G protein coupled receptors.

Analysis of many G protein-coupled receptors by site-directed mutagenesis has been guided by the model of receptor-ligand interactions that evolved from studies of β–adrenergic receptors. As the model assigns distinct roles for aspartic acid residues in TD domains II and III, and conserved serine residues in TDV, and these residues are highly conserved among G protein-coupled receptors, it is logical to mutate these residues in order to determine receptor function. Indeed, most studies of adrenergic, muscarinic cholinergic, and dopaminergic receptors indicate that the carboxylate side group of the aspartate in TDIII which corresponds to the β_2-adrenergic receptor aspartate-113 acts as the primary counterion for the positively charged substituents of ligands for these receptors (Neve et al., 1993). Substitution of a residue with an uncharged side group for aspartate leads to a loss of affinity for ligands to such an extent that the receptors are not detected in radioligand binding assays. Restoring the negative charge by substitution with glutamate restores considerable function (Strader et al., 1988).

Sensitivity to sodium is a common feature of Gi-coupled receptors. D2 receptors are coupled to Gi proteins and are regulated by sodium. The affinity of D2 receptors for substituted benzamide antagonists is increased in the presence of sodium

(Neve et al., 1990). Substituted benzamide affinity for D2 receptors is also altered by varying [H+] (Neve, 1991). In D2 receptors, substitution of aspartate-80 in TDII with an alanine (Ala80-D2) results in a reduced affinity for substituted benzamides (Neve et al., 1991a) (Appendix I). We surmised that the reduced affinity of Ala80-D2 for substituted benzamide ligands was due to an alteration in the sensitivity of D2 receptors to sodium and pH. This hypothesis was confirmed by our finding that the Ala80-D2 mutant is insensitive to changes in sodium concentration, and has decreased senstivity to changes in pH (Neve et al., 1991a). Furthermore, the affinity of the butyrophenone spiperone for D2 receptors, which is not sensitive to sodium or pH, was not affected by the aspartate-80 mutation.

In addition to being less sensitive to sodium and pH in radioligand binding assays, the Ala80-D2 mutant is incapable of inhibiting adenylyl cyclase activity, whereas the wild-type D2 receptor mediates inhibition of isoproterenol-stimulated adenylyl cyclase activity. The conservative substitution of glutamic acid for aspartate-80 (Glu80-D2) reduces, but does not eliminate, the capacity to inhibit adenylyl cyclase. The uncoupling of Ala80-D2 receptors from adenylyl cyclase is likely to be due to the inability of the mutant receptors to interact productively with G proteins, since GTP-sensitive high-affinity binding of dopamine to Ala-80 D2 is not detectable (Neve et al., 1993). These results are in agreement with the hypothesis that aspartate-80 serves to maintain a particular receptor conformation needed for interaction with G proteins and signal transduction, and suggest that the interaction of monovalent cations with the aspartic acid residue modulates receptor conformation.

Most of the phenomena described above for Ala80-D2 are also characteristic of other G protein-coupled receptors in which the corresponding aspartic acid residue is mutated. For example, aspartate-79 is necessary for allosteric regulation of α_2–adrenergic receptors by sodium (Horstman et al., 1990), and aspartate-71 of m_1

muscarinic receptors is involved in the binding of allosteric antagonists (Lee et al., 1992). Loss of GTP-sensitive binding of agonists as a result of substitution for this conserved aspartate residue has been described for α_2-adrenergic receptors (Wang et al., 1991; Surprenant et al., 1992) and β_2–adrenergic receptors. Substitutions made to the aspartate-79 residue in α_2-adrenergic receptors and corresponding aspartate residues in human β_2–adrenergic and m_1 muscarinic receptors greatly diminish the receptors' ability to inhibit (Wang et al., 1991) or stimulate (Chung et al., 1988) adenylyl cyclase activity, and to stimulate phospholipase C activity (Fraser et al., 1989).

Is the role of the serines in TDV conserved amongst catecholamine receptors? As it has been demonstrated with mutational studies examining the aspartate residues that there is conservation of function among G protein-coupled receptors, the question arises whether or not function is conserved for the serines in TDV amongst catecholamine receptors. It was one of the aims of this thesis to examine the role of these serines in DA D2 receptors, the question being, do the serines in TDV have a function in D2 receptors that is identical to the function proposed for Ser-204, Ser-203, or Ser-207 in β–adrenergic receptors? That is, are there two serines in TDV that specifically interact with the *meta*- and *para*-hydroxyls of catecholamines? To approach this problem, four serines, 193, 194, 197, and 391 of the $D2_{415}$ receptor were substituted with alanines. These mutants were stably expressed in C6 glioma cells and their ligand binding properties were compared to those of wild-type D2 receptors. It was found that the serines are important for ligand binding, but that the model as proposed for β–adrenergic receptors is not strictly conserved (Cox et al., 1992)(manuscript#1).

Another aim of this thesis was to characterize D3 receptors and compare their ligand binding, function, and regulation to D2 receptors. D3 receptors differ from D2 receptors in pharmacology and regional distribution in brain. The

pharmacological profile of D3 receptors has largely been determined using recombinant D3 receptors expressed in cell lines (Sokoloff et al., 1990; Sokoloff et al., 1992; Seabrook et al., 1992; Boundy et al., 1993; Malmberg et al., 1993). Through these studies, the means of selectively labeling D3 receptors in brain was identified, making it possible to confirm the presence of D3 receptors in brain. Some D2 radioligands are potent at D3 receptors and can therefore be used to study D3 receptors. In manuscript #2, it is shown that the D2 radioligand [^{125}I]epidepride binds with high affinity to D3 receptors and can be used to characterize endogenous D3 receptors in the rat.

In the cell lines used to express D3 receptors thus far, little interaction of D3 receptors with G proteins has been detected. It has been proposed that D3 receptors couple weakly to G proteins. However, it could be that the cell lines used lacked the correct G protein. To address this question, in manuscript # 3 a D3 receptor cDNA was cloned from rat olfactory tubercle, and the cDNA was stably expressed in C6 glioma cells. Functional studies were performed to assess D3 receptor effector pathways in these cell lines. I also wished to compare the regulation of D3 and D2 receptors. In particular, I wished to examine the effect of prolonged treatment of agonists on receptor density.

For my dissertation, I present an examination of the ligand binding role of the serines in TDV of the D2 receptor (manuscript #1). I also present a characterization of the D3 receptor (manuscript #2) using the radioligand [^{125}I]epidepride to identify a small population of binding sites in rat brain that can be characterized as D3 binding sites. I used D3 receptors stably expressed in C6 glioma cells to study functional and regulatory properties of D3 receptors.

Additionally, I have appended a manuscript on which I am second author which examines the role of the amino acid residue aspartate-80 in D2 receptors. I have referred to this work in the introduction and also in the discussion. I contributed largely to this paper and have included it to indicate that there were a whole series of mutational studies, some of which had interesting results. As these manuscripts have several co-authors, I would like

to specify my contribution. My work entailed the generation and maintenance of the transient and stable cell lines used in these studies, northern analyses, radioligand binding and functional studies, data analysis, and manuscript preparation.

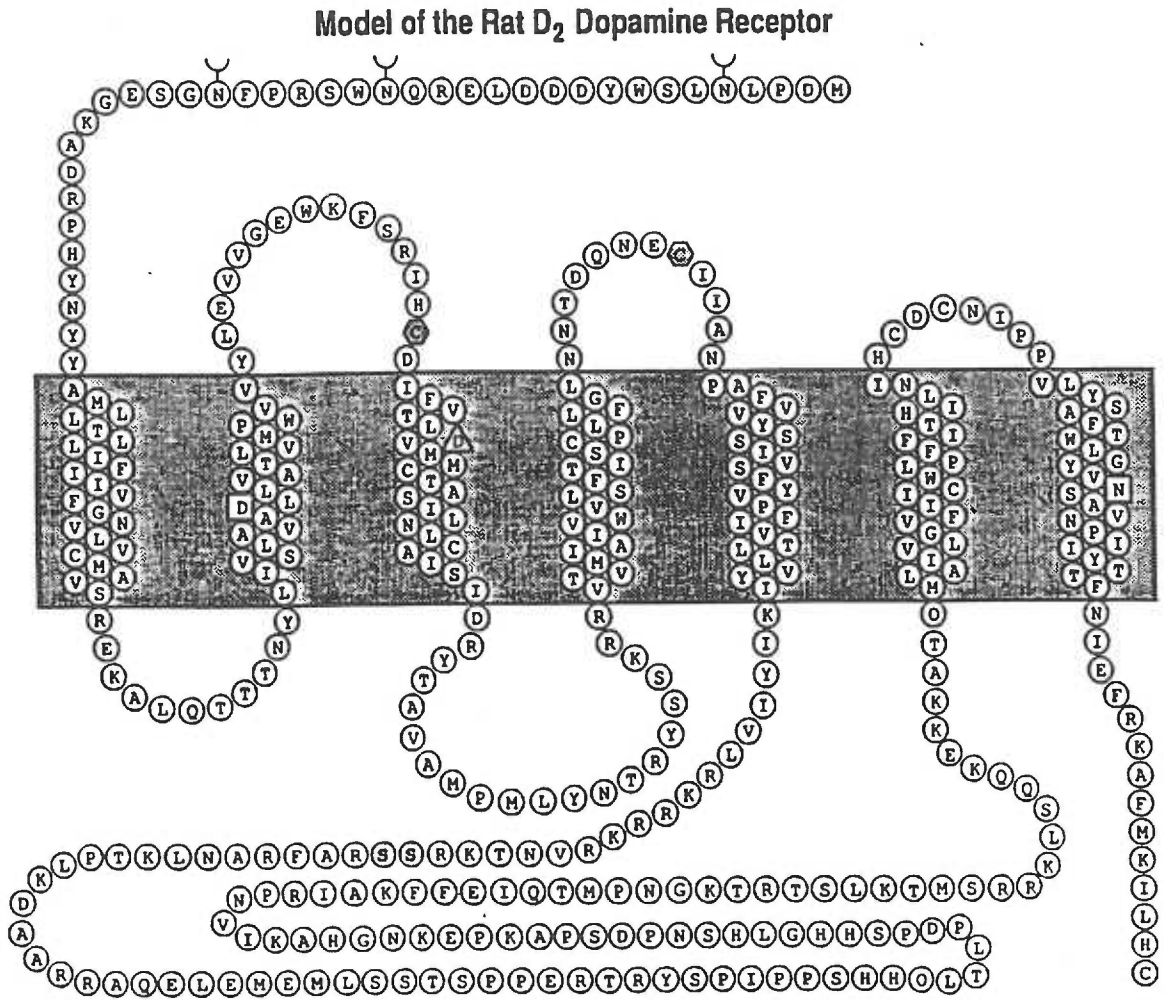

Figure 1. Model of the rat $D2_{415}$ dopamine receptor as determined from the hydropathy plot of the amin acid sequence. Amino acid residues aspartate-80 and aspartate-114 are found in the second and third transmembrane domain respectively. The serines -193, -194, and -197 are all found in the fifth transmembrane domain.

Table 1.

Properties of DA receptors (Philip Seeman {1992} Dopamine receptor sequences; therapeutic levels of neuroleptics occupy D2 receptors, clozapine occupies D4. *Neuropsychopharmacology* 7, 261-284.)

	D1 GROUP		D2 GROUP		
	D1	D5	D2	D3	D4
Found alone in:	Parathy.	–	ant. pit.	–	–
Adenylate cyclase	stimulates	stimulates	inhibits	inhibits	inhibits?
P-inositol metab.	–	–	may inhibit or stimulate		
Amino acids	446	475-477	414-444	400-446	387
Introns in gene	No	No	Yes	Yes	Yes
Chromosome (C)	5q34.5	4p15.2	11q22.5	3q	11p.tip
Pseudogenes		on C1,C2			
Polymorphism	EcoR1	none	Taq I		HinCII
Agonist K at high-affinity state (NaCl absent):					
	nM	nM	nM	nM	nM
Apomorphine	0.7	?	0.66	?	4.1
" at low state:	~450	363	127	73	
Bromocriptine*	~700	454	4.8	7.4	340
Dopamine	0.8	?	7.5	3.9	28
" at low state:	~2,000	228	4300	73	450
Fenoldopam-R	1.6	?	2.8	?	?
" at low state:	~39	15	1000	?	321
Pergolide	0.8	?	0.75	?	?
" at low state:	~800	918	60	0.6 to 2	?
(+)PHNO	75	?	0.98	?	?
" at low state:	5000	?	645	?	79
Quinpirole	1900	?	3.9	?	?
" at low state:	42000	50000	3680	5 to 39	46
SKF 38393	1 to 6	?	157	?	?
" at low state:	~200	~100	8800	5000	1800
SKF 81297	3.6	?	320	?	?
" at low state:	~60	?	8000	?	?
Antagonist K (with or without Na):					
	nM	nM	nM	nM	nM
Chlorpromazine	96	133	8.5	~6	37
Clozapine (no Na)	–	–	86	–	–
" (with Na)	172	250	182	479	9
Haloperidol	60	48	1.3	3 to 10	5.1
Raclopride	18000	?	2.9	3.5	237
Remoxipride	?	?	447	2300	3685
SCH 23390	0.37	0.3	1430	?	3560
Spiperone	258	4500	0.08	0.6	0.08
Sulpiride-S-(-)	34500	77270	14.7	~23	52
Sulpiride-R-(+)	25800	28636	868	422	?

* Bromocriptine has same affinity for high and low states.
K values for D1 from Sunahara et al. (1990) and this lab.
K values for D3 from Sokoloff et al. (1990,1992).
K values for D4 from Van Tol et al. (1991).
K values for D5 from Sunahara et al. (1991).

REFERENCES

Autelitano D.J., Snyder L., Sealfon S.C., and Roberts J.L. (1989) Dopamine D_2-receptor messenger RNA is differentially regulated by dopaminergic agents in rat anterior and neurointermediate pituitary. *Mol. Cell. Endocrinol.* **67**, 101-105.

Boundy V.A., Luedtke R.R., Gallitano A.L., Smith J.E., Filtz T.M., Kallen R.G., and Molinoff P.B. (1993) Expression and characterization of the rat D_3 dopamine receptor: pharmacologic properties and development of antibodies. *J. Pharmacol. Exp. Ther.* **264**, 1002-1011.

Bouthenet M.-L., Souil E., Martres M.-P., Sokoloff P., Giros B., and Schwartz J.-C. (1991) Localization of dopamine D_3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. *Brain Res.* **564**, 203-219.

Bunzow J.R., Van Tol H.H.M., Grandy D.K., Albert P., Salon J., Christie M., Machida C.A., Neve K.A., and Civelli O. (1988) Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature* **336**, 783-787.

Chung F.-Z., Wang C.-D., Potter P.C., Venter J.C., and Fraser C.M. (1988) Site-directed mutagenesis and continuous expression of human β-adrenergic receptors: Identification of a conserved aspartate residue involved in agonist binding and receptor activation. *J. Biol. Chem.* **263**, 4052-4055.

Cox B.A., Henningsen R.A., Spanoyannis A., Neve R.L., and Neve K.A. (1992) Contributions of conserved serine residues to the interactions of ligands with dopamine D2 receptors. *J. Neurochem.* **59**, 627-635.

Dal Toso R., Sommer B., Ewert M., Herb A., Pritchett D.B., Bach A., Shivers B.D., and Seeburg P.H. (1989) The dopamine D_2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* **8**, 4025-4034.

De Camilli P., Macconi D., and Spada A. (1979) Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. *Nature (Lond.)* **278**, 252-254.

Dearry A., Gingrich J.A., Falardeau P., Fremeau R.T., Bates M.D., and Caron M.G. (1990) Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature (Lond.)* **347**, 72-75.

Emorine L.J., Marullo S., Briend-Sutren M.-M., Patey G., Tate K., Delavier-Klutchko C., and Strosberg A.D. (1989) Molecular characterization of the human β_3-adrenergic receptor. *Science* **245**, 1118-1121.

Felder R.A., Blecher M., Eisner G.M., and Jose P.A. (1984) Cortical tubular and glomerular dopamine receptors in the rat kidney. *Amer. J. Physiol.* **246**, F557-568.

Fraser C.M., Wang C.-D., Robinson D.A., Gocayne J.D., and Venter J.C. (1989) Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**, 840-847.

Freedman J.E. and Weight F.F. (1988) Single K^+ channels activated by D_2 dopamine receptors in acutely dissociated neurons from rat corpus striatum. *Proc. Natl. Acad. Sci. USA* **85**, 3618-3622.

Ganz M.B., Pachter J.A., and Barber D.L. (1990) Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **265**, 8989-8992.

Gingrich J.A. and Caron M.G. (1993) Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci.* **16299-321**,

Giros B., Sokoloff P., Martres M.-P., Riou J.-F., Emorine L.J., and Schwartz J.-C. (1989) Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature (Lond.)* **342**, 923-926.

Giros B., Martres M.-P., Pilon C., Sokoloff P., and Schwartz J.-C. (1991) Shorter variants of the D_3 dopamine receptor produced through various patterns of alternative splicing. *Biochem. Biophys. Res. Comm.* **176**, 1584-1592.

Grandy D.K., Marchionni M.A., Makam H., Stofko R.E., Alfano M., Frothingham L., Fischer J.B., Burke-Howie K.J., Bunzow J.R., Server A.C., and Civelli O. (1989) Cloning of the cDNA and gene for a human D_2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **86**, 9762-9766.

Grandy D.K., Zhang Y., Bouvier C., Zhou Q.-Y., Johnson R.A., Allen L., Buck K., Bunzow J.R., Salon J., and Civelli O. (1991) Multiple human D_5 receptor genes: A functional receptor and two pseudogenes. *Proc. Natl. Acad. Sci. USA* **88**, 9175-9179.

Horstman D.A., Brandon S., Wilson A.L., Guyer C.A., Cragoe E.J., and Limbird L.E. (1990) An aspartate conserved among G-protein receptors confers allosteric regulation of alpha$_2$-adrenergic receptors by sodium. *J. Biol. Chem.* **265**, 21590-21595.

Lacey M.G., Mercuri N.B., and North R.A. (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of rat substantia nigra zona compacta. *J. Physiol. (Lond.)* **392**, 397-416.

Lee N.H., Hu J.R., and Elfakahany E.E. (1992) Modulation by Certain Conserved Aspartate Residues of the Allosteric Interaction of Gallamine at the m1-Muscarinic Receptor. *J. Pharmacol. Exp. Ther.* **262**, 312-316.

Lledo P.-M., Legendre P., Zhang J., Israel J.-M., and Vincent J.-D. (1990) Effects of dopamine on voltage-dependent potassium currents in identified rat lactotroph cells. *Neuroendocrinology* **52**, 545-555.

Lledo P.M., Homburger V., Bockaert J., and Vincent J.-D. (1992) Differential G protein-mediated coupling of D_2 dopamine receptors to K^+ and Ca^{2+} currents in rat anterior pituitary cells. *Neuron* **8**, 455-463.

Malgaroli A.L., Vallar L., Elahi F.R., Pozzan T., Spada A., and Meldolesi J. (1987) Dopamine inhibits cytosolic Ca^{2+} increases in rat lactotroph cells: Evidence of a dual mechanism of action. *J. Biol. Chem.* **262**, 13920-13927.

Malmberg Å., Jackson D.M., Eriksson A., and Mohell N. (1993) Unique binding characteristics of antipsychotic agents interacting with human dopamine D_{2A}, D_{2B}, and D_3 receptors. *Mol. Pharmacol.* **43**, 749-754.

Mansour A., Meng F., Meadorwoodruff J.H., Taylor L.P., Civelli O., and Akil H. (1992) Site-directed mutagenesis of the human dopamine-D2 receptor. *Eur. J. Pharmacol-Molec. Pharm.* **227**, 205-214.

Meador-Woodruff J.H., Mansour A., Bunzow J.R., Van Tol H.H.M., Watson S.J., and Civelli O. (1989) Distribution of D_2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. USA* **86**, 7625-7628.

Monsma F.J., Brassard D.L., and Sibley D.R. (1989a) Identification and characterization of D_1 and D_2 dopamine receptors in cultured neuroblastoma and retinoblastoma clonal cell lines. *Brain Res.* **492**, 314-324.

Monsma F.J., McVittie L.D., Gerfen C.R., Mahan L.C., and Sibley D.R. (1989b) Multiple D_2 dopamine receptors produced by alternative RNA splicing. *Nature* **342**, 926-929.

Monsma F.J., Mahan L.C., McVittie L.D., Gerfen C.R., and Sibley D.R. (1990) Molecular cloning and expression of a D_1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**, 6723-6727.

Montmayeur J.-P. and Borrelli E. (1991) Transcription mediated by a cAMP-responsive promoter element is reduced upon activation of dopamine D_2 receptors. *Proc. Natl. Acad. Sci. USA* **88**, 3135-3139.

Mountjoy K.G., Robbins L.S., Mortrud M.T., and Cone R.D. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248-1251.

Najlerahim A., Barton A.J.L., Harrison P.J., Heffernan J., and Pearson R.C.A. (1989) Messenger RNA encoding the D_2 dopaminergic receptor detected by in situ hybridization histochemistry in rat brain. *FEBS Lett.* **255**, 335-339.

Neve K.A., Henningsen R.A., Kinzie J.M., De Paulis T., Schmidt D.E., Kessler R.M., and Janowsky A. (1990) Sodium-dependent isomerization of dopamine D-2 receptors characterized using [^{125}I]epidepride, a high-affinity substituted benzamide ligand. *J. Pharmacol. Exper. Ther.* **252**, 1108-1116.

Neve K.A. (1991) Regulation of dopamine D2 receptors by sodium and pH. *Mol. Pharmacol.* **39**, 570-578.

Neve K.A., Cox B.A., Henningsen R.A., Spanoyannis A., and Neve R.L. (1991a) Pivotal role for aspartate-80 in regulation of D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* **39**, 733-739.

Neve K.A., Neve R.L., Fidel S., Janowsky A., and Higgins G.A. (1991b) Increased abundance of alternatively spliced forms of D-2 receptor mRNA after denervation. *Proc. Natl. Acad. Sci. USA* **88**, 2802-2806.

Neve K.A., Kozlowski M.R., and Rosser M.P. (1992) Dopamine D2 receptor stimulation of Na^+/H^+ exchange assessed by quantification of extracellular acidification. *J. Biol. Chem.* **267**, 25748-25753.

Neve, K.A., Henningsen, R.A., Kozell, L.B., Cox, B.A. and Neve, R.L. Structure-function analysis of dopamine-D_2 receptors by *in vitro* mutagenesis. In: *Dopamine receptor function and pharmacology*, edited by Niznik, H.B. New York: Marcel Dekker, Inc., 1993, p. 299-321.

Nussinovitch I. and Kleinhaus A.L. (1992) Dopamine inhibits voltage-activated calcium channel currents in rat pars intermedia pituitary cells. *Brain Res.* **574**, 49-55.

Onali P., Olianas M.C., and Gessa G.L. (1985) Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28**, 138-145.

Piomelli D., Pilon C., Giros B., Sokoloff P., Martres M.-P., and Schwartz J.-C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. *Nature* **353**, 164-167.

Seabrook G.R., Patel S., Marwood R., Emms F., Knowles M.R., Freedman S.B., and Mcallister G. (1992) Stable expression of human D_3 dopamine receptors in GH_4C_1 pituitary cells. *FEBS Lett.* **312**, 123-126.

Selbie L.A., Hayes G., and Shine J. (1989) The major dopamine D2 receptor: Molecular analysis of the human $D2_A$ subtype. *DNA* **8**, 683-689.

Sokoloff P., Giros B., Martres M.-P., Bouthenet M.-L., and Schwartz J.-C. (1990) Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature* **347**, 146-151.

Sokoloff P., Andrieux M., Besançon R., Pilon C., Martres M.-P., Giros B., and Schwartz J.-C. (1992) Pharmacology of human dopamine D_3 receptor expressed in a mammalian cell line: comparison with D_2 receptor. *Eur. J. Pharmacol-Molec. Pharm.* **225**, 331-337.

Strader C.D., Sigal I.S., Candelore M.R., Rands E., Hill W.S., and Dixon R.A.F. (1988) Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263**, 10267-10271.

Strader C.D., Candelore M.R., Hill W.S., Sigal I.S., and Dixon R.A.F. (1989a) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.* **264**, 13572-13578.

Strader C.D., Sigal I.S., and Dixon R.A.F. (1989b) Structural basis of β-adrenergic receptor function. *FASEB J.* **3**, 1825-1832.

Sunahara R.K., Niznik H.B., Weiner D.M., Stormann T.M., Brann M.R., Kennedy J.L., Gelernter J.E., Rozmahel R., Yang Y., Israel Y., Seeman P., and O'Dowd B.F. (1990) Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5. *Nature* **347**, 80-83.

Sunahara R.K., Guan H.-C., O'Dowd B.F., Seeman P., Laurier L.G., Ng G., George S.R., Torchia J., Van Tol H.H.M., and Niznik H.B. (1991) Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature* **350**, 614-619.

Surprenant A., Horstman D.A., Akbarali H., and Limbird L.E. (1992) A point mutation of the α_2-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science* **257**, 977-980.

Tiberi M., Jarvie K.R., Silvia C., Falardeau P., Gingrich J.A., Godinot N., Bertrand L., Yang-Feng T.L., Fremeau R.T., and Caron M.G. (1991) Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D_1 dopamine receptor subtype: Differential expression pattern in rat brain compared with the D_{1A} receptor. *Proc. Natl. Acad. Sci. USA* **88**, 7491-7495.

Vallar L., Muca C., Magni M., Albert P., Bunzow J., Meldolesi J., and Civelli O. (1990) Differential coupling of dopaminergic D_2 receptors expressed in different cell types: Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in Ltk^- fibroblasts, hyperpolarization, and cytosolic-free Ca^{2+} concentration decrease in GH_4C_1 cells. *J. Biol. Chem.* **265**, 10320-10326.

Van Tol H.H.M., Bunzow J.R., Guan H.-C., Sunahara R.K., Seeman P., Niznik H.B., and Civelli O. (1991) Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**, 610-614.

Van Tol H.H.M., Wu C.M., Guan H.-C., Ohara K., Bunzow J.R., Civelli O., Kennedy J., Seeman P., Niznik H.B., and Jovanovic V. (1992) Multiple dopamine D4 receptor variants in the human population. *Nature* **358**, 149-152.

Wang C.-D., Buck M.A., and Fraser C.M. (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40**, 168-179.

Weiner D.M., Levey A.I., Sunahara R.K., Niznik H.B., O'Dowd B.F., Seeman P., and Brann M.R. (1991) D_1 and D_2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. USA* **88**, 1859-1863.

Weiner D.M. and Brann M.R. (1989) The distribution of dopamine D2 receptor mRNA in rat brain. *FEBS Lett.* **253**, 207-213.

Weinshank R.L., Adham N., Macchi M., Olsen M.A., Branchek T.A., and Hartig P.R. (1991) Molecular cloning and characterization of a high affinity dopamine receptor (D_{1B}) and its pseudogene. *J. Biol. Chem.* **266**, 22427-22435.

Zhou Q.-Y., Grandy D.K., Thambi L., Kushner J.A., Van Tol H.H.M., Cone R., Pribnow D., Salon J., Bunzow J.R., and Civelli O. (1990) Cloning and expression of human and rat D_1 dopamine receptors. *Nature* **347**, 76-80.

Manuscript #1

Contributions of Conserved Serine Residues to the Interactions of Ligands with Dopamine D2 Receptors

Barbara A. Cox[Ψ1], Robert A. Henningsen, Athena Spanoyannis*, Rachael L. Neve*, and Kim A. Neve

Medical Research Service, Veterans Affairs Medical Center and Departments of Pharmacology and [Ψ]Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon; *Department of Psychobiology, University of California, Irvine, California, USA

Address proofs and reprint requests to:

Kim A. Neve
Medical Research Service (151LL)
VA Medical Center
3710 SW US Veterans Hospital Rd.
Portland, OR 97207 USA
Tel. (503) 721-7911
FAX (503) 721-7839

Abbreviations used: $D2_{415}$, short (415-amino acid) form of D2 receptors; G protein, GTP-binding protein; NPA, *N*-propylnorapomorphine; PCR, polymerase chain reaction; SSC, standard sodium citrate.

[1]formerly Barbara Tester

Abstract: Four dopamine D2 receptor mutants were constructed, in each of which an alanine residue was substituted for one of four conserved serine residues, Ser-193, Ser-194, Ser-197, and Ser-391. Wild-type and mutant receptors were expressed transiently in COS-7 cells and stably in C_6 glioma cells for analysis of ligand-receptor interactions. In radioligand binding assays, the affinity of D2 receptors for dopamine was decreased 50-fold by substitution of alanine for Ser-193, implicating this residue in the binding of dopamine. Each mutant had smaller decreases in affinity for one or more of the ligands tested, with no apparent relationship between the class of ligand and the pattern of mutation-induced changes in affinity, except that the potency of agonists was decreased by substitution for Ser-193. The potency of dopamine for inhibition of adenylyl cyclase was substantially reduced by substitution of alanine for Ser-193 or Ser-197. Mutation of Ser-194 led to a complete loss of efficacy for dopamine and *para*-tyramine, which would be consistent with an interaction between Ser-194 and the *para*-hydroxyl substituent of dopamine that is necessary for activation of the receptors to occur.

Since mutation of the corresponding residues of β_2-adrenergic receptors has very different consequences, we conclude that although the position of these serine residues is highly conserved among catecholamine receptors, and the residues as a group are important in ligand binding and activation of receptors by agonists, the function of each of the residues considered separately varies among catecholamine receptors.

Running Title: Serine Residues and D2 Receptor Ligand Binding

Key words: dopamine, dopamine D2 receptors, cDNA, agonist

The molecular cloning of numerous receptors that are coupled to guanine nucleotide-binding proteins (G proteins) has revealed that these G protein-coupled receptors comprise a superfamily of genes encoding receptors with common structural features and significant conservation of amino acid sequence, particularly in the presumed transmembrane domains. Conservation of primary structure of G protein-coupled receptors is an important aid to analysis of structure-function relationships by site-directed mutagenesis, because functionally significant amino acid residues or domains are likely to be conserved. When a function becomes associated with a conserved amino acid residue in a specific receptor, this may become the basis for postulating a similar role for the corresponding residue in other receptors of the same sub-family. Some highly conserved amino acids do function similarly in a number of receptors; the amino acid corresponding to Asp-80 of dopamine D2 receptors, for example, is required for modulation of signaling pathways by D2 (Neve et al., 1991), β_2-adrenergic (Chung et al., 1988; Strader et al., 1988), m_1 muscarinic receptors (Fraser et al., 1989), and α_2-adrenergic receptors (Wang et al. 1991).

Catecholamine receptors have in common a cluster of serine residues in transmembrane region V. Serine residues corresponding to Ser-193, Ser-194, and Ser-197 of the rat D2 receptor are present in all known β-adrenergic receptors (Emorine et al., 1989, and references therein), dopamine receptors (Bunzow et al., 1988; Sokoloff et al., 1990; Dearry et al., 1990; Zhou et al.; 1990; Van Tol et al., 1991; Sunahara et al., 1991), and α-adrenergic receptors (Lomasney et al., 1991; Lanier et al., 1991; see references therein), although in some α_2-adrenergic receptor subtypes the two adjacent serine residues are separated from the third serine by four residues (Lanier et al., 1991; Wang et al., 1991). Ser-391 in transmembrane region VII is also highly conserved among G protein-coupled receptors. The contribution of these conserved serine residues to the interactions of ligands with receptors was determined first for β_2-adrenergic receptors. Strader et al. (1989) developed a model in which the *meta*- and

para-catechol hydroxyl groups of catecholamine transmitters interact with Ser-204 and Ser-207 of the hamster β_2-adrenergic receptor, residues corresponding to Ser-194 and Ser-197 of the rat D2 receptor. For human α_{2A}-adrenergic receptors, which do not have precisely the same alignment of serine residues as β_2-adrenergic receptors, ligand binding and functional studies provide some evidence for an interaction between the *para*-hydroxyl substituent and Ser-204 (Wang et al., 1991), corresponding to Ser-207 of β_2-adrenergic receptors. To determine if either model also describes binding of agonists to dopamine receptors, we have constructed four dopamine D2 receptor mutants in which an alanine residue was substituted for one of the conserved serine residues at positions 193, 194, 197, and 391 of $D2_{415}$, the short form of D2 receptors (Bunzow et al., 1988). Our results indicate that, as shown for β_2-adrenergic receptors, some conserved serine residues contribute substantially and selectively to the binding of catecholamine agonists and to activation of D2 receptors. The contribution of each serine residue, however, differs from the contribution of the corresponding residue in β_2-adrenergic receptors.

MATERIALS AND METHODS

Materials

All radioisotopes were purchased from Dupont-New England Nuclear (Boston, MA). Spiperone (Janssen), quinpirole (Lilly), (*S*)-(-)-*N*-[(1-ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide and eticlopride (Dr. T. de Paulis, Vanderbilt University), and a rat $D2_{415}$ cDNA (Dr. O. Civelli, Oregon Health Sciences University) were generous gifts. Sulpiride, R(-)-propylnorapomorphine (NPA), and 3-hydroxyphenethylamine (*meta*-tyramine) were purchased from Research Biochemicals, Inc. (Natick, MA). Dopamine (3-hydroxytyramine) and 4-hydroxyphenethylamine (*para*-tyramine) were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction and Expression of D2 Receptor Mutants

A full-length $D2_{415}$ cDNA (Bunzow et al., 1988) was cloned into pRSV (Gorman et al., 1983) modified by the addition of a polylinker containing several unique restriction sites. Trans-PCR was used to join DNA fragments containing a region of overlap into which the desired base substitutions were introduced, as described previously (Neve et al., 1991). To create the alanine substitutions for Ser-193 and Ser-194, we synthesized the oligonucleotide primers 5'-GTCTACGCCTCCATTGTCTCATTC-3', 5'-GTCTACTCCGCCATTGTCTCATTC-3', and their reverse complements, in both sets of primers altering TCC (Ser) to GCC (Ala). To substitute alanine for Ser-197 we used the oligonucleotide 5'-GTCTACTCCTCCATTGTCGCATTC-3' and its reverse complement, changing TCA (Ser) to GCA (Ala). Alanine (GCT) was substituted for Ser-391 (AGT) using the primer 5'-GGCTATGTCAACGCTGCCGTCAAC-3' and its reverse complement. Confirmation of the mutations was obtained by cDNA sequence analysis.

COS-7 cells, used for transient expression of D2 receptor cDNAs, were maintained at 37° in Dulbecco's modified Eagle's medium supplemented with 8% iron-supplemented calf bovine serum and 2% fetal bovine serum. Cells were seeded in 20 ml of medium in a 150-mm diameter plate 24 hr prior to transfection. Transfection was carried out by calcium phosphate precipitation (Chen and Okayama, 1988). Plasmid DNA (30 μg) was mixed with 1.0 ml of 0.25 M $CaCl_2$. An equal volume of 2X BES-buffered saline [50 mM *N,N*-bis(hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95) was added and the mixture was incubated at room temperature for 15 min before being added by drops to the medium in the plate. The plate was incubated 20-24 hr in an atmosphere of 3% CO_2/97% air, washed with phosphate-buffered saline, then re-fed and incubated in an atmosphere of 10% CO_2 for 48 hr before harvesting for analysis of RNA or for receptor binding assays.

Stable expression of mutant and wild-type D2 receptors in C_6 glioma cells was obtained by calcium phosphate precipitation exactly as described previously (Neve et al., 1991) except that D2 receptor cDNA (15 μg) was co-transfected with pBabe Puro (2 lg), conferring resistance to puromycin (Morgenstern and Land, 1990). Puromycin-resistant cells were isolated by selection at a concentration of 2 μg/ml, a concentration that kills control C_6 cells within 1 week (unpublished observations).

RNA Blot Hybridization

A 1.3-kilobase *Xho*I-*Bgl*II fragment of $D2_{415}$ was ^{32}P-labeled using a Boehringer Mannheim random-primed DNA labeling kit. Methods of RNA isolation, electrophoresis in formaldehyde/agarose gels, and transfer to Biotrans membrane were as described (Neve et al., 1986). Hybridizations were carried out overnight in 5X standard saline citrate (SSC)/50% formamide at 42°, followed by washes taken to 0.1X SSC at 55°. The washed membrane was exposed to Kodak X-OMAT film for 24 hr at -70°.

Radioligand Binding Assays

[^{125}I]Epidepride (NCQ 219, (*S*)-(-)-*N*-[(1-ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-[^{125}I]iodo-benzamide) was prepared from its tributyltin precursor using a modification (Neve et al., 1991) of the method of Clanton et al. (1991). Tissue preparation and radioligand binding assays were carried out essentially as described (Neve et al., 1990; Neve et al., 1991). Aliquots of the membrane preparation (3-20 μg 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, [^{125}I]epidepride (2,200 Ci/mmol), and appropriate drugs. Spiperone (1 μM) was used to define nonspecific binding, because spiperone has extremely low affinity for α_2-adrenergic receptors, which may be labeled by [^{125}I]epidepride in some tissues

(Neve et al., 1990). GTP (100 μM) was added to assays in experiments assessing the binding of agonists, to ensure that the ligand-receptor interaction was assessed without effects due to receptor coupling with G proteins. Saturation and competition experiments were carried out in a volume of 0.5 ml and 0.25 ml, respectively.

Cyclic AMP Accumulation

The accumulation of cyclic AMP in intact cells was measured using the method of Shimizu et al. (1969) as described previously (Neve et al., 1991). The ability of D2 receptor agonists to inhibit cyclic AMP accumulation stimulated by the ß-adrenergic receptor agonist isoproterenol was determined. Results are expressed as the percentage of [^{3}H]ATP converted to [^{3}H]cAMP.

Data Analysis

Data were analyzed by nonlinear regression using the program GraphPAD. In saturation binding experiments, the free concentration of [^{125}I]epidepride was calculated as the concentration added minus the concentration bound. In all competition experiments the concentration of [^{125}I]epidepride ranged from 74 to 120 pM. K_I values were calculated from experimentally determined IC_{50} values as described by Munson and Rodbard (1988). Averages for K_I, K_D, and EC_{50} values are expressed as the geometric means [the antilogarithm of mean logarithms (Fleming et al., 1972)] followed by the 95% confidence interval of the mean in parentheses. Statistical comparison of the mean logarithms was carried out using Student's *t*-test, with Dunnett's correction for multiple comparisons. The standard Gibbs free energy change (ΔG^o) was calculated from the equation $\Delta G^o = RT\ln K_D$, where R is the gas constant and T is the temperature in degrees Kelvin. Protein was measured by the method of Peterson (1977).

RESULTS

Analysis of mutant and wild-type D2 receptor RNA in COS-7 cells

Four different point mutations were introduced into the $D2_{415}$ cDNA by the PCR, using trans-PCR. Each mutation changed one conserved serine residue to an alanine residue, yielding the mutants Ala193-D2, Ala194-D2, Ala197-D2, and Ala391-D2. Transfection of the mutant and wild-type cDNAs into COS-7 cells resulted in the expression of high levels of recombinant D2 receptor mRNA, whereas there was no detectable hybridization of a D2 receptor cDNA probe to RNA from control COS-7 cells (Fig. 1), or to RNA from cells transfected with pRSVneo (data not shown). As shown in Figure 1, 2 forms of D2 receptor mRNA were present in COS-7 cells expressing wild-type or mutant D2 receptors. The shorter band, corresponding to the band present in RNA prepared from rat striatum, represents full-length D2 receptor mRNA (approximately 2.6 kb), whereas the upper band probably consists of D2 receptor mRNA that includes the SV40 3' untranslated region and polyadenylation site (Gorman et al., 1983).

Binding of [^{125}I]epidepride to wild-type and mutant D2 receptors

COS-7 cells transfected with wild-type or mutant D2 receptor cDNA had high densities of receptors labeled by the selective D2-receptor ligand, [^{125}I]epidepride (Table 1), whereas control cells or cells transfected with pRSVneo had no detectable specific binding of [^{125}I]epidepride (data not shown). [^{125}I]Epidepride bound to transiently expressed wild-type D2 receptors with a mean K_D of 46 pM (Table 2), a value close to that determined using rat striatal membranes or recombinant $D2_{415}$ stably expressed in mammalian cells (Neve et al., 1990; Neve et al., 1991). The affinity of Ala193-D2 and Ala197-D2 for [^{125}I]epidepride was similar to that of $D2_{415}$. The affinity of Ala194-D2 and Ala391-D2 for [^{125}I]epidepride was decreased 4- to 5-fold, compared that of wild-type receptors

Competition analysis of the affinity of wild-type and mutant D2 receptors for drugs

The apparent affinity of several agonist and antagonist ligands for D2 receptor mutants was determined by inhibition of the binding of [^{125}I]epidepride (Tables 2 and 3). Although Ala194-D2 and Ala391-D2 had moderately decreased affinity for [^{125}I]epidepride, all four mutants had affinities for the prototypical D2 antagonist spiperone that were indistinguishable from that of $D2_{415}$. To determine if the greater sensitivity of [^{125}I]epidepride to replacement of conserved serine residues was a characteristic of substituted benzamide derivatives, inhibition of the binding of [^{125}I]epidepride by the substituted benzamides sulpiride and eticlopride was assessed. None of the mutations altered the binding of eticlopride, whereas the potency of sulpiride for Ala197-D2 and Ala391-D2 was slightly decreased.

The apparent affinity of D2 receptors for dopamine was decreased 50-fold by substitution of an alanine residue for Ser-193, and moderately decreased by substitution for Ser-391 (Fig. 2, Tables 2 and 3). The affinities of Ala194-D2 and Ala197-D2 for dopamine differed slightly or not at all from $D2_{415}$. Two other D2 receptor agonists were affected differentially by the mutations; the affinities of Ala194-D2 and Ala193-D2 for NPA were decreased approximately 15-fold and the potency of NPA for Ala197-D2 was slightly reduced compared to $D2_{415}$, whereas the potency of the non-catecholamine agonist quinpirole for Ala194-D2 and Ala193-D2 was slightly reduced.

To explore the hypothesis that catechol hydroxyl groups interact with conserved serine residues, the affinities of tyramine (*para*-tyramine, 4-hydroxyphenethylamine) and its isomer *meta*-tyramine (3-hydroxyphenethylamine) were determined (Table 2, Fig. 3). Compared to dopamine, *meta*-tyramine and *para*-tyramine were approximately 10- and 100-fold less potent, respectively, at inhibiting the binding of [^{125}I]epidepride to $D2_{415}$ (Table 2). None of the mutations resulted in substantial

changes in affinity for either isomer of tyramine, although the affinities of Ala193-D2 for *para*-tyramine and Ala391-D2 for *meta*-tyramine were decreased slightly.

Mutation-induced changes in drug potency were quantified by calculating the difference between free energy changes associated with ligand binding to mutant and wild-type receptors (Table 3). The greatest decrease in the free energy change was observed for binding of the catecholamine agonists dopamine (2.33 kcal/mol) and NPA (1.72 kcal/mol) to Ala193-D2.

Inhibition of Adenylyl Cyclase

To assess the importance of interactions between catechol hydroxyl groups and conserved serine residues in transmembrane region V for dopamine-induced inhibition of adenylyl cyclase, wild-type and mutant receptors were stably expressed in C_6 glioma cells. The average density of receptors on the cell lines used in these studies is shown in Table 1. The affinity of each receptor for [^{125}I]epidepride, determined by saturation analysis (Fig. 4A and 4B), was as follows: $D2_{415}$, 43 pM; Ala193-D2, 40 pM; Ala194-D2, 226 pM, Ala197-D2, 55 pM; Ala391-D2, 198 pM. These values are virtually identical to those determined using COS-7 cells transiently expressing the receptors.

The effect of alanine substitutions on EC_{50} values for inhibition of cyclic AMP accumulation by dopamine were in qualitative agreement with results from radioligand binding assays. Dopamine was 200-fold less potent at Ala193-D2 than at wild-type receptors, and 18-fold less potent at Ala-197-D2 (Table 4, Fig. 5A). Although *meta*- and *para*-tyramine were both considerably less potent than dopamine at activating wild-type receptors, the potency of the monohydroxylated analogs was not markedly altered by substitution for any of the serine residues (Table 4, Fig. 5B and 5C). At wild-type D2 receptors, both *meta*- and *para*-tyramine were partial agonists, as shown by maximal inhibition of enzyme activity that was 76% and 65%, respectively, of the maximal inhibition observed for dopamine. None of the mutations markedly altered

the efficacy of dopamine, *meta*-tyramine, or *para*-tyramine, except for substitution for Ser-194. Both dopamine and *para*-tyramine were completely ineffective at inhibiting cyclic AMP accumulation in cells expressing Ala194-D2.

DISCUSSION

Much of the free energy of binding of catecholamines to β_2-adrenergic receptors results from interactions of the agonists with three amino acid residues, Asp-113, Ser-204, and Ser-207 (Strader et al., 1988; Strader et al., 1989). Other conserved serine residues make important contributions to β_2-adrenergic receptor function, because substitution for Ser-319 decreases the affinity of catecholamines, whereas substitution for Ser-203 prevents the appearance of immunoreactive protein (Strader et al., 1989). The contributions of Ser-204 and Ser-207 have received considerable attention, however, because specific hydrogen-bonding interactions between those residues and the *meta*- and *para*-hydroxyl groups of catecholamines have been identified. On the basis of this model describing the binding of catecholamines to β_2-adrenergic receptors, similar interactions involving the corresponding serine residues have been proposed for dopamine D1 (O'Dowd et al., 1990; Dearry et al., 1990), D2 (Hibert et al., 1991; cover, *FASEB J.* 4, issue 10, 1990), D3 (Sokoloff et al., 1990), D4 (Van Tol et al., 1991), and D5 receptors (Sunahara et al., 1991). While our data support the importance of the conserved serine residues in transmembrane region V, the data also show substantial differences between dopamine D2, β_2-adrenergic, and other catecholamine receptors in the functions of the residues.

The most striking difference between D2 and β_2-adrenergic receptors is that transfection of COS-7 cells by each of the D2 receptor mutants resulted in high levels of expression of mRNA and receptor protein, in contrast to the lack of detectable protein after substitution of alanine for Ser-203 of β_2-adrenergic receptors (Strader et al., 1989). Mutation of the corresponding residue of dopamine D1 receptors, Ser-198, also yielded functional receptors, although with reduced affinity for both agonists and

antagonists (D. Frail, personal communication). The lack of effect of the serine substitutions on the affinity of dopamine D2 receptors for the ligands spiperone and eticlopride indicates that none of the mutations caused gross structural changes in the expressed protein.

A second major difference between D2 and β_2-adrenergic receptors is that, whereas Ser-204 and Ser-207 contribute substantially to the binding of catecholamines to β_2-adrenergic receptors, substitution for the corresponding residues in D2 receptors had little effect on the binding of dopamine. Substitution of alanine for Ser-194 had no effect on the potency of dopamine in radioligand binding assays, and substitution for Ser-197 decreased the potency of dopamine only slightly. In contrast, substitution for Ser-193 of D2 receptors decreased the potency of dopamine 50-fold. Mutation of Ser-391, like Ser-319 of β_2-adrenergic receptors, moderately decreased the affinity of D2 receptors for the natural ligand.

The relative contributions of Ser-193 and Ser-197 to the binding of dopamine were confirmed in functional studies of inhibition of adenylyl cyclase, in which the potency of dopamine was reduced more at Ala193-D2 than at Ala197-D2. Interestingly, dopamine had no detectable agonist activity when Ser-194 was replaced by alanine. Although dopamine binds equally well to wild-type D2 receptors or Ala194-D2, Ser-194 is necessary for activation of the receptors by dopamine. The absolute requirement for one serine residue for activation of a receptor by the native agonist has not been observed for β_2-adrenergic receptors (Strader et al., 1989), α_{2A}-adrenergic receptors (Wang et al., 1991), or dopamine D1 receptors (D. Frail, personal communication). It could by hypothesized that the contrast between the unchanged affinity of Ala194-D2 for dopamine and the loss of agonist activity results from a separation of the residues involved in ligand binding and activation of the receptors. A second possibility is that Ser-194 is normally involved in the binding of dopamine as

well as activation of the receptors, but that in the absence of Ser-194 dopamine binds to a distinct site with equal affinity but a loss of efficacy.

On the basis of structure-activity relationships for D2 receptor agonists, it has been proposed that agonists must have a hydrogen-bonding group to interact with a primary hydrogen-bonding site on the receptor (Seeman, 1980). According to this model, the *meta*-hydroxyl group at position 3 on the ring for dopamine interacts with the primary hydrogen-bonding site, whereas the *para*-hydroxyl group interacts with an accessory site. Our data support this model in some respects, and it is tempting to assign the role of the primary hydrogen-bonding site to Ser-193. Thus, D2 receptors had higher affinity for *meta*-tyramine than for *para*-tyramine. Also, substitution for Ser-193 substantially decreased the potency of the catecholamine agonists dopamine and NPA, but was not additive with substitution of hydrogen for the *meta*-hydroxyl group of dopamine (*para*-tyramine).

Other data, however, do not support the hypothesis that the primary hydrogen-bonding site is Ser-193. For example, if the 3-hydroxyl group of catecholamines interacts with Ser-193, substitution for that residue might be expected to decrease the potency of *meta*-tyramine. The slight decrease in affinity of Ala193-D2 for *meta*-tyramine observed in the present experiments was not statistically significant, however, and the rank order of potency for dopamine and *meta*-tyramine at Ala193-D2 is reversed compared to the rank order at $D2_{415}$. In addition, the binding of quinpirole was only slightly altered by substitution for Ser-193, and was decreased more by substitution for Ser-194. Each of these issues merits further consideration.

The use of the two isomers of tyramine in these studies was based on the assumption that if a hydrogen bond exists between specific groups on the ligand and the receptor, binding of the ligand will be disrupted to the same extent whether the hydrogen-bonding group is removed from the ligand or from the receptor. This assumption may not be valid if receptors recognize the stereoelectronic structure of

ligands rather than specific atoms (Collin et al., 1989). Removal of a hydroxyl group from the catechol ring of a ligand would certainly cause changes in the overall stereoelectronic structure that extend beyond the simple removal of a hydrogen-bonding group. Our data suggest that the stereoelectronic features of *meta*-tyramine that are recognized by D2 receptors are quite different from the significant stereoelectronic features of dopamine. This is supported by the effects of dopamine and the tyramine isomers on adenylyl cyclase. The finding that neither dopamine nor *para*-tyramine retained agonist activity at Ala194-D2 suggests that an interaction between the *para*-hydroxyl group and Ser-194 is required for activation of the receptors by these compounds. The agonist activity of *meta*-tyramine, on the other hand, was not diminished by substitution for any of the serine residues. The lack of effect of the mutations on binding to or activation of the receptors by *meta*-tyramine is in agreement with the view that *meta*-tyramine and dopamine differ substantially in the way in which they interact with D-2 receptors.

The second issue concerns our finding that quinpirole may interact with a distinct hydrogen-bonding site, since the potency of quinpirole was only slightly decreased by substitution for Ser-194 or Ser-193, and unaltered by the other mutations. Our data, including those described above for *meta*-tyramine, suggest that ligands, even structurally related compounds, interact with unique but overlapping sites on receptors. As shown in Table 3, substitution for Ser-193 greatly or moderately decreased the potency of dopamine and NPA, and slightly decreased the potency of *para*-tyramine and quinpirole. Substitution for Ser-194 decreased the potency of [^{125}I]epidepride, NPA, and quinpirole. Ala197-D2 had slightly decreased affinity for dopamine, NPA, and sulpiride. Ala391-D2 had slightly-to-moderately decreased affinity for [^{125}I]epidepride, dopamine, sulpiride, and *meta*-tyramine. These data support the findings of Marullo et al. (1990), who concluded on the basis of studies with chimeric β_1-/β_2-adrenergic receptors that each ligand has structural properties that determine

different sites of interaction with the receptor, so that it may not always be possible to define, for example, a distinct agonist binding site, or to define a particular receptor-ligand interaction associated with pharmacological specificity.

In conclusion, our results demonstrate that, whereas the position of several serine residues in transmembrane domain V is highly conserved among catecholamine receptors, the individual contribution of each residue to the binding of ligands is not conserved among catecholamine receptors. Ser-193 contributes importantly to the binding of dopamine and other agonists; our data do not, however, unambiguously support an interaction between Ser-193 and a particular hydrogen-bonding group of dopamine, or support a role for Ser-193 as the primary hydrogen-bonding site for all agonists. Ser-194 is absolutely required for activation of D2 receptors by some agonists, perhaps as a result of bonding with the *para*-hydroxyl group of catecholamines.

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REFERENCES

Bunzow J.R., Van Tol H.H.M., Grandy D.K., Albert P., Salon J., Christie M., Machida C.A., Neve K.A., and Civelli O. (1988) Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature* **336**, 783-787.

Chen C.A. and Okayama H. (1988) Calcium phosphate-mediated gene tranfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques* **6**, 632-638.

Chung F.-Z., Wang C.-D., Potter P.C., Venter J.C., and Fraser C.M. (1988) Site-directed mutagenesis and continuous expression of human ß-adrenergic receptors: Identification of a conserved aspartate residue involved in agonist binding and receptor activation. *J. Biol. Chem.* **263**, 4052-4055.

Clanton J.A., de Paulis T., Schmidt D., Ansari M., Manning R.G., Baldwin R., and Kessler R.M. (1991) Preparation of [123I]- and [125I]-epidepride: a dopamine D2 receptor antagonist radioligand. *J. Labelled Compounds Radiopharmaceut.* **29**, 745-751.

Collin S., Evrard G., Vercauteren D.P., Durant F., Carrupt P.-A., Van de Waterbeemd H., and Testa B. (1989) Stereoelectronic study of zetidoline, a dopamine D2 receptor antagonist. *J. Med. Chem.* **32**, 38-42.

Dearry A., Gingrich J.A., Falardeau P., Fremeau R.T., Bates M.D., and Caron M.G. (1990) Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature* **347**, 72-75.

Emorine L.J., Marullo S., Briend-Sutren M.-M., Patey G., Tate K., Delavier-Klutchko C., and Strosberg A.D. (1989) Molecular characterization of the human β_3-adrenergic receptor. *Science* **245**, 1118-1121.

Fleming W.W., Westfall D.P., De La Lande I.S., and Jellett L.B. (1972) Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmacol. Exper. Ther.* **181**, 339-345.

Fraser C.M., Wang C.-D., Robinson D.A., Gocayne J.D., and Venter J.C. (1989) Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**, 840-847.

Gorman C., Padmanabhan R., and Howard B.H. (1983) High efficiency DNA-mediated transformation of primate cells. *Science* **221**, 551-553.

Hibert M.F., Trumpp-Kallmeyer S., Bruinvels A., and Hoflack J. (1991) Three-dimensional models of neurotransmitter G-binding protein-coupled receptors. *Mol. Pharmacol.* **40**, 8-15.

Lanier S.M., Downing S., Duzic E., and Homcy C.J. (1991) Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor: identification of a unique receptor subtype. *J. Biol. Chem.* **266**, 10470-10478.

Lomasney J.W., Cotecchia S., Lorenz W., Leung W.-Y., Schwinn R.J., Yang-Feng T.L., Brownstein M., Lefkowitz R.J., and Caron M.G. (1991) Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor: the gene for which is located on human chromosome 5. *J. Biol. Chem.* **266**, 6365-6369.

Marullo S., Emorine L.J., Strosberg A.D., and Delavier-Klutchko C. (1990) Selective binding of ligands to β1, β2 or chimeric β1/β2-adrenergic receptors involves multiple subsites. *EMBO J.* **9**, 1471-1476.

Morgenstern, J.P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl. Acids Res.* **18**, 3587-3596.

Munson P.J. and Rodbard D. (1988) An exact correction to the "Cheng-Prusoff" correction. *J. Recept. Res.* **8**, 533-546.

Neve K.A., Cox B.A., Henningsen R.A., Spanoyannis A., and Neve R.L. (1991) Pivotal role for aspartate-80 in regulation of D2 receptor affinity for drugs and coupling to adenylyl cyclase. *Mol. Pharmacol.* **39**, 733-739.

Neve K.A., Henningsen R.A., Kinzie J.M., de Paulis T., Schmidt D.E., Kessler R.M., and Janowsky A. (1990) Sodium-dependent isomerization of dopamine D-2 receptors characterized using [^{125}I]epidepride, a high-affinity substituted benzamide ligand. *J. Pharmacol. Exper. Ther.* **252**, 1108-1116.

Neve R.L., Harris P., Kosik K.S., Kurnit D.M., and Donlon T.A. (1986) Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Mol. Brain Res.* **1**, 271-280.

O'Dowd B.F., Nguyen T., Tirpak A., Jarvie K.R., Israel Y., Seeman P., and Niznik H.B. (1990) Cloning of two additional catecholamine receptors from rat brain. *FEBS Lett.* **262**, 8-12.

Peterson G.L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 346-356.

Seeman P. (1980) Brain dopamine receptors. *Pharmacol. Rev.* **32**, 229-313.

Shimizu H., Daly J.W., and Creveling C.R. (1969) A radioisotopic method for measuring the formation of adenosine 3',5'-monophosphate in incubated slices of brain. *J. Neurochem.* **16**, 1609-1619.

Sokoloff P., Giros B., Martres M.-P., Bouthenet M.-L., and Schwartz J.-C. (1990) Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature* **347**, 146-151.

Strader C.D., Candelore M.R., Hill W.S., Sigal I.S., and Dixon R.A.F. (1989) Identification of two serine residues involved in agonist activation of the ß-adrenergic receptor. *J. Biol. Chem.* **264**, 13572-13578.

Strader C.D., Sigal I.S., Candelore M.R., Rands E., Hill W.S., and Dixon R.A.F. (1988) Conserved aspartic acid residues 79 and 113 of the ß-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263**, 10267-10271.

Sunahara R.K., Guan H.-C., O'Dowd B.F., Seeman P., Laurier L.G., Ng G., George S.R., Torchia J., Van Tol H.H.M., and Niznik H.B. (1991) Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature* **350**, 614-619.

Van Tol H.H.M., Bunzow J.R., Guan H.-C., Sunahara R.K., Seeman P., Niznik H.B., and Civelli O. (1991) Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**, 610-614.

Wang C.-D., Buck M.A., Fraser, C.M. (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40**, 168-179.

Zhou Q.-Y., Grandy D.K., Thambi L., Kushner J.A., Van Tol H.H.M., Cone R., Pribnow D., Salon J., Bunzow J.R., and Civelli O. (1990) Cloning and expression of human and rat D_1 dopamine receptors. *Nature* **347**, 76-80.

TABLE 1. *Density of wild-type and mutant D2 receptors*

	B_{max} (fmol/mg of protein)				
Drug	D2-415	Ala193-D2	Ala194-D2	Ala197-D2	Ala391-D2
COS-7 cells	1103 ± 258	1202 ± 371	529 ± 74	683 ± 18	713 ± 277
C_6 cells	144 ± 11	746 ± 68	1079 ± 288	534 ± 11	105 ± 23

The density of binding sites was determined by saturation analysis of the binding of [^{125}I]epidepride in membranes prepared from cells expressing wild-type or mutant D2 receptors transiently (COS-7 cells) or stably (C_6 glioma cells). Results are given as the mean of 3 (C_6 cells) or 5-8 (COS-7 cells) independent experiments ± SEM.

TABLE 2. *Drug affinity for wild-type and mutant D2 receptors*

	Affinity				
Drug	D2-415	Ala193-D2	Ala194-D2	Ala197-D2	Ala391-D2
epidepride (pM)	46 (35-59)	54 (30-99)	167 (141-198)	30 (22-42)	165 (101-271)
spiperone (pM)	50 (35-72)	51 (37-70)	31 (23-42)	40 (28-56)	22 (16-30)
eticlopride (pM)	37 (25-53)	27 (10-70)	41 (28-62)	30 (15-60)	74 (18-305)
sulpiride (nM)	11 (9-13)	5 (4-6)	8 (4-16)	44 (38-51)	58 (28-119)
NPA (nM)	20 (14-30)	350 (210-590)	280 (50-170)	83 (65-107)	17 (3-81)
dopamine (μM)	2.7 (2.1-3.5)	129 (94-176)	3.2 (2.4-4.1)	7.1 (5.4-9.4)	10 (5.5-18)
quinpirole (μM)	4.1 (3.4-5.0)	8.4 (5.9-12)	18 (6-57)	5.1 (3.1-8.2)	2.9 (1.0-8.5)
m-tyramine (μM)	20 (14-27)	36 (23-57)	19 (7-51)	17 (7-41)	44 (35-55)
p-tyramine (μM)	160 (120-210)	320 (310-340)	160 (25-989)	160 (130-190)	220 (180-260)

The apparent affinity (K_I) of the indicated drugs was determined by inhibition of the binding of [^{125}I]epidepride to membranes prepared from COS-7 cells transiently expressing $D2_{415}$ or mutant D2 receptors. Affinity values for epidepride are K_D values determined by saturation analysis of the radioligand, and were used to calculate K_I values for the other drugs. Mean affinity values from 2-8 independent experiments are followed by the 95% confidence limits of the mean in parentheses. Experiments with agonists were carried out in the presence of 100 μM GTP. Affinity values are in the units specified for each drug.

TABLE 3. *Mutation-induced shifts in free energy change of ligand binding*

	$\Delta(\Delta G^o)$			
Drug	Ala193-D2	Ala194-D2	Ala197-D2	Ala391-D2
epidepride	--	0.82	--	0.81
sulpiride	--	--	0.84	1.01
dopamine	2.33	--	0.59	0.79
NPA	1.72	1.59	0.85	--
quinpirole	0.43	0.89	--	--
m-tyramine	--	--	--	0.49
p-tyramine	0.42	--	--	--

The magnitude of the shift in affinity resulting from the substitution of an alanine for a serine residue was determined from the data in Table 2. The free energy change of binding (ΔG^o) was calculated for each drug-receptor interaction, and the decrease in ΔG^o, or $\Delta(\Delta G^o)$, associated with the mutation of a particular serine residue was determined from the difference between ΔG^o for the mutant receptor and for $D2_{415}$. Values are expressed as kcal/mol. Results are shown only for statistically significant decreases in affinity ($p < 0.05$), with -- indicating no significant difference. Results for eticlopride and spiperone are not included, since binding of these ligands was not altered by any of the mutations.

TABLE 4. *Effects of mutations on inhibition of isoproterenol-stimulated cyclic AMP accumulation by D2 receptor agonists*

	$D2_{415}$	Ala193-D2	Ala194-D2	Ala197-D2
dopamine				
EC_{50}	5 nM (2-20)	1.1 μM (0.7-16)	nd[a]	90 nM (51-160)
Max	92 ± 2%	81 ± 7%	nd[a]	90 ± 2%
m-tyramine				
EC_{50}	4 μM (1-26)	1.0 μM (0.1-9.8)	2 μM (1-3)	13 μM (10-19)
Max	70 ± 6%	67 ± 8%	74 ± 12%	61 ± 4%
p-tyramine				
EC_{50}	7 μM (2-30)	12 μM (4-32)	nd[a]	4 μM (0.5-40)
Max	60 ± 13%	57 ± 4%	nd[a]	46 ± 4%

Results are shown from 3-5 independent experiments in which inhibition of isoproterenol-stimulated cyclic AMP accumulation was determined using intact C_6 cells expressing wild-type or mutant D2 receptors. The concentration at which 50% of maximal inhibition was observed (EC_{50}) was determined by nonlinear regression. Values given are the geometric means followed by the 95% confidence limits of the mean. The maximal inhibition observed (Max) is expressed as a percentage of total isoproterenol-stimulated activity. The mean ± SEM is given.

[a]no detectable inhibition of adenylyl cyclase was observed.

FIGURE LEGENDS

Fig. 1. Northern blot analysis demonstrates specific hybridization of a D2 receptor cDNA probe to wild-type and mutant D2 receptors. Thirty μg of total RNA was electrophoresed in each lane. Lane 1, $D2_{415}$; lane 2, Ala197-D2; lane 3, Ala194-D2; lane 4, Ala193-D2; lane 5, Ala391-D2; lane 6, control COS-7 cells; lane 7, rat neostriatum. The presence of 2 bands in transfected COS-7 cells is probably due to the presence of two polyadenylation sites, as explained further in *Results*.

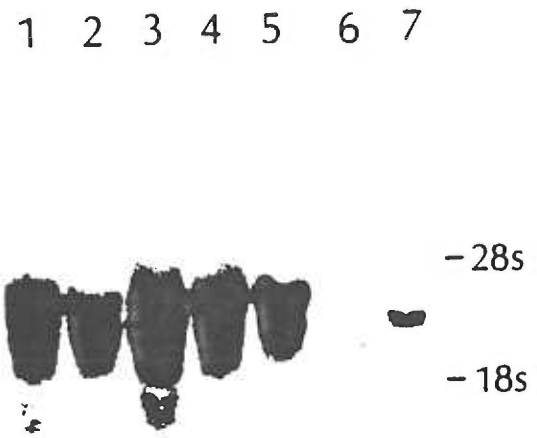
Fig. 2. The binding of [^{125}I]epidepride to wild-type and mutant D2 receptors was inhibited by multiple concentrations of dopamine. Each curve is representative of 3-8 independent experiments. Results are plotted as radioligand bound, expressed as a percentage of specific binding in the absence of inhibitor, *vs.* the logarithm of the concentration of dopamine. In the *legend*, S193A designates the mutant in which Ser-193 was replaced by an alanine residue (Ala193-D2), and so on.

Fig. 3. The chemical structures of the agonists used in the present study are shown.

Fig. 4. A. Saturation isotherms of the binding of [^{125}I]epidepride to membranes prepared from C_6 cells expressing $D2_{415}$ or mutant D2 receptors are shown. Results, from one of three independent experiments, are plotted as radioligand bound, expressed as a percentage of B_{max} for each tissue, *vs.* the corrected free concentration of [^{125}I]epidepride (total radioligand added minus amount bound). In this experiment, affinity (K_D) and B_{max} values, respectively, for each tissue were 37 pM and 126 fmol/mg of protein (D-2_{415}), 33 pM and 702 fmol/mg of protein (Ala193-D2), 219 pM and 930 fmol/mg of protein (Ala194-D2), 56 pM and 551 fmol/mg of protein (Ala197-D2), and 168 pM and 144 fmol/mg of protein (Ala391-D2). In the *legend*, S193A designates the mutant in which Ser-193 was replaced by an alanine residue

(Ala193-D2), and so on. B. Data are transformed and plotted as Bound (fmol/mg of protein) *vs*. Bound/Free radioligand (pM).

Fig. 5 Data and computer-generated curves are shown for inhibition of isoproterenol-stimulated cyclic AMP accumulation by the indicated D2 receptor agonists. Each curve is representative of 3-5 independent experiments. Results are plotted as the percentage of total isoproterenol-stimulated conversion of [^{3}H]ATP to [^{3}H]cyclic AMP *vs*. the logarithm of the concentration of agonist. In the *legend*, S193A designates the mutant in which Ser-193 was replaced by an alanine residue (Ala193-D2), and so on. A. Inhibition of adenylyl cyclase by dopamine. No curve was drawn for the data shown for mutant Ala194-D2, as there was no detectable inhibition of activity. B. Inhibition of adenylyl cyclase by *meta*-tyramine. C. Inhibition of adenylyl cyclase by *para*-tyramine.



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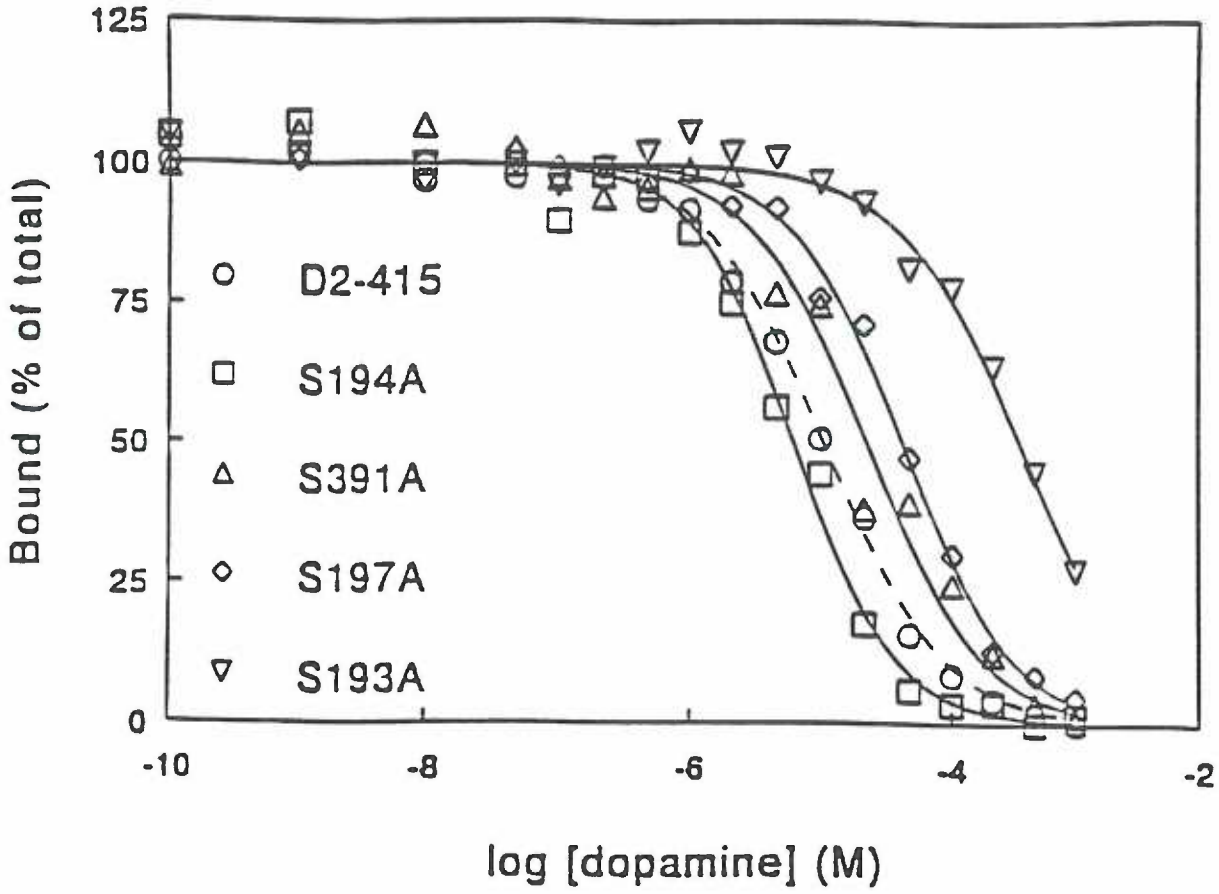

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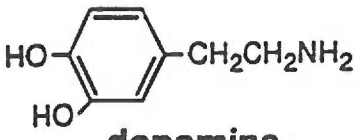
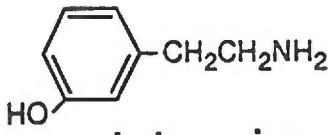
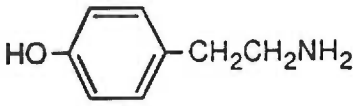


dopamine



meta-tyramine



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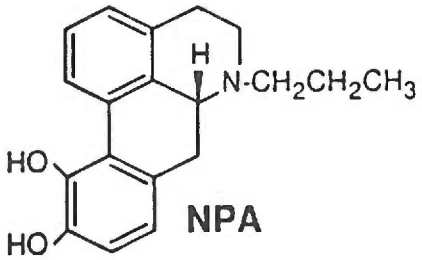
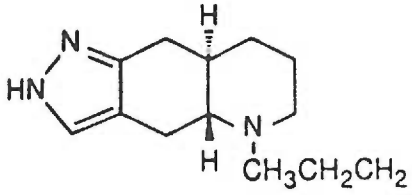
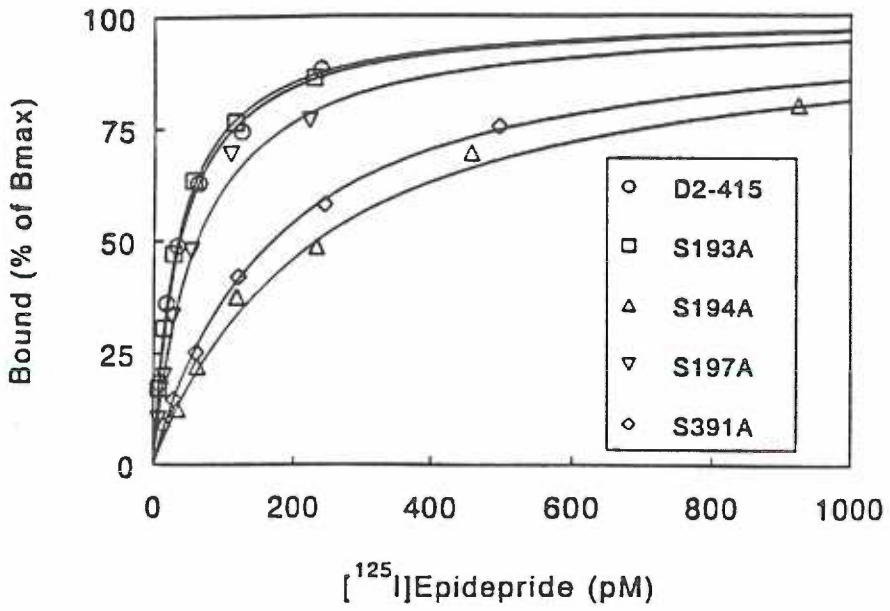


NPA



quinpirole

Fig. 3. The chemical structures of the agonists used in the present study are shown.



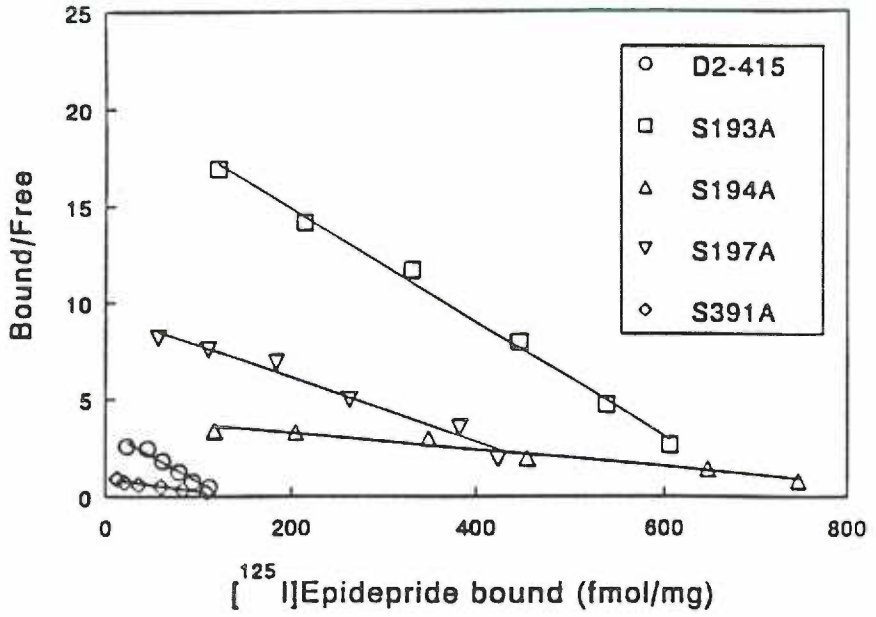


Fig. 4. A. Saturation isotherms of the binding of [^{125}I]epidepride to membranes prepared from C_6 cells expressing $D2_{415}$ or mutant D2 receptors are shown. Results, from one of three independent experiments, are plotted as radioligand bound, expressed as a percentage of B_{max} for each tissue, *vs.* the corrected free concentration of [^{125}I]epidepride (total radioligand added minus amount bound). In this experiment, affinity (K_D) and B_{max} values, respectively, for each tissue were 37 pM and 126 fmol/mg of protein ($D\text{-}2_{415}$), 33 pM and 702 fmol/mg of protein (Ala193-D2), 219 pM and 930 fmol/mg of protein (Ala194-D2), 56 pM and 551 fmol/mg of protein (Ala197-D2), and 168 pM and 144 fmol/mg of protein (Ala391-D2). In the *legend*, S193A designates the mutant in which Ser-193 was replaced by an alanine residue (Ala193-D2), and so on. B. Data are transformed and plotted as Bound (fmol/mg of protein) *vs.* Bound/Free radioligand (pM).

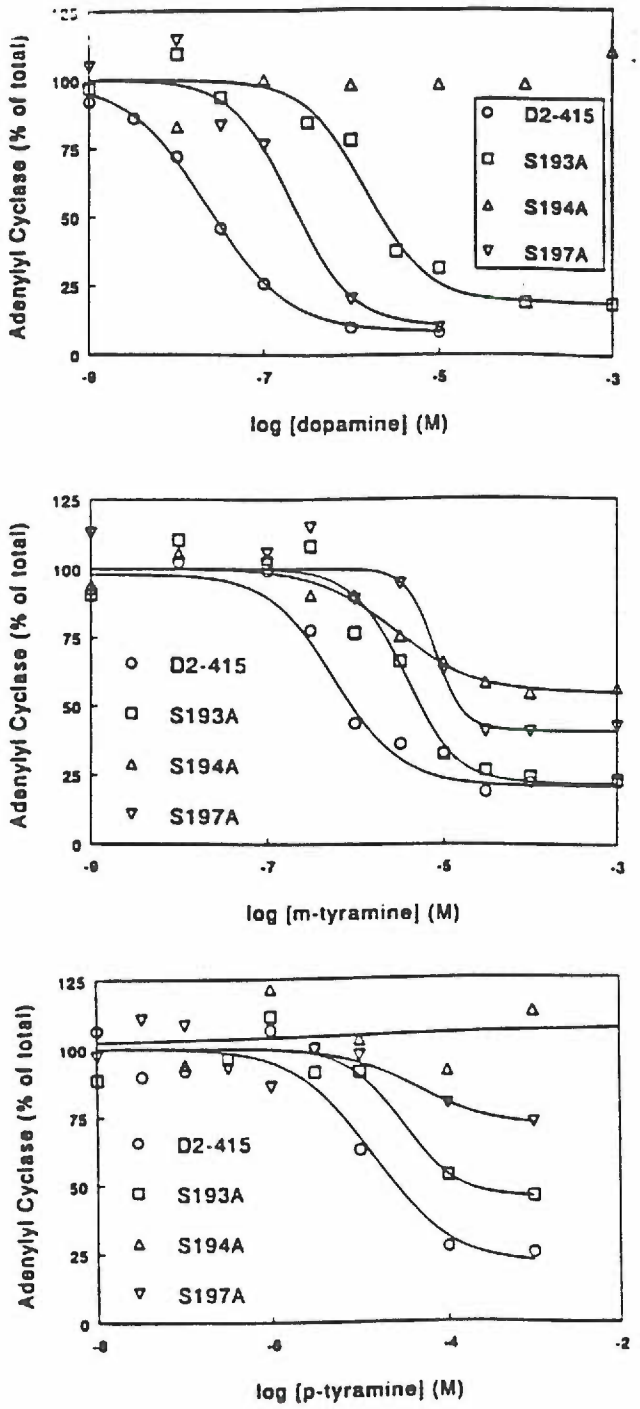


Fig. 5 Data and computer-generated curves are shown for inhibition of isoproterenol-stimulated cyclic AMP accumulation by the indicated D2 receptor agonists. Each curve is representative of 3-5 independent experiments. Results are plotted as the percentage of total isoproterenol-stimulated conversion of [^{3}H]ATP to [^{3}H]cyclic AMP *vs.* the logarithm of the concentration of agonist. In the *legend*, S193A designates the mutant in which Ser-193 was replaced by an alanine residue (Ala193-D2), and so on. A. Inhibition of adenylyl cyclase by dopamine. No curve was drawn for the data shown for mutant Ala194-D2, as there was no detectable inhibition of activity. B. Inhibition of adenylyl cyclase by *meta*-tyramine. C. Inhibition of adenylyl cyclase by *para*-tyramine.

Manuscript #2

Characterization and Regulation of Dopamine D3 Receptors Expressed in Mammalian Cells[1]

Barbara A. Cox, Mary P. Rosser, Michael R. Kozlowski, Robert A. Henningsen, Shannon L. Smiley, Kevin Duwe, Rachael L. Neve, and Kim A. Neve

Departments of Psychiatry and Pharmacology (K.A.N.) and Biochemistry and Molecular Biology (B.A.C.), Oregon Health Sciences University and Veterans Affairs Medical Center (B.A.C., R.A.H., S.L.S., K.A.N), Portland, Oregon; Department of Screening and Biochemical Research, Bristol-Myers Squibb Pharmaceutical Research Institute (M.P.R., M.R.K.); Molecular Genetics Laboratory, McLean Hospital (K.D., R.L.N.), Belmont, Massachussetts

Abstract

We have stably expressed a rat D3 receptor cDNA in C_6 glioma cells (C6-D3 cells) and characterized the binding of the D2 radioligand [^{125}I]epidepride, which also has a high affinity for D3 receptors (K_d = 60-120 pM). The binding of [^{125}I]epidepride to recombinant D3 and D2 receptors was compared to binding to membranes prepared from two regions of rat brain, the neostriatum and the ventral forebrain from which the neostriatum had been removed (extrastriatal basal forebrain). Two site analysis of the data indicated that approximately 20% of the binding sites for [^{125}I]epidepride in the rat extrastriatal basal forebrain had a higher affinity for the agonist quinpirole and lower affinity for the antagonists spiperone and domperidone than was observed for binding to recombinant D2 receptors, but very close to affinity values determined in membranes from C6-D3 cells. In C6-D3 cells, the agonists dopamine and quinpirole maximally inhibited isoproterenol-stimulated adenylyl cyclase activity by 37% and 45% respectively. Quinpirole also induced a 9-16% increase in the rate of extracellular acidification by C6-D3 cells. The acidification was inhibited by the D3 antagonist epidepride and by the Na^+/H^+ antiporter inhibitors amiloride and methylisobutylamiloride, but pertussis toxin treatment had no effect on quinpirole-induced extracellular acidification. Overnight treatment of C6-D3 cells with agonists dopamine, quinpirole, and resulted in large concentration-dependent increases (over 500%) in the density of D3 receptors on membranes prepared from cells. Antagonists had no effect on the density of D3 receptors in C6-D3 cells, except for domperidone, which caused a small increase in the density of D3 receptors. Treatment with pertussis toxin had no effect on the agonist-induced receptor proliferation, indicating that an interaction with pertussis toxin-sensitive G proteins was not required. Densitometry analysis of northern blots of RNA showed no significant *N*-propylnorapomorphine-induced increase in D3 receptor message over that of control. Pretreatment of C6-D2 cells with 10 μM DA resulted in a substantial heterologous sensitization, in which isoproterenol-stimulated cyclase activity was

enhanced more than two-fold. Sensitization of β–adrenergic receptor-stimulated adenylyl cyclase by D2 receptors was prevented by simultaneous treatment with 10 μM dopamine and 1 μM spiperone. In contrast, isoproterenol-stimulated enzyme activity was inhibited by greater than 50% in C6-D3 cells pre-treated with dopamine.

Dopamine (DA) receptors, members of the super family of receptors capable of binding to guanine nucleotide binding proteins (G proteins), have classically been defined as being either DA D1 receptors or DA D2 receptors based on their pharmacology and their respective ability to stimulate or inhibit adenylyl cyclase (De Camilli et al., 1979). In fact, both endogenous and recombinant D2 receptors are known to modulate a number of signaling pathways such as potassium channels (Lacey et al., 1987; Freedman and Weight, 1988; Lledo et al., 1990), calcium mobilization and polyphosphoinositide hydrolysis (Malgaroli et al., 1987; Nussinovitch and Kleinhaus, 1992; Lledo et al., 1992; Vallar et al., 1990), and Na^+/H^+ exchange (Ganz et al., 1990). Recombinant D2 receptors also potentiate arachidonic acid release (Felder et al., 1984; Piomelli et al., 1991) and stimulate Na^+/H^+ exchange (Neve et al., 1992). The cloning of a cDNA for the DA D2 receptor by Bunzow et al. (1988) led the way for the cloning of cDNAs for D1 (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990), D1B/D5 (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991; Weinshank et al., 1991), D3 (Sokoloff et al., 1990), and D4 (Van Tol et al., 1991) receptors. DA receptors are now classified as being either D2-like (D2, D3, D4) or D1-like (D1A, D1B) based on their sequence identity, pharmacology and function.

D2 and D3 receptors have approximately 50% overall homology at the amino acid level, and 75% homology within the seven transmembrane domains (Sokoloff et al., 1990). These two D2-like receptors differ in pharmacology and regional distribution in brain. The pharmacological profile of D3 receptors has been largely determined using recombinant D3 receptors expressed in cell lines (Sokoloff et al., 1990; Sokoloff et al., 1992; Seabrook et al., 1992; Boundy et al., 1993; Malmberg et al., 1993). Through these studies, the means of selectively labeling D3 receptors was identifed, making it possible to confirm the presence of D3 receptors in brain. The distribution and pharmacological profile of D3 receptors in brain have been determined using either agonist radioligands such as [^{3}H]7-OH-DPAT or [^{3}H]CV 205 502 (Lévesque et al., 1992; Landwehrmeyer et

al., 1993) which bind to D3 receptors more potently than to D2 receptors, or using radiolabeled substituted benzamide derivatives (Schotte et al., 1992; Murray et al., 1992; Lévesque et al., 1992), some of which have approximately equal potency for D2 and D3 receptors, but will preferentially label D3 receptors if combined with an unlabeled D2-selective ligand. The distribution of D3 binding sites is in general agreement with the distribution of D3 receptor mRNA (Sokoloff et al., 1990; Bouthenet et al., 1991; Landwehrmeyer et al., 1993). D3 receptors or message are most abundant in the ventral forebrain, with olfactory tubercle having the highest densities, and also present in other areas such as the hippocampus, septum, and substantia nigra. Except for in the islands of Calleja, where the density of D3 receptors is quite high, D3 receptors and message are generally present at much lower levels than D2 receptors. The finding that a number of agonists and "autoreceptor-selective" antagonists are more potent at D3 receptors than at D2 receptors, together with the presence of D3 receptor message in dopaminergic neurons, causes some speculation that D3 receptors may act as autoreceptors (Sokoloff et al., 1990; Bouthenet et al., 1991).

D3 receptors appear to couple only weakly to G proteins in several cell lines. Whereas high affinity binding of agonists is frequently observed, the proportion of high affinity sites is often lower, and addition of guanyl nucleotides causes smaller or partial decreases in the affinity of agonists (Sokoloff et al., 1992; Lévesque et al., 1992; Seabrook et al., 1992; Castro and Strange, 1993). The regional specificity of D3 receptors, differing pharmacology and apparently weak coupling to G proteins distinguish D3 receptors from D2 receptors, and, therefore, they may participate uniquely in the dopaminergic system.

We now report that the D2 radioligand [^{125}I]epidepride (Neve et al., 1990) also binds with high affinity to D3 receptors and can be used to characterize endogenous D3 receptors in the rat. Additionally, we have cloned a D3 receptor cDNA from rat olfactory tubercle, and have stably expressed in the cDNA in C_6 glioma cells. Using these C6-D3 cells, we have also examined the function and regulation of D3 receptors.

Methods

Construction of Flag-D3 receptors. We obtained a rat D3 cDNA (gift of P. Sokoloff) which was cloned into pRSV (Gorman et al., 1983). We were unable to obtain high expression of D3 receptors using this construct in either C6 or COS-7 cells, although abundant mRNA was present (unpublished observations). Since we had been able to obtain high expression of a D2 receptor cDNA to which we had attached the Flag sequence at the 5' end (Prickett et al., 1989; Tester et al., 1991), we attached the same sequence to the D3 receptor cDNA. The D3 receptor cDNA was released from pGEM3 with *Eco*RI, after which the ends were filled in with T4 DNA polymerase and *Cla*I linkers were ligated on. Following digestion of the cDNA with *Cla*I, the cDNA was ligated with *Cla*I-digested pGEM7/FLAG (pGEM7 into which the FLAG-encoding sequence had been inserted) so that the initial methionine was in-frame with the FLAG amino acid sequence. The FLAG-D3 receptor cassette was then released from pGEM7 by first digesting with *Nsi*I, blunting the end, then digesting with *Xba*I. This fragment was cloned into *Sal*I/*Xba*I-cut pRSV in which the *Sal*I end had been made blunt. With this construct we obtained receptor densities of several hundred fmoles/mg when stably expressed in C6 cells. Some of these studies, such as those in figures 2 and 5, used the Flag-D3 receptor cell line. We subsequently cloned a D3 receptor cDNA as described below, which was used in the remaining studies. As we observed for D2 receptors (Tester et al., 1991), the presence of the Flag sequence at the amino terminus, did not appear to alter the properties of the receptor.

Cloning of rat D3 receptor cDNA. A cDNA encoding the rat D3 receptor was isolated by reverse transcription followed by the polymerase chain reaction, using RNA isolated from rat olfactory tubercle. First-strand cDNA was synthesized using a primer (primer 4) complementary to a 25-nucleotide segment of the rat D3 sequence that begins with the stop codon and extends into the 3'-UTR (Giros et al., 1991). A *Hind*III restriction site was added to the 5'-end of the primer. The 3'-half of the cDNA was amplfied by the PCR

using the antisense primer described above together with a 25-base sense primer (primer 3) beginning with nucleotide 786 of the sequence published by Giros *et al.*, (1991). The 5'-half of the cDNA was amplified using a primer complementary to primer 3 (primer 2) and primer 1, incorporating the 25 bases immediately before the translational start codon, with an *Eco*RI site attached to the 5' end. Both fragments were amplified under standard conditions (7 min at 95° C, followed by the addition of Taq polymerase and 40 cycles: 94° C for 2 min, 60° C for 2 min, 72° C for 3 min). DMSO (10%) was included in the reaction. Amplified fragments were purified and digested with appropriate enzymes. The 3'-fragment was digested with *BamH*I and *Hind*III. The 5'-fragment was partially digested with *Bam*HI, then cut with *Eco*RI. The two fragments were ligated and subcloned into pRSV for stable expression of D3 receptors in C6 cells.

DNA-mediated expression of D3 receptors in C6 cells. C6 cells used for stable expression of D3 receptor cDNAs were maintained as described previously (Neve et al., 1985) except that the medium was supplemented with 5% iron-supplemented calf bovine serum and 5% fetal bovine serum (Hyclone, Logan,UT). DNA mediated transfection of C6 cells was carried out by calcium phosphate precipitation (Chen and Okayama, 1988) exactly as described previously (Cox et al., 1992), transfecting D3 receptor cDNA (15 μg) with pBabe Puro (2 μg), to confer resistance to puromycin (Morgenstern and Land, 1990). Briefly, exponentially growing cells were seeded in 10 ml of Dulbecco's modification of Eagles medium (DMEM)/10 cm plate and incubated overnight. Plasmid DNA was mixed with 0.5ml of 0.25 M $CaCl_2$. An equal volume of 2X BBS (50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM $NaHPO_4$) was added, and the mixture was incubated at room temperature for twenty minutes, then added dropwise to the medium in the plate. The plates were incubated 20-24 hours in an atmosphere of 3% CO_2, washed with phosphate buffered saline, then refed and incubated in an atmosphere of 10% CO_2. On the second day after the addition of DNA, cells were split into ten plates and incubated overnight before beginning selection with puromycin (2

μg/ml). Clonal puromycin-resistant cells were isolated after approximately ten days of puromycin treatment by trypsinization within a 10 μl cloning ring. Clones were transferred to 24-well plates and then expanded into duplicate 60 mm plates. Cell lines positive for D3 receptors were determined by radioligand binding assays using [^{125}I]epidepride as the radioligand. These positive cell lines were then subcloned by the method of dilution and stock cell lines were maintained under selection. C6-D2 cells, used in some experiments, expressed the rat long form ($D2_{444}$), as described previously (Neve et al., 1992).

RNA blot hybridization. A 0.68-kb *Hind*III fragment of D3 was ^{32}P-labeled by random hexamer-primed DNA synthesis (Boehringer-Mannheim). Methods of RNA isolation, electrophoresis in formaldehyde/agarose gels, and transfer to Biotrans membrane were as described (Neve et al., 1986). Hybridizations were carried out overnight in 5X standard saline citrate (SSC)/50% formamide at 42° C, followed by washes taken to 0.1X SSC at 55° C. The washed membrane was exposed to Kodak X-OMAT film for 24 h at -70° C. To control for variability in loading the gel, blots were also probed with a ^{32}P-labeled cyclophilin cDNA (Danielson et al., 1988). RNA abundance was quantified using optical density peak area determined by a Quantiscan densitometer and a JAVA image analysis system.

Radioligand binding assays. [^{125}I]Epidepride [NCQ 219, (S)-(-)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2,3-dimethoxy-5-[^{125}I]iodobenzamide] was prepared from its tributyltin precursor using a modification (Neve et al., 1991) of the method of Clanton et al. (1991). Tissue preparation and radioligand binding assays were carried out essentially as described (Neve et al., 1990; Neve et al., 1991). Aliquots of the membrane preparation (3-40 μg of protein) were added to duplicate assay tubes containing the following (final concentrations): assay buffer [50 mM Tris-HCl, pH 7.4 with 155 mM NaCl (Tris-buffered saline), 0.001 % bovine serum albumen, [^{125}I]epidepride (2000 Ci/mmol), and appropriate drugs. Spiperone (2 or 5 μM) was used to define nonspecific binding for

competition or saturation assays; respectively. Idazoxan (100 nM) was included in the assays using rat brain tissue to inhibit binding of radioligand to α_2-adrenergic receptors (Neve et al., 1990). Incubations were carried out at 37° C for 90 min, which are non-equilibrium conditions for D2 receptors (Kessler et al., 1991). Saturation and competition experiments were carried out in a volume of 0.5 ml and 0.25 ml, respectively.

Cyclic AMP accumulation. The measurement of cyclic AMP in intact cells was measured as previously described (Neve et al., 1985). Cells were grown in six-well cluster dishes at a density of 18,000/cm^2, and on day 3 growth medium was replaced by 1.5 ml of HEPES-buffered L15 medium. For experiments to assess heterologous sensitization, drugs were added in triplicate and cells were incubated in air at 37° C for two hours. At the end of the incubation, cells were kept on ice and were washed with 3 x 1.5 ml of ice-cold PBS and resuspended in 1.5 ml ice-cold HEPES-buffered L15 medium. [^{3}H]Adenine (1 μCi/well) and isoproterenol (1 μM) were added and cells were incubated for 10 min at 37° C. Incubation was terminated by two rinses with ice-cold phosphate-buffered saline, then adding 3% trichloroacetic acid. [^{3}H]ATP and[^{3}H]cAMP were separated using successive Dowex and alumina columns. Results are expressed as the percentage of [^{3}H]ATP converted to [^{3}H]cAMP. For measurements of D3 receptor-mediated inhibition of isoproterenol-stimulated adenylyl cyclase activity, no drugs were added during the two hour incubation in L15. Cells were taken directly from the air incubator and set in a 37° C water bath. [^{3}H]Adenine was added 15 min prior to the addition of isoproterenol and increasing concentrations of DA agonists. After 7 min the assay was stopped by rinsing twice with 1.5 ml ice-cold phosphate-buffered saline.

Measurement of extracellular acidification. Extracellular acidification was quantified as described by Neve et al. (1992). Serum-deprived (24 hr in serum-free medium) C6-D3 cultures in disposable cell capsules were loaded into the chambers of the microphysiometer (Molecular Devices Corp.). The chambers were perfused with culture medium lacking bicarbonate and with a low buffering capacity (1 mM sodium phosphate),

supplemented with 1mg/ml bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin, hereafter referred to as modified medium. The modified medium also contained drugs as indicated.

Acidification was measured in 4 min cycles, in each of which cells were perfused with modified medium for 210s. The flow was interrupted for an interval of 30s, during which the rate of acidification was measured and recorded. The flow was then resumed and the next cycle begun. For purposes of quantification, the rate of acidification caused by experimental medium from a given reservoir was determined 2500 s after opening the valve for the reservoir.

Drug-induced proliferation of D3 receptors. All drug solutions were freshly prepared and sterilized by filtration prior to use. Indicated drugs were diluted 100-fold by addition to cells grown on 10 cm plates in DMEM. At the end of the incubation, cells were washed with calcium-, magnesium-free phosphate-buffered saline, refed with DMEM, and returned to the incubator. After ten minutes, cells were washed three times with growth medium and then harvested. To harvest, cells were lysed 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 from the plate and spun at 24,000 x *g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkman Polytron homogenizer at setting 6 for 10 sec, in Tris-buffered saline. This membrane preparation was then spun again at 24,000 x *g* and resuspended. For measurement of mRNA levels, agonist treatments were the same except that cells were grown in 15 cm plates.

Data analysis. Data were analyzed by nonlinear regression using the program GraphPAD. In saturation binding experiments, the free concentration of [^{125}I]epidepride was calculated as the concentration added minus the concentration bound. In all competition experiments, the concentration of [^{125}I]epidepride ranged from 120-276 pM. Potencies for the inhibition studies in tables 1 and 2 were left as IC_{50} values, because assay

conditions were not at equilibrium for D2 receptors. Averages for K_d and IC_{50} values are the geometric means [the antilogarithm of mean logarithms (Fleming et al., 1972)].

Results

Although binding of [^{125}I]epidepride to D2 receptors is unstable at 37° C (Kessler et al., 1991), we observed that binding to D3 receptors approached equilibrium slowly and was stable at 37° C (data not shown). Therefore, assays for D3 receptors were carried out for 90 min at 37° C. Saturation analyses of the binding of the D2 receptor antagonist [^{125}I]epidepride to membranes prepared from C6-D3 cells showed that D3 receptors have high affinity for [^{125}I]epidepride (fig.1) with K_d values ranging from 60-120 pM. Several cell lines were used in these studies, with D3 receptor densities ranging from 100 to 1500 fmoles/mg protein.

Comparison of recombinant D3 receptors to rat extrastriatal basal forebrain. Drug potencies were determined by inhibition studies using [^{125}I]epidepride. Values determined in membrane preparations of rat basal forebrain from which the neostriatum was removed (extrastriatal basal forebrain) were compared to those using membrane preparations from rat neostriatum and C6-D3 and C6-D2 cells. Two site analysis of the data indicated that a small population of sites in the extrastriatal basal forebrain (table 1) had high affinity for the agonist quinpirole (fig.2A) and low affinity for the antagonists spiperone (fig.2B) and domperidone. For neostriatum, however, curves for these drugs are best fit to only one site with a pharmacological profile indistinguishable from data using C6-D2 cells, indicating that only D2 receptors were being labeled in the neostriatum (table 2). The competitions curves for haloperidol, clozapine, (-)3-PPP, sulpiride and (+)-butaclamol all were best fit to one site in both regions of rat brain tested (table 2).

Inhibition of adenylyl cyclase by recombinant D3 receptors. We obtained variable results in demonstrating inhibition of adenylyl cyclase activity by recombinant D3 receptors. In some cases, we observed modest concentration-dependent inhibition by DA

or quinpirole (fig. 3), whereas in other experiments we did not observe clear inhibition of adenylyl cyclase by D3 receptors even when using the receptors at a density of 1500 fmoles/mg of membrane. We tried to ascertain the reason for this variability. Reliable adenylyl cyclase inhibition by quinpirole or DA occurred when the density of D3 receptors was above 150 fmoles/mg membrane and cells were grown to confluence (9 out of 10 experiments for quinpirole, 3/4 for DA). At lower cell densities no agonist inhibition of adenylyl cyclase activity occurred (0/5). The average maximal inhibition by quinpirole was 45 ± 6% (EC_{50} = 12 nM, N = 9), whereas maximal inhibition by DA was 37 ± 5% (EC_{50} = 23 nM, N = 3) of isoproterenol-stimulated adenylyl cyclase activity.

D3 receptor-stimulated extracellular acidification. Addition of 100 nM quinpirole to the medium accelerated the rate of extracellular acidification by C6-D3 cells but not by control C6 cells. The increase in the rate of acidification induced by quinpirole was 8.6 ± 0.9% (N = 3) greater than the rate of acidification before addition of quinpirole. Although epidepride (100 nM) had no effect on the basal rate of acidification, the antagonist inhibited quinpirole-induced acidification, so that the increase in the rate in the presence of quinpirole and epidepride was 1.8 ± 4% (N = 3) of the rate before addition of drugs. To ascertain if Na^+/H^+ exchange mediated the increased rate of acidification, the effect of the inhibitors amiloride and methylisobutylamiloride was determined (fig. 4A). Both compounds inhibited the quinpirole-induced extracellular acidification. C6-D3 cells were treated with pertussis toxin (100 ng/ml) for 18 h prior to testing in the microphysiometer, to inactivate pertussis toxin-sensitive G proteins. The pertussis toxin treatment had no effect on quinpirole-induced extracellular acidification (fig. 4B).

Agonist-induced proliferation of D3 receptors. Treatment of C6-D3 cells for 24 hours with DA, quinpirole, or N-propylnorapomorphine (NPA) resulted in large concentration-dependent increases (over 500%) in the density of D3 receptors on membranes prepared from the cells (fig. 5). There was no significant effect of drug treatment on the affinity of D3 receptors for [^{125}I]epidepride; mean K_d values for control or drug-treated conditions

ranged from 90 to 160 pM. As shown in figure 6, the receptor proliferation induced by 10 μM NPA was maximal after 10 h of treatment. Longer treatments caused no additional increase in the density of receptors (data not shown). The effect was reversible, as washout experiments showed a return to control levels within 24-36 h (data not shown). The dopamine D2 antagonists epidepride and sulpiride had no effect on the density of D3 receptors in C6-D3 cells (table 3), whereas domperidone treatment at concentrations between 100 nM and 10 μM increased the density of D3 receptors. The magnitude of the domperidone-induced receptor proliferation was considerably less than the proliferation induced by agonists. High concentrations of some of the antagonists decreased the affinity of D3 receptors for [^{125}I]epidepride (table 3), presumably due to incomplete washout of the drugs.

Treatment with pertussis toxin had no effect on agonist-induced receptor proliferation. Receptor density after treatment with 10 μM NPA (1166 ± 217 fmol/mg protein, $N = 3$) did not differ significantly from cells incubated with 10 μM NPA after overnight treatment with 100 μg/ml of pertussis toxin (1249 ± 186 fmol/mg protein). The density of receptors in cells treated only with pertussis toxin (254 ± 86 fmol/mg, $N = 3$) did not differ significantly from those of control cells (243 ± 74 fmol/mg).

RNA was prepared from control C6-D3 cells and cells that had been treated with 10 μM NPA for 2 or 24 h. On northern blots, the density of hybridization to D3 receptor mRNA was expressed as a ratio of the abundance of RNA for cyclophilin. This ratio was compared for control and NPA-treated C6-D3 cells from 3 independent northern analyses, each with 3 replicate lanes for each condition. The D3/cyclophilin ratio in cells treated for 2 h with NPA was 102 ± 4 % of the ratio in control cells, and the D3/cyclophilin ratio in cells treated 24 h with NPA was 133 ± 13% of control cells ($N = 3$, $P > 0.05$).

Heterologous regulation of adenylyl cyclase. C6 cells, C6-D3, and C6-D2 cells were pretreated with 10 μM DA for 2 h prior to measurement of adenylyl cyclase activity in the presence of 1 μM isoproterenol, a concentration of the β–adrenergic receptor agonist that

maximally stimulates enzyme activity. Pretreatment of C6-D2 cells with DA substantially increased isoproterenol-stimulated adenylyl cyclase activity (fig. 7), an effect completely prevented by simultaneous pretreatment with 1 μM spiperone. Whereas DA pretreatment had only a modest effect on wild type (untransfected) C6 cells, isoproterenol-stimulated enzyme activity was inhibited by greater than 50% in C6-D3 cells that had been pretreated with DA (fig. 7). Spiperone partially prevented the heterologous desensitization induced by D3 receptors.

Discussion

We expressed rat D3 receptor cDNAs in C6 glioma cells and compared the binding of the radioligand [^{125}I]epidepride to these cells and to rat brain. We found that [^{125}I]epidepride labeled a small population of receptors in the extrastriatal basal forebrain, but not in the neostriatum, that had the pharmacological profile of recombinant D3 receptors. Quinpirole was 35-fold selective for D3 receptors, whereas the antagonists spiperone and domperidone were 24- and 56-fold selective for D2 receptors, respectively. Haloperidol, (+)-butaclamol, clozapine, (-)3-PPP, and sulpiride were also selective for recombinant D2 receptors, but the selectivity with these ligands was not great enough to distinguish between D2 and D3 receptors in competition analyses using rat brain tissue.

In confluent C6-D3 cells, both DA and quinpirole inhibited isoproterenol-stimulated adenylyl cyclase activity. Although in many cell lines D3 receptors appear not to inhibit adenylyl cyclase, we have found that in C6-D3 cells inhibition was usually observed if cells were grown to confluence. Compared to D2 receptors, however, inhibition was modest (Cox et al., 1992). Sokoloff et al.(1992) also reported modest and inconsistent inhibition by the human D3 receptor expressed in CHO cells.

DA D2 receptors expressed in C6 cells or L-fibroblasts mediate enhanced extracellular acidification (Neve et al., 1992). Several lines of evidence indicate that the enhanced rate of acidification is due to stimulation of Na^+/H^+ exchange. Thus, acidification is prevented by the Na^+/H^+ antiporter inhibitors amiloride and

methylisobutylamiloride. Furthermore, removal of extracellular sodium, which is necessary for extrusion of protons by the antiporter, prevents D2 receptor-stimulated extrusion of acid. Pertussis toxin-sensitive G proteins appear not to be necessary for stimulation of Na^+/H^+ exchange by D2 receptors. Our present results demonstrate that quinpirole also enhanced the rate of extracellular acidification *via* D3 receptors in C6-D3 cells. The accelerated extracellular acidification was sensitive to the D3 receptor antagonist epidepride and to inhibitors of Na^+/H^+ exchange, but not sensitive to inactivation of pertussis toxin-sensitive G proteins.

According to the classical model of regulation of receptor sensitivity by agonist, increased stimulation of receptors decreases receptor sensitivity and responsiveness, whereas decreased stimulation of receptors increases receptor density and responsiveness. Surprisingly, Ivins et al., (1991) observed a proliferation of endogenous D2 receptors on SUP1 cells when they were subjected to prolonged incubation with agonists. These results were confirmed in preliminary reports using cell lines expressing recombinant D2 receptors (Kozell et al., 1992; Zhang et al., 1992; Molinoff et al., 1992). In the present studies, the agonists quinpirole, DA, and NPA all induced a robust proliferation of D3 receptors. Of three antagonists tested, only domperidone caused a modest increase in the density of D3 receptors. Agonist-induced receptor proliferation on cultured cells is unusual, but not unprecedented. The densities of $5HT_2$ receptors on cultured cerebellar neurons and β_3-adrenergic receptors on 3T3 cells are increased by persistent stimulation with agonists, apparently due to an increased abundance of receptor mRNA (Akiyoshi et al., 1993; Thomas et al., 1992). The density of vitamin D_3 receptors is also increased by agonists, although the mechanism of proliferation appears to be receptor stabilization, *i.e.*, a decreased rate of degradation of receptors that are occupied by agonist (Costa and Feldman, 1987; Wiese et al., 1992). The abundance of D3 receptor mRNA in C6-D3 cells was not significantly increased by short- or long-term treatment with NPA, demonstrating that either receptor synthesis was increased by a post-translational mechanism, or that

agonist-induced proliferation was due to a decreased rate of degradation of D3 receptors. The receptor proliferation apparently did not require an interaction with pertussis toxin-sensitive G proteins, as agonist-induced receptor proliferation was unaffected by pretreatment with pertussis toxin.

One mechanism that has been proposed (Ivins et al., 1991) for agonist-induced proliferation of D2 receptors is increased intracellular cAMP as a result of the increased level of adenylyl cyclase activity that occurs after prolonged stimulation of D2 receptors (Ivins et al., 1991; Bates et al., 1991). In C_6 cells, we find that stimulation of D2 receptors with DA for 2 h greatly enhanced adenylyl cyclase activity. This is, however, unlikely to be the mechansim for the proliferation of D3 receptors, because prolonged activation of D3 receptors reduced the subsequent stimulation of adenylyl cyclase activity by isoproterenol. This heterologous desensitization of β–adrenergic receptors appears to be a novel functional consequence of stimulation of D3 receptors, because it was inhibited by spiperone and not observed in wild type C_6 cells.

In summary, we have stably expressed DA D3 receptors in C_6 glioma cell lines and determined that the radioligand [^{125}I]epidepride bound to D3 receptors with high affinity. Comparing the pharmacological profile of recombinant D3 and D2 receptors to those in rat extrastriatal basal forebrain and neostriatum, we discerned a small population of binding sites in rat forebrain that could be characterized as D3 binding sites. Recombinant D3 receptors expressed in C_6 cells inhibited adenylyl cyclase activity weakly and stimulated extracellular acidification. Prolonged treatment with agonists increased the density of D3 receptors and desensitized β–adrenergic receptors.

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References

Akiyoshi J., Hough C., and Chuang D.-M. (1993) Paradoxical increase of 5-hydroxytryptamine$_2$ receptors and 5-hydroxytryptamine$_2$ receptor mRNA in cerebellar granule cells after persistent 5-hydroxytryptamine$_2$ receptor stimulation. *Mol. Pharmacol.* **43**, 349-355.

Bates M.D., Senogles S.E., Bunzow J.R., Liggett S.B., Civelli O., and Caron M.G. (1991) Regulation of responsiveness at D_2 dopamine receptors by receptor desensitization and adenylyl cyclase sensitization. *Mol. Pharmacol.* **39**, 55-63.

Boundy V.A., Luedtke R.R., Gallitano A.L., Smith J.E., Filtz T.M., Kallen R.G., and Molinoff P.B. (1993) Expression and characterization of the rat D_3 dopamine receptor: pharmacologic properties and development of antibodies. *J. Pharmacol. Exp. Ther.* **264**, 1002-1011.

Bouthenet M.-L., Souil E., Martres M.-P., Sokoloff P., Giros B., and Schwartz J.-C. (1991) Localization of dopamine D_3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. *Brain Res.* **564**, 203-219.

Bunzow J.R., Van Tol H.H.M., Grandy D.K., Albert P., Salon J., Christie M., Machida C.A., Neve K.A., and Civelli O. (1988) Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature* **336**, 783-787.

Castro S.W. and Strange P.G. (1993) Coupling of D_2 and D_3 dopamine receptors to G-proteins. *FEBS Lett.* **315**, 223-226.

Chen C.A. and Okayama H. (1988) Calcium phosphate-mediated gene tranfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques* **6**, 632-638

Clanton J.A., De Paulis T., Schmidt D., Ansari M., Manning R.G., Baldwin R., and Kessler R.M. (1991) Preparation of [^{123}I]- and [^{125}I]-epidepride: a dopamine D2

receptor antagonist radioligand. *J. Labelled Compounds Radiopharmaceut.* **29**, 745-751.

Costa E.M. and Feldman D. (1987) Measurement of 1,25-dihydroxyvitamin D_3 receptor turnover by dense amino acid labeling: changes during receptor up-regulation by vitamin D metabolites. *Endocrinology* **120**, 1173-1178.

Cox B.A., Henningsen R.A., Spanoyannis A., Neve R.L., and Neve K.A. (1992) Contributions of conserved serine residues to the interactions of ligands with dopamine D2 receptors. *J. Neurochem.* **59**, 627-635.

Danielson P.E., Forss-Petter S., Brow M.A., Calavetta L., Douglass J., Milner R.J., and Sutcliffe J.G. (1988) p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **7**, 261-267.

De Camilli P., Macconi D., and Spada A. (1979) Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. *Nature (Lond.)* **278**, 252-254.

Dearry A., Gingrich J.A., Falardeau P., Fremeau R.T., Bates M.D., and Caron M.G. (1990) Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature (Lond.)* **347**, 72-75.

Felder R.A., Blecher M., Eisner G.M., and Jose P.A. (1984) Cortical tubular and glomerular dopamine receptors in the rat kidney. *Amer. J. Physiol.* **246**, F557-568.

Fleming W.W., Westfall D.P., De La Lande I.S., and Jellett L.B. (1972) Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmacol. Exper. Ther.* **181**, 339-345.

Freedman J.E. and Weight F.F. (1988) Single K^+ channels activated by D_2 dopamine receptors in acutely dissociated neurons from rat corpus striatum. *Proc. Natl. Acad. Sci. USA* **85**, 3618-3622.

Ganz M.B., Pachter J.A., and Barber D.L. (1990) Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **265**, 8989-8992.

Giros B., Mestikawy S.E., Bertrand L., and Caron M.G. (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett.* **295**, 149-154.

Gorman C., Padmanabhan R., and Howard B.H. (1983) High efficiency DNA-mediated transformation of primate cells. *Science* **221**, 551-553.

Grandy D.K., Zhang Y., Bouvier C., Zhou Q.-Y., Johnson R.A., Allen L., Buck K., Bunzow J.R., Salon J., and Civelli O. (1991) Multiple human D_5 receptor genes: A functional receptor and two pseudogenes. *Proc. Natl. Acad. Sci. USA* **88**, 9175-9179.

Ivins K.J., Luedtke R.R., Artymyshyn R.P., and Molinoff P.B. (1991) Regulation of dopamine D2 receptors in a novel cell line (SUP1). *Mol. Pharmacol.* **39**, 531-539.

Kessler H.A., Ansari S.M., Schmidt D.E., De Paulis T., Clanton J.A., Innis R., Al-Tikriti M., and Manning R.G. (1991) High affinity dopamine D2 receptor radioligands. 2. [^{125}I]Epidepride, a potent and specific radioligand for the characterization of striatal and extrastriatal dopamine D2 receptors. *Life Sci.* **49**, 617-628.

Kozell L.B., Starr S., Machida C.A., Neve R.L., and Neve K.A. (1992) Agonist-induced changes in density of D1, D2, and chimeric D1/D2 receptors. *Soc. Neurosci. Abstr.* **18**, 276.

Lacey M.G., Mercuri N.B., and North R.A. (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of rat substantia nigra zona compacta. *J. Physiol. (Lond.)* **392**, 397-416.

Landwehrmeyer B., Mengod G., and Palacios J.M. (1993) Differential visualization of dopamine D_2 and D_3 receptor sites in rat brain. A comparative study using *in situ*

hybridization histochemistry and ligand binding autoradiography. *Eur. J. Neurosci.* **5**, 145-153.

Lévesque D., Diaz J., Pilon C., Martres M.P., Giros B., Souil E., Schott D., Morgat J.L., Schwartz J.C., and Sokoloff P. (1992) Identification, characterization, and localization of the dopamine D_3 receptor in rat brain using 7-[^{3}H]hydroxy-*N,N*-di-*n*-propyl-2-aminotetralin. *Proc. Natl. Acad. Sci. USA* **89**, 8155-8159.

Lledo P.-M., Legendre P., Zhang J., Israel J.-M., and Vincent J.-D. (1990) Effects of dopamine on voltage-dependent potassium currents in identified rat lactotroph cells. *Neuroendocrinology* **52**, 545-555.

Lledo P.M., Homburger V., Bockaert J., and Vincent J.-D. (1992) Differential G protein-mediated coupling of D_2 dopamine receptors to K^+ and Ca^{2+} currents in rat anterior pituitary cells. *Neuron* **8**, 455-463.

Malgaroli A.L., Vallar L., Elahi F.R., Pozzan T., Spada A., and Meldolesi J. (1987) Dopamine inhibits cytosolic Ca^{2+} increases in rat lactotroph cells: Evidence of a dual mechanism of action. *J. Biol. Chem.* **262**, 13920-13927.

Malmberg Å., Jackson D.M., Eriksson A., and Mohell N. (1993) Unique binding characteristics of antipsychotic agents interacting with human dopamine D_{2A}, D_{2B}, and D_3 receptors. *Mol. Pharmacol.* **43**, 749-754.

Molinoff P.B., Filtz T.M., and Artymyshyn R.P. (1992) Regulation of D2 receptors in cultured cells. *Proc. 7th International CA Symp.* 218.

Monsma F.J., Mahan L.C., McVittie L.D., Gerfen C.R., and Sibley D.R. (1990) Molecular cloning and expression of a D_1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**, 6723-6727.

Morgenstern J.P. and Land H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl. Acids Res.* **18**, 3587-3596.

Murray A.M., Ryoo H., and Joyce J.N. (1992) Visualization of dopamine D3-like receptors in human brain with [^{125}I]epidepride. *Eur. J. Pharmacol-Molec. Pharm.* **227**, 443-445.

Neve K.A., Barrett D.A., and Molinoff P.B. (1985) Selective regulation of *beta*-1 and *beta*-2 adrenergic receptors by atypical agonists. *J. Pharmacol. Exper. Ther.* **235**, 657-664.

Neve K.A., Henningsen R.A., Kinzie J.M., De Paulis T., Schmidt D.E., Kessler R.M., and Janowsky A. (1990) Sodium-dependent isomerization of dopamine D-2 receptors characterized using [^{125}I]epidepride, a high-affinity substituted benzamide ligand. *J. Pharmacol. Exper. Ther.* **252**, 1108-1116.

Neve K.A., Cox B.A., Henningsen R.A., Spanoyannis A., and Neve R.L. (1991) Pivotal role for aspartate-80 in regulation of D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* **39**, 733-739.

Neve K.A., Kozlowski M.R., and Rosser M.P. (1992) Dopamine D2 receptor stimulation of Na^+/H^+ exchange assessed by quantification of extracellular acidification. *J. Biol. Chem.* **267**, 25748-25753.

Neve R.L., Harris P., Kosik K.S., Kurnit D.M., and Donlon T.A. (1986) Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Mol. Brain Res.* **1**, 271-280.

Nussinovitch I. and Kleinhaus A.L. (1992) Dopamine inhibits voltage-activated calcium channel currents in rat pars intermedia pituitary cells. *Brain Res.* **574**, 49-55.

Piomelli D., Pilon C., Giros B., Sokoloff P., Martres M.-P., and Schwartz J.-C. (1991) Dopamine activation of the arachidonic acid cascade as a basis for D1/D2 receptor synergism. *Nature* **353**, 164-167.

Prickett K.S., Amberg D.C., and Hopp T.P. (1989) A calcium-dependent antibody for identification and purification of recombinant proteins. *BioTechniques* **7**, 580-589.

Schotte A., Janssen P.F.M., Gommeren W., Luyten W.H.L.M., and Leysen J.E. (1992) Autoradiographic evidence for the occlusion of rat brain dopamine D_3 receptors *in vivo*. *Eur. J. Pharmacol.* **218**, 373-375.

Seabrook G.R., Patel S., Marwood R., Emms F., Knowles M.R., Freedman S.B., and Mcallister G. (1992) Stable expression of human D_3 dopamine receptors in GH_4C_1 pituitary cells. *FEBS Lett.* **312**, 123-126.

Sokoloff P., Giros B., Martres M.-P., Bouthenet M.-L., and Schwartz J.-C. (1990) Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature* **347**, 146-151.

Sokoloff P., Andrieux M., Besançon R., Pilon C., Martres M.-P., Giros B., and Schwartz J.-C. (1992) Pharmacology of human dopamine D_3 receptor expressed in a mammalian cell line: comparison with D_2 receptor. *Eur. J. Pharmacol-Molec. Pharm.* **225**, 331-337.

Sunahara R.K., Niznik H.B., Weiner D.M., Stormann T.M., Brann M.R., Kennedy J.L., Gelernter J.E., Rozmahel R., Yang Y., Israel Y., Seeman P., and O'Dowd B.F. (1990) Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5. *Nature* **347**, 80-83.

Sunahara R.K., Guan H.-C., O'Dowd B.F., Seeman P., Laurier L.G., Ng G., George S.R., Torchia J., Van Tol H.H.M., and Niznik H.B. (1991) Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature* **350**, 614-619.

Tester B.A., Henningsen R.A., Spanoyannis A., Neve R.L., and Neve K.A. (1991) Flag-$D2_{415}$ and Flag-$D2_{444}$, unique mutants of dopamine D2 receptors $D2_{415}$ and $D2_{444}$, offer a novel approach for rapid purification of D2 receptors. *Soc. Neurosci. Abstr.* **17**, 815.

Thomas R.F., Holt B.D., Schwinn D.A., and Liggett S.B. (1992) Long-term agonist exposure induces upregulation of β_3-adrenergic receptor expression via multiple cAMP response elements. *Proc. Natl. Acad. Sci. USA* **89**, 4490-4494.

Tiberi M., Jarvie K.R., Silvia C., Falardeau P., Gingrich J.A., Godinot N., Bertrand L., Yang-Feng T.L., Fremeau R.T., and Caron M.G. (1991) Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D_1 dopamine receptor subtype: Differential expression pattern in rat brain compared with the D_{1A} receptor. *Proc. Natl. Acad. Sci. USA* **88**, 7491-7495.

Vallar L., Muca C., Magni M., Albert P., Bunzow J., Meldolesi J., and Civelli O. (1990) Differential coupling of dopaminergic D_2 receptors expressed in different cell types: Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in Ltk^- fibroblasts, hyperpolarization, and cytosolic-free Ca^{2+} concentration decrease in GH_4C_1 cells. *J. Biol. Chem.* **265**, 10320-10326.

Van Tol H.H.M., Bunzow J.R., Guan H.-C., Sunahara R.K., Seeman P., Niznik H.B., and Civelli O. (1991) Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**, 610-614.

Weinshank R.L., Adham N., Macchi M., Olsen M.A., Branchek T.A., and Hartig P.R. (1991) Molecular cloning and characterization of a high affinity dopamine receptor ($D_{1\beta}$) and its pseudogene. *J. Biol. Chem.* **266**, 22427-22435.

Wiese R.J., Uhland-Smith A., Ross T.K., Prahl J.M., and Deluca H.F. (1992) Up-regulation of the vitamin D receptor in response to 1, 25-dihydroxyvitamin D_3 results from ligand-induced stabilization. *J. Biol. Chem.* **267**, 20082-20086.

Zhang L.-J., Smyk-Randall E.M., Monsma F.J., and Sibley D.R. (1992) Regulation of the D_{2L} receptor in stably transfected CHO cells. *Soc. Neurosci. Abstr.* **18**, 663.

Zhou Q.-Y., Grandy D.K., Thambi L., Kushner J.A., Van Tol H.H.M., Cone R., Pribnow D., Salon J., Bunzow J.R., and Civelli O. (1990) Cloning and expression of human and rat D_1 dopamine receptors. *Nature* **347**, 76-80.

Table 1.

Competitive inhibition of the binding of [^{125}I]epidepride to rat extrastriatal basal forebrain.

IC_{50} values for inhibition of the binding of [^{125}I]epidepride by a number of drugs were determined as described in Methods. Results shown are geometric means from 3 or 4 independent experiments, expressed as nM, with the 95% confidence limits given in parentheses. The mean ± S.E. of the percentage of total specific binding represented by D2 or D3 receptors is shown.

Drug	Receptor	IC50 (nM)	% of total
spiperone	D2	0.03 (0.003 - 0.3)	77 ± 8
	D3	2.2 (1.0 - 5.0)	23 ± 8
domperidone	D2	2.1 (1.6 - 2.9)	81 ± 2
	D3	139 (69 - 279)	19 ± 2
quinpirole	D2	9300 (6800 - 12800)	79 ± 3
	D3	52 (21 - 132)	21 ± 3

Table 2

Comparison of the binding of ligands to recombinant D2 and D3 receptors and rat brain

IC_{50} values for inhibition of the binding of [^{125}I]epidepride by a number of drugs were determined as described in Methods, using membranes prepared from C6-D3 cells, C6-D2 cells, rat neostriatum, or extrastriatal basal forebrain (forebrain). Results shown are geometric means from 2 to 7 independent experiments, expressed as nM, with the 95% confidence limits given in parentheses. For the three drugs that yielded two-site fits in the extrastriatal basal forebrain, the mean IC_{50} values for the two sites (D2 IC_{50}/D3 IC_{50}, values taken from table 1) are shown for purposes of comparison to values in the other tissues.

Drug	IC_{50} (nM)			
	C6-D2	Neostriatum	Forebrain	C6-D3
spiperone	0.3 (0.2-0.5)	0.1 (0.04-0.3))	0.03/2.2	7.1 (4.6-111)
(+)-butaclamol	4.2 (4.0-4.5)	2.4 (1.6-3.5)	3 (2.7-3-3)	40 (37-44)
domperidone	3.1 (1.8-5.4))	6.3 (1.8-22)	2.1/139	175 (85-395
haloperidol	8.2 (4.7-14)	7.4 (4.7-12)	2.9 (0.1-57)	163 (108-246)
sulpiride	102 (80-129)	72 (65-81)	74 (66-84)	254 (156-415)
clozapine	267 (183-390)	245 (83-721)	725 (601-875)	962 (442-2090)
(-)3-PPP	1400 (478-4120)	2420 (1740-3370)	2200 (2070-2340)	3470 (2940-4080)
quinpirole	6680 (3450-12900)	10400 (6010-18500)	9340/52	191 (144-254)

Table 3

Effects of antagonists on D3 receptor density and affinity

C6-D3 cells were treated with the indicated drugs for 14 h (overnight), then membranes were prepared and the density of D3 receptors was determined as described in Methods. The number of independent experiments is in parentheses after each drug concentration, and 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 these experiments, the K_d of [^{125}I]epidepride in untreated cells was 93 pM (69-124 pM) The density of receptors is the mean ± S.E., expressed as a percentage of the control density of receptors.

Drug	Density (%)	Affinity
epidepride		
10 nM (6)	92 ± 8	115 (99-135)
100 nM (6)	90 ± 14	159 (120-209)
1 μm (5)	102 ± 20	*408 (233-715)
sulpiride		
100 nM (5)	79 ± 13	90 (55-148)
1 μM (6)	101 ± 14	93 (53-163)
10 μM (6)	109 ± 17	94 (51-173)
domperidone		
100 nM (6)	*152 ± 13	58 (37-89)
1 μM (6)	*159 ± 17	73 (53-99)
10 μM (3)	*221 ± 16	277 (111-689)

*$P < 0.05$, significant increase relative to control.

Figure Legends

Fig. 1. Saturation analysis of the specific binding of [^{125}I]epidepride to membranes prepared from C6-D3 cells was carried out. Nonspecific binding was defined as binding not displaced by 5 μM spiperone. The results shown are from a representative experiment conducted with duplicate determinations. The affinity (K_d) of D3 receptors for [^{125}I]epidepride and the density of binding sites (B_{max}) were determined by nonlinear regession analysis.

Fig. 2. Results are shown from representative experiments in which the binding of [^{125}I]epidepride was assessed in membranes prepared from C6-D3 cells, rat basal forebrain from which the neostriatum had been removed (extrastriatal basal forebrain,), neostriatum and C6-D2 cells. Averaged results from all experiments are presented in tables 1 and 2. Data are plotted as the percentage of total binding *vs.* the logarithm of the concentration of inhibitor. A, data for quinpirole inhibition of [^{125}I]epidepride binding to C6-D3 cells and C6-D2 cells were fit to a curve that assumes the presence of only one class of binding sites. For binding to extrastriatal basal forebrain membranes, the computer-drawn curve was best fit to a two-site model. In this experiment, IC_{50} values for quinpirole were 0.2, 4.0, and 17 μM for C6-D3, forebrain, and C6-D2, respectively. B, data for spiperone inhibition of [^{125}I]epidepride binding to neostriatum and C6-D3 cells were fit to a curve that assumes the presence of only one class of binding sites. For binding to extrastriatal basal forebrain membranes, the computer-drawn curve was best fit to a two-site model. In this experiment, IC_{50} values for spiperone were 0.15, 0.22, and 5.8 nM for neostriatum, forebrain, and C6-D3, respectively.

Fig. 3. Adenylyl cyclase activity in C6-D3 cells was assessed by measuring the conversion of [^{3}H]ATP to [^{3}H]cyclic AMP. The curves shown are from a single experiment in which each point is the average of triplicate determinations. Data are plotted as adenylyl cyclase activity, expressed as a percentage of total enzyme activity stimulated by 1 μM isoproterenol, *vs.* the concentration of agonist. Analysis of the curves by nonlinear

regression indicated that the EC_{50} and maximal inhibition by DA were 67 nM and 38%, respectively,and 0.2 nM and 48% for quinpirole.

Fig. 4. The effect of Na^+/H^+ antiporter inhibitors and pertussis toxin treatment on quinpirole-induced extracellular acidification by C6-D3 cells was determined. Results are plotted as the mean ± S.E. of the increase in rate of extracellular acidification, expressed as a percentage of the rate of acidification prior to addition of drug. Data were taken from the rate of acidification determined at 2500 s after initiation of flow of medium from a given reservoir. A, in 4 independent experiments, C6-D3 cells were incubated with either 100 nM quinpirole, or the indicated inhibitor of Na^+/H^+ exchange (10 μM amiloride or 1 μM methylisobutyl amiloride, MIA), or quinpirole together with the inhiibitor (QUIN+INHIB). B, control C6-D3 cells (CONTROL) or cells that had been treated with pertussis toxin (100 ng/ml overnight) were incubated with 100 nM quinpirole or vehicle, as indicated. ($N = 3$)

Fig. 5. Treatment of C6-D3 cells with agonists caused a concentration-dependent proliferation of D3 receptors. Cells were treated overnight with DA, quinpirole, or NPA at the indicated concentrations, before determination of the density of binding sites as described in Methods. Each bar represents the mean ± S.E. of 4 independent experiments except for 10 μM NPA ($N = 3$).

Fig. 6. The time course of proliferation of D3 receptors during treatment with 10 μM NPA was determined in 4 independent experiments in which C6-D3 cells were harvested at the indicated time after initiation of the incubation with NPA. The density of binding sites was determined as described in Methods. Longer incubations (12-24 hrs) caused no further increase in the density of receptors.

Fig. 7. Wild-type C_6 cells (C6), or C_6 cells expressing recombinant D3 (C6-D3) or D2 (C6-D2) receptors were treated with 10 μM DA (DA) or DA + 1 μM spip (DA/Spip) for 2 h. Cells were washed, and β–adrenergic receptor-stimulated adenylyl cyclase activity was determined by stimulation with 1 μM isoproterenol, as described in Methods. Results

shown are the mean ± S.E. of 3-7 independent experiments, expressed as a percentage of isoproterenol-stimulated activity in untreated cells.

*$P < 0.05$ compared to control cells.

$^{+}P < 0.05$ compared to DA-treated cells.

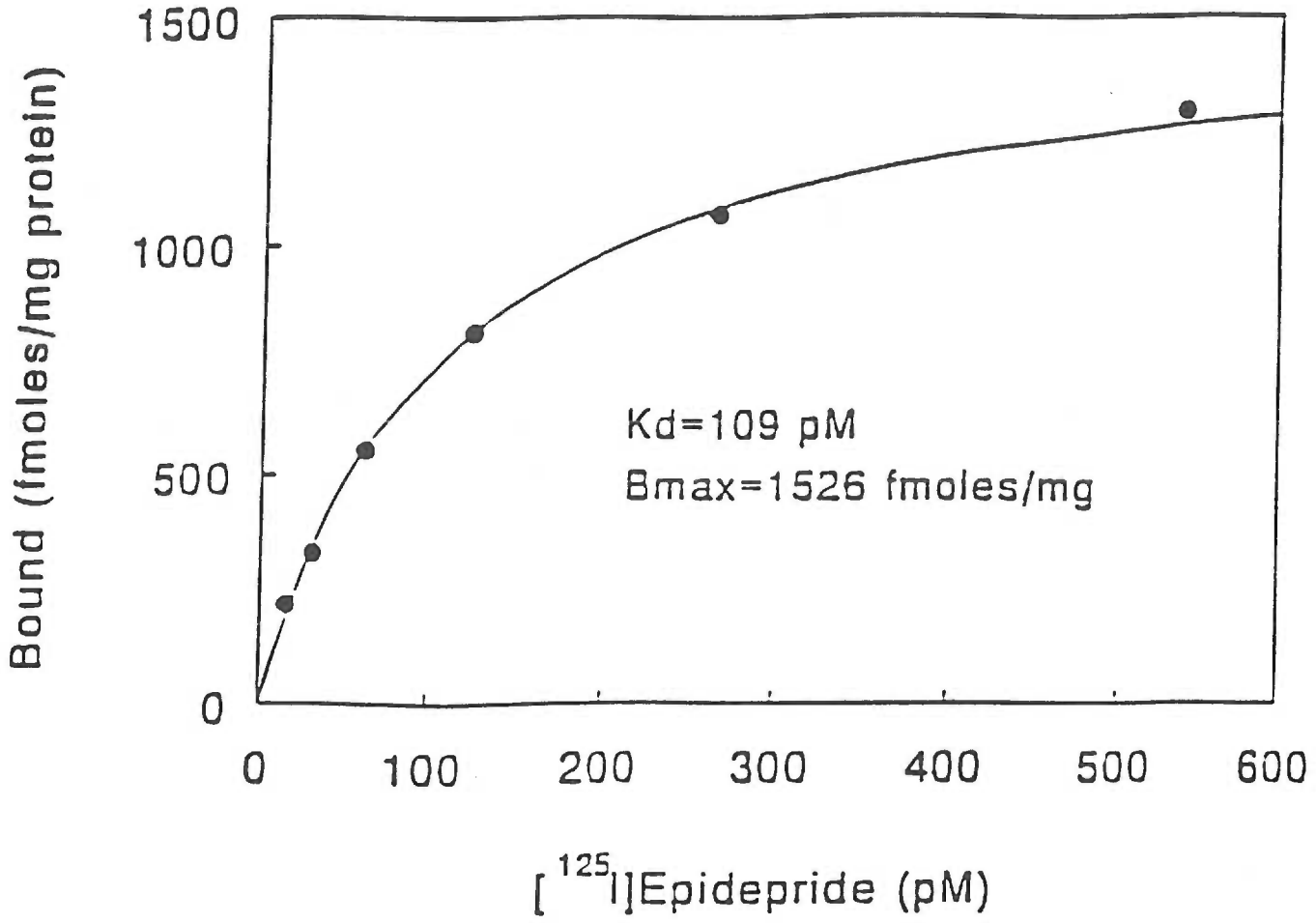

Fig. 1. Saturation analysis of the specific binding of [^{125}I]epidepride to membranes prepared from C6-D3 cells was carried out. Nonspecific binding was defined as binding not displaced by 5 μM spiperone. The results shown are from a representative experiment conducted with duplicate determinations. The affinity (K_d) of D3 receptors for [^{125}I]epidepride and the density of binding sites (B_{max}) were determined by nonlinear regession analysis.

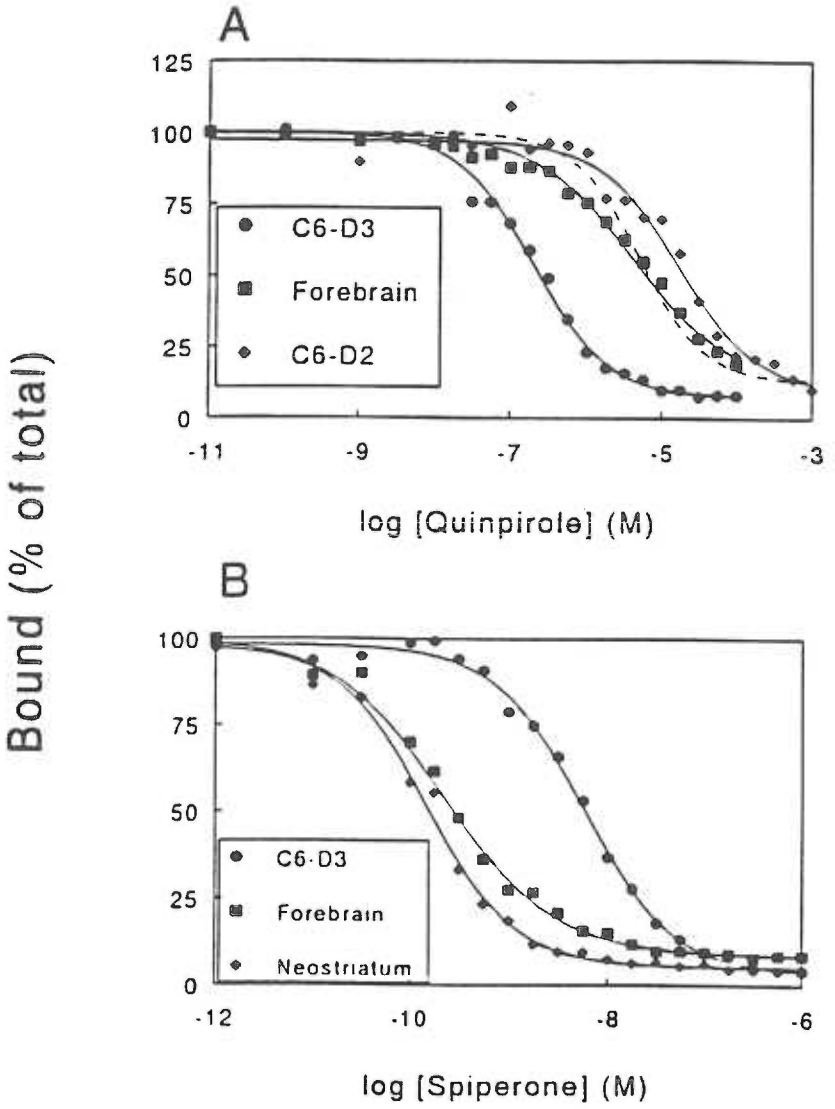

Fig. 2. Results are shown from representative experiments in which the binding of [^{125}I]epidepride was assessed in membranes prepared from C6-D3 cells, rat basal forebrain from which the neostriatum had been removed (extrastriatal basal forebrain,), neostriatum and C6-D2 cells. Averaged results from all experiments are presented in tables 1 and 2. Data are plotted as the percentage of total binding *vs.* the logarithm of the concentration of inhibitor. A, data for quinpirole inhibition of [^{125}I]epidepride binding to C6-D3 cells and C6-D2 cells were fit to a curve that assumes the presence of only one class of binding sites. For binding to extrastriatal basal forebrain membranes, the computer-drawn curve was best fit to a two-site model. In this experiment, IC_{50} values for quinpirole were 0.2, 4.0, and 17 μM for C6-D3, forebrain, and C6-D2, respectively. B, data for spiperone inhibition of [^{125}I]epidepride binding to neostriatum and C6-D3 cells were fit to a curve that assumes the presence of only one class of binding sites. For binding to extrastriatal basal forebrain membranes, the computer-drawn curve was best fit to a two-site model. In this experiment, IC_{50} values for spiperone were 0.15, 0.22, and 5.8 nM for neostriatum, forebrain, and C6-D3, respectively

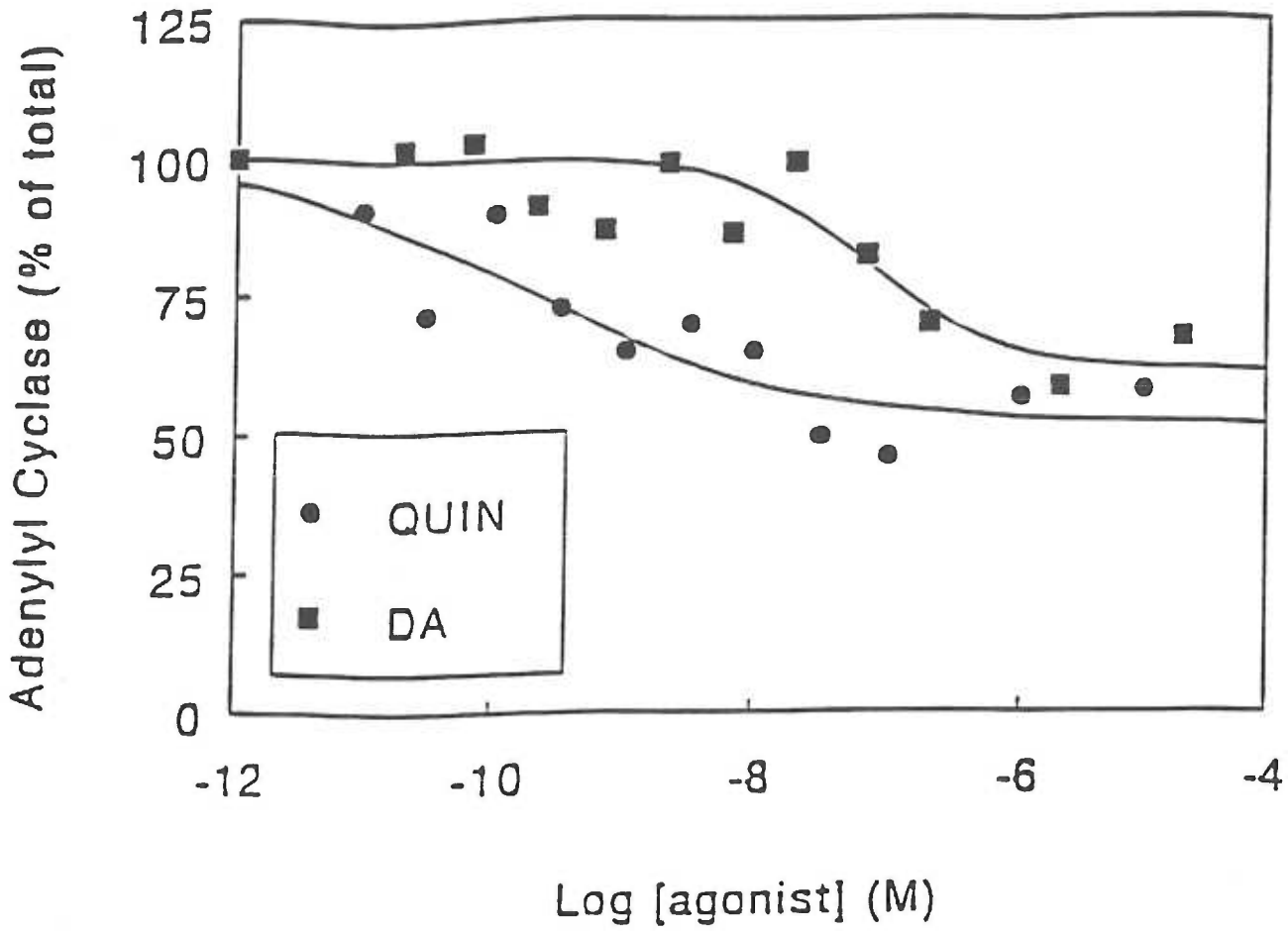

Fig. 3. Adenylyl cyclase activity in C6-D3 cells was assessed by measuring the conversion of [^{3}H]ATP to [^{3}H]cyclic AMP. The curves shown are from a single experiment in which each point is the average of triplicate determinations. Data are plotted as adenylyl cyclase activity, expressed as a percentage of total enzyme activity stimulated by 1 μM isoproterenol, *vs.* the concentration of agonist. Analysis of the curves by nonlinear regression indicated that the EC_{50} and maximal inhibition by DA were 67 nM and 38%, respectively,and 0.2 nM and 48% for quinpirole.

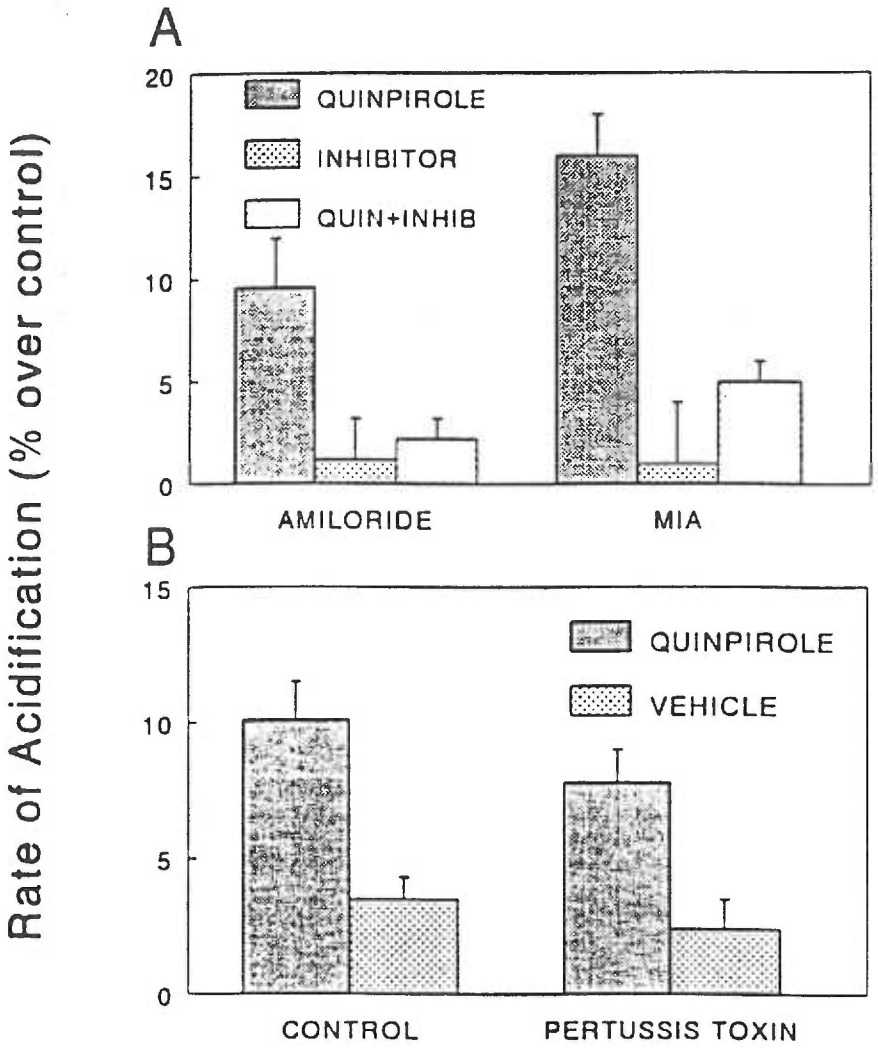

Fig. 4. The effect of Na^+/H^+ antiporter inhibitors and pertussis toxin treatment on quinpirole-induced extracellular acidification by C6-D3 cells was determined. Results are plotted as the mean ± S.E. of the increase in rate of extracellular acidification, expressed as a percentage of the rate of acidification prior to addition of drug. Data were taken from the rate of acidification determined at 2500 s after initiation of flow of medium from a given reservoir. A, in 4 independent experiments, C6-D3 cells were incubated with either 100 nM quinpirole, or the indicated inhibitor of Na^+/H^+ exchange (10 μM amiloride or 1 μM methylisobutyl amiloride, MIA), or quinpirole together with the inhiibitor (QUIN+INHIB). B, control C6-D3 cells (CONTROL) or cells that had been treated with pertussis toxin (100 ng/ml overnight) were incubated with 100 nM quinpirole or vehicle, as indicated. ($N = 3$)

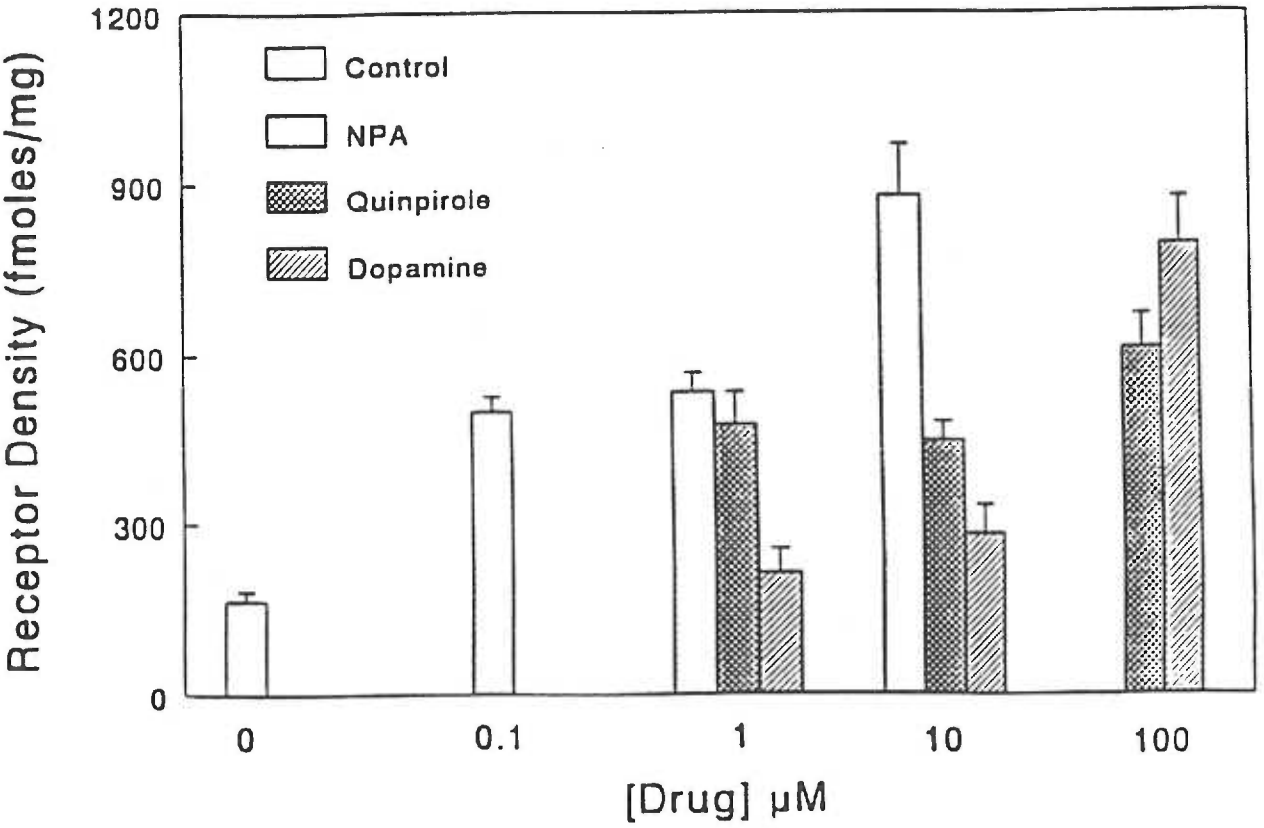

Fig. 5. Treatment of C6-D3 cells with agonists caused a concentration-dependent proliferation of D3 receptors. Cells were treated overnight with DA, quinpirole, or NPA at the indicated concentrations, before determination of the density of binding sites as described in Methods. Each bar represents the mean ± S.E. of 4 independent experiments except for 10 μM NPA ($N = 3$).

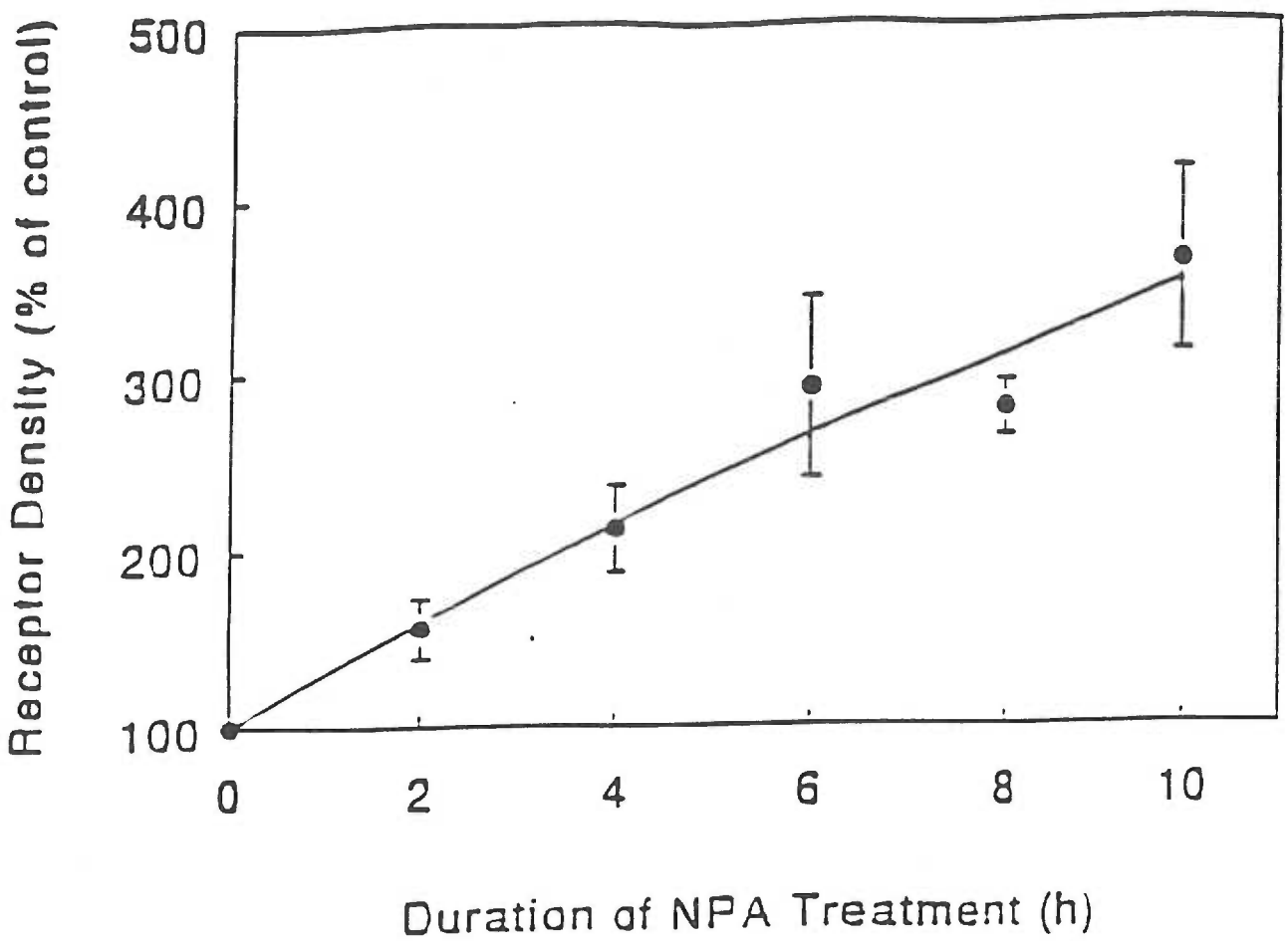

Fig. 6. The time course of proliferation of D3 receptors during treatment with 10 μM NPA was determined in 4 independent experiments in which C6-D3 cells were harvested at the indicated time after initiation of the incubation with NPA. The density of binding sites was determined as described in Methods. Longer incubations (12-24 hrs) caused no further increase in the density of receptors.

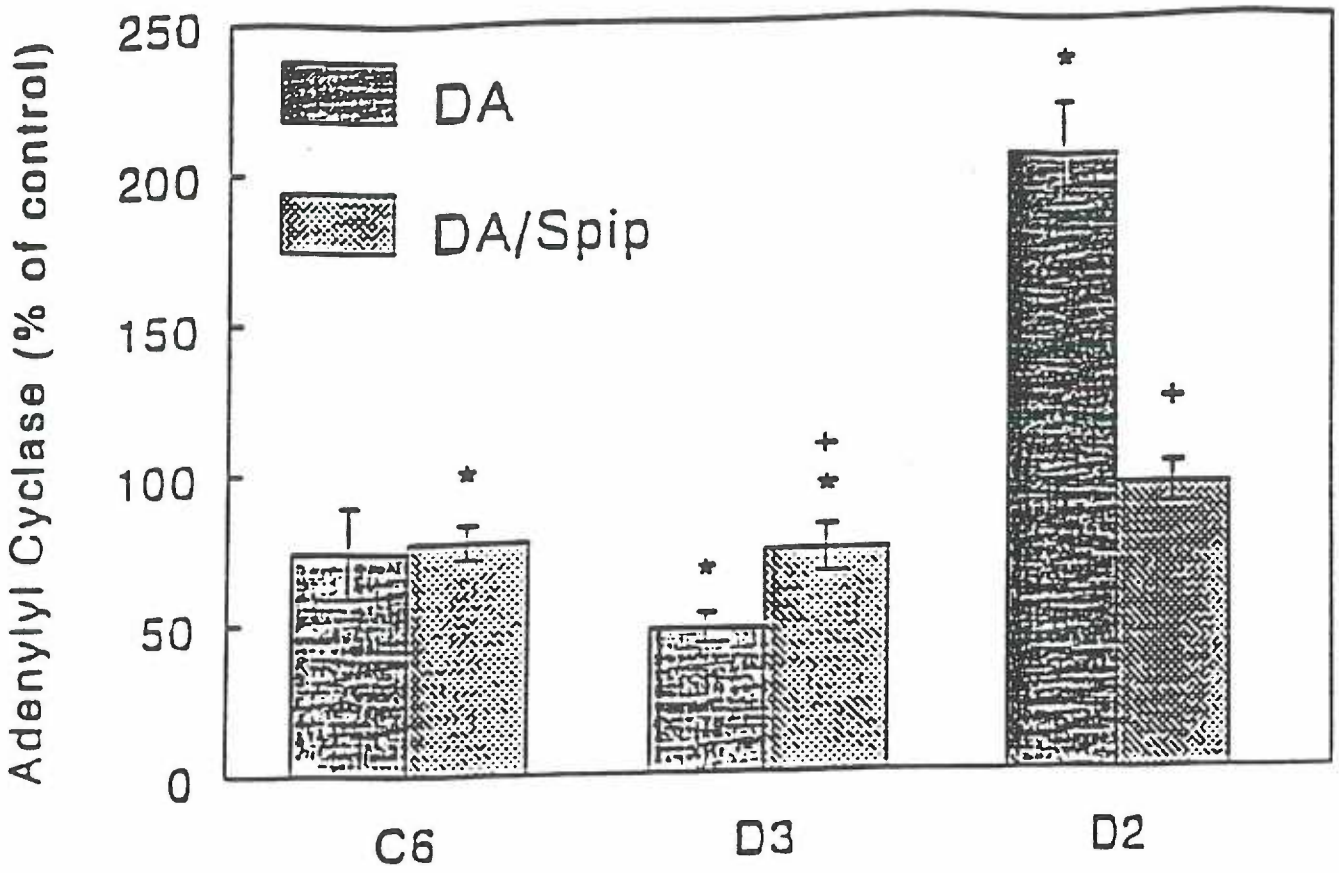

Fig. 7. Wild-type C_6 cells (C6), or C_6 cells expressing recombinant D3 (C6-D3) or D2 (C6-D2) receptors were treated with 10 μM DA (DA) or DA + 1 μM spip (DA/Spip) for 2 h. Cells were washed, and β–adrenergic receptor-stimulated adenylyl cyclase activity was determined by stimulation with 1 μM isoproterenol, as described in Methods. Results shown are the mean ± S.E. of 3-7 independent experiments, expressed as a percentage of isoproterenol-stimulated activity in untreated cells.

*$P < 0.05$ compared to control cells.

Discussion and Conclusions

Site-directed mutagenesis of D2 receptors

Initially, we set out to characterize DA D2 receptors through the use of site-directed mutagenesis. The conservation of primary structure of G protein-coupled receptors helped in the analysis of structure-function relationships by site-directed mutagenesis. Association of function with conserved amino acid residues in a specific receptor suggests a similar role for the corresponding residues in other receptors of the same subfamily.

We designed a number of mutations based on studies of the β-adrenergic receptor. We examined the roles of aspartates-80 and -114. Aspartate-80 and aspartate-114 mutants yielded results that corroborate studies done with other receptors in that these amino acid residues are important for ligand binding, receptor conformation, sensitivity to monovalent cations, and activation. A manuscript is appended which addresses the role of aspartate-80.

We also looked at the results of cysteine mutations, but these results, though interesting to us, were not interesting enough for publication. And, we looked at possible changes in ligand-binding and receptor function that might occur when deletions were made in the third cytoplasmic loop of both D2 isomers, $D2_{444}$ and $D2_{415}$. These results were presented in abstracts at meetings, but have not been published in a peer-reviewed journal.

For my discussion, I will include results of the aspartate-80 and aspartate-114 studies along with the studies involving the conserved serines in TDV. These studies are interesting and contribute to the understanding of D2 receptor and function.

The conserved aspartate residues

First, we examined the aspartate residues. We constructed and characterized mutant D2 receptors in which aspartate-80 (Asp-80) and aspartate-114 (Asp-114), corresponding to aspartate-79 and aspartate-113 of β–adrenergic receptors, were changed to glutamate (Glu80-D2 and Glu-114-D2), or changed to alanine (Ala80-D2) or asparagine (Asn114-D2).

When Asp-80 was substituted for an alanine (Ala-80), the binding of substituted benzamide derivatives, normally sensitive to sodium and pH, lost sensitivity to these monvalent cations and had reduced affinity for the mutated receptors (Neve et al., 1991). Spiperone, which has no sensitivity to sodium or pH, suffered no loss of affinity with the mutation (Neve et al., 1991). Substitution at aspartate-80 with an alanine eliminated D2 receptor-mediated inhibition of adenylyl cyclase (Neve et al., 1991). These data suggest that the aspartate-80, through an interaction with cations, is responsible for maintaining a conformation of the receptor conducive to G protein coupling and substituted benzamide binding.

When we replaced aspartate-114 with an asparagine, we could not detect any receptors in radioligand binding assays. We attribute this to a loss of affinity for the ligand. When we restored the negative charge by conservatively substituting with a glutamate, we were able to detect binding activity (Neve et al., 1993). These data argue for a role for this aspartate as a primary counterion for the positively charged substituents of ligands for the D2 receptor. This is in agreement with the β–adrenergic receptor model.

We would conclude from these data that the roles of the residues aspartate-80 and aspartate-114 are conserved among D2 and many other receptors, including β- and α–adrenergic and m_1 muscarinic receptors.

The conserved serines in TDV

We then wanted to ascertain the role of the serines in TDV. Does their role in ligand binding strictly adhere to the β–adrenergic receptor model in that two of the serines in TDV interact specifically with the two hydroxyls of the catecholamine ring? The contribution of each of the conserved serines in TDV to receptor function was assessed by constructing three mutant receptors, Ala193-D2, Ala194-D2, and Ala-197-D2, in each of which a serine was converted to alanine. These serines are conserved among catecholamine receptors, with some variation in the number of residues between the first pair and the third serine.

We found that the mutations had only slight effects on the antagonist ligands spiperone and eticlopride. The affinity of Ala194-D2 for epidepride was reduced slightly compared to wild-type receptors, as was the affinity of Ala197-D2 for sulpiride.

The affinity of several agonists was more severely affected by substitution for serine residues. The affinity of Ala193-D2 for DA was markedly decreased compared to wild-type D2 receptors. Substitution of an alanine for Ser-193 also had the greatest effect on the binding of norapomorphine (NPA). The binding of NPA was also markedly reduced by substitution for Ser-194 and Ser-197. Ala193-D2 and Ala194-D2 both had modestly reduced activity for the non-catechol agonist quinpirole.

These data suggest that Ser-193 is a primary hydrogen-bonding site for D2 receptor agonists. On the basis of agonist structure-activity relationships, it has been proposed that the *meta*-hydroxyl group of DA interacts with a primary hydrogen-bonding site, wheras the *para*-hydroxyl group interacts with an accessory site (Seeman, 1980). We sought to test this hypothesis using the mono-hydroxylated DA analogs tyramine (*para*-tyramine) and *meta*-tyramine. According to the rationale of Strader et al.(1989), if the *meta*-hydroxyl group of dopamine forms a bond with Ser-193, for example, substitution for that serine ought to decrease the potency of *meta*-tyramine. On the other hand, substitution for the serine that bonds with the *para*-hydroxyl group will not affect the binding of *meta*-tyramine, but would be expected to decrease receptor affinity for *para*-

tyramine. We were unable to confirm the hypothesis about the interaction of the *meta*- and *para*-catechol hydroxyl groups and any of the serine residues because the binding of *meta*-tyramine was not altered by substitution for any serine residues, and only Ala193-D2 had modestly reduced afiinity for *para*-tyramine.

Next, we measured inhibition of isoproterenol-stimulated adenylyl cyclase activity by DA, *meta*-tyramine, and *para*-tyramine. The potency of DA for Ala193-D2 was markedly reduced compared to wild-type D2 receptors, whereas a moderate reduction in potency was seen for Ala197-D2. Consistent with the binding data, *meta*-tyramine and *para*-tyramine were approximately equipotent and equally efficacious at $D2_{415}$, Ala193-D2, and Ala197-D2. However, Ala194-D2 was unable to mediate inhibition of adenylyl cyclase by either DA or *para*-tyramine, whereas, *meta*-tyramine was as efficacious and potent at Ala194-D2 as at $D2_{415}$.

From these data, we conclude that Ser-193 contributes importantly to the binding of DA and other agonists. Ser-194 is absolutely required for the activation of D2 receptors by some agonists. This requirement of Ser-194 for activation may be because there is a formation of a hydrogen bond with the *para*-catechol hydroxyl group. The contrast between the unchanged affinity of Ala194-D2 for DA and the loss of agonist activity could result from a separation between the residues involved in ligand binding and activation of the receptors. It could also be that Ser-194 is normally involved in the binding of DA and is also involved in the activation of the receptors, but in the absence of Ser-194, DA and *para*-tyramine bind to a distinct site with equal affinity, but with a loss of efficacy. Over all, the data indicate that ligands interact with unique but overlapping sites on D2 receptors.

Our results from site-directed mutagenesis of serine residues agreed with results of a recent molecular modeling study of the binding of DA to TD III-V of the D2 receptor. Moereels and Leysen (1993) calculated two minimized complexes, one starting with DA in a position corresponding to binding of catecholamines to β–adrenergic receptors, so

that the *meta*- and *para*-catechol hydroxyl groups bond to Ser-194 and Ser-197, and the second complex beginning with DA tilted by about 90° relative to the starting position for complex one, with no interaction assumptions. In both minimized complexes, there was no interaction between Ser-194 and DA. Complex 1 had hydrogen bonds between the *meta*- and *para*-catechol hydroxyl groups and Ser-193 and Ser-197, whereas in complex 2, which was more stable than complex 1, Ser-193 interacted with the phenyl ring of DA and Ser-197 interacted with the NH_3^+ group. This modeling study does not explain the inability of DA to activate Ala194-D2.

Comparison of the role of TDV serine residues in D2 receptors vs. other catecholamine receptors

Strader et al. (1989) first assessed the contributions of conserved serine residues to the interactions of ligands with β_2-adrenergic receptors, obtaining compelling data in support of hydrogen-bonding interactions between the *meta*- and *para*-catechol hydroxyl groups of agonists and Ser-204 and Ser-207, respectively. Interestingly, just as DA and *para*-tyramine bound to Ala194-D2 with unchanged affinity but no apparent efficacy, Strader and coworkers observed that Ala207-βAR and wild-type β–adrenergic receptors have similar affinities for a compound with only one hydroxyl substitution in the *para* position on the phenyl ring, although the mono-hydroxylated compound was unable to activate Ala207-βAR. These authors postulated that in the absence of Ser-207 hydrogen-bonding can occur between the *para*-catechol hydroxyl group and Ser-204, but that this interaction does not lead to activation of β–adrenergic receptors. β_2-Adrenergic receptors in which Ser-203 (corresponding to Ala-193 of D2 receptors) was changed to alanine are apparently not inserted into the cell membrane, as determined by lack of immunoreactivity.

In one mutagenesis study of α_2-adrenergic receptors, serines 200 and 204 (presumably corresponding to Ser-194 and Ser-197 of D2 receptors) were changed to

alanine (Wang et al., 1991). Ser-199 (perhaps corresponding to Ser-193 of D2 receptors) was not mutated. Mutation of either Ser-200 or Ser-204 decreases the affinity of epinephrine or the *meta*-catechol hydroxyl-substituted agonist phenylephrine, whereas the affinity of the *para*-catechol hydroxyl-substituted isomer synephrine is unaffected in assays of radioligand binding. These results are consistent with an interaction of either serine with the *meta*-hydroxyl group, whereas the modest decrease in the ability of Ala204-α_2-adrenergic receptors to mediate inhibition of adenylyl cyclase activity by synephrine points to an interaction between the para-catechol hydroxyl group and Ser-204 that is important in activation of the receptors.

Pollock et al. (1992) mutated serines 198, 199, and 202 of DA D1 receptors, corresponding to Ser-193, 194, and 197 of D2 receptors. Mutation of Ser-199 to alanine reduced the affinity of a number of agonists and antagonists, and also decreases the maximal stimulation of adenylyl cyclase activity by SKF-38393, but not DA. Mutation of Ser-202 has little effect on the binding of most ligands, except that Ala202-D1 has markedly decreased affinity for DA. The maximal stimulation of adenylyl cyclase activity by DA is not decreased by mutation of Ser-202, although stimulation by SKF-38393 is decreased. Mutation of Ser-198 greatly decreases the affinity of a number of agonist and antagonist ligands. As observed for the other two mutants, the maximal accumulation of cyclic AMP stimulated by SKF-38393, but not by DA, is reduced. One problem with assessing activation of receptors in this way, and a possible explanation for the finding by these authors that stimulation by SKF-38393 is selectively diminished at all three mutants, is that a receptor reserve could mask a diminution of the efficacy of DA. Loss of efficacy for SKF-38393, a partial agonist (Machida et al., 1992), would be evident before loss of efficacy of DA. A receptor reserve would be particularly likely to be present when receptors are expressed at the high densities (approximately 5 pmol/mg of protein) reported by Pollock *et al.* (1992). It is also possible that the amino acids involved in activation of D1 receptors by DA are different from those involved in activation by SKF-

38393. These results using mutant D1 receptors demonstrate that serine residues in TDV are important for ligand binding and activation of the receptors, and that different ligands may have distinct binding sites.

In summary, these results from four receptors confirm in a general sense the important role ascribed to serine residues in TDV in the model of Strader et al. (1989). The contribution of each serine considered independently, however, is less well conserved across classes of receptors. The serine corresponding to Ser-193 of D2 receptors is required for proper synthesis and processing of β_2–adrenergic receptors, but not for D1 or D2 receptors. Of the three serine residues, Ser-193 contributes most to the affinity of D1 and D2 receptors for DA. Ser-197 also contributes to the affinity of both of these classes of receptors for DA. The serine residues corresponding to Ser-194 and Ser-197 both contribute to the binding of catecholamines to β_2– and α_2–adrenergic receptors. Mutation of any of the serines of D1 receptors decreases maximal stimulation of adenylyl cyclase activity by SKF-38393, whereas mutation of the residue corresponding to Ser-197 only reduces α_2–adrenergic receptor activation by a *para*-catechol hydroxyl-substituted agonist, and mutation of Ser-194 prevent activation of D2 receptors by DA and *para*-tyramine.

Characterization of D2 receptor subtypes $D2_{444}$, $D2_{415}$, and D3

We were also interested in differences among the D2 receptor subtypes. Originally, we wanted to determine differences, if any, between $D2_{444}$ and $D2_{415}$, the two molecular forms of D2 receptor generated by alternative splicing of a single gene (Selbie et al., 1989; Monsma et al., 1989; Giros et al., 1989; Dal Toso et al., 1989). It was thought by many that there might be distinct differences between these two isoforms, but, if there are differences, they are subtle. $D2_{415}$ and $D2_{444}$ expressed in C6 cells have similar affinities for [^{125}I]epidepride and other antagonists that we have tested, and respond similarly to changes in pH or sodium concentrations (Neve et al., 1991;

appended). Both forms inhibit adenylyl cyclase activity and activate Na+/H+ exchange (Neve et al., 1992), although we, and others, have reported that $D2_{415}$ inhibits adenylyl cyclase more efficiently than $D2_{444}$ (Tester et al., 1991; Montmayeur and Borrelli, 1991; Montmayeur et al., 1993; Hayes et al., 1992). The affinities of $D2_{415}$ and $D2_{444}$ for DA in the presence or absence of GTP are similar, although in the absence of GTP the proportion of $D2_{444}$ receptors with high affinity for DA is somewhat lower than the proportion of $D2_{415}$ receptors with high affinity for DA, in agreement with the finding of others (Tester et al., 1991; Castro and Strange, 1993). In addition, the human short form may be more sensitive than the long form to desensitization by protein kinase C (Liu et al., 1992). Therefore, despite early speculation, we and other investigators have been unable to detect major differences between $D2_{444}$ and $D2_{415}$.

The D3 receptor

Finally, we characterized the D2-like receptor D3. We used C6 glioma cells stably transfected with D2 and D3 receptor cDNA, C6-D2 and C6-D3, to characterize D3 receptors. We found that the D2 radioligand [^{125}I]epidepride bound with high affinity to D3 receptors. We compared the binding of [^{125}I]epidepride to C6-D2 and C6-D3 cells, and to rat brain. We were able to identify a small population of receptors in the extrastriatal basal forebrain, but not in the neostriatum, that had the pharmacological profile of recombinant D3 receptors. The agonist quinpirole was selective for D3 receptors, and the antagonists spiperone and domperidone were selective for D2 receptors, consistent with the reported pharmacological profile for D3 receptors.

DA and quinpirole inhibited isoproterenol-stimulated cyclase activity in confluent C6-D3 cells. The inhibition was modest and was consistent only when cells were grown to confluence. In many cell lines D3 receptors appear not to inhibit adenylyl cyclase although inconsistent inhibition has been observed by human D3 receptors expressed in CHO cells (Sokoloff et al., 1992). As assessed by GTP-sensitive binding of agonists, D3

receptors appear to couple less efficiently than D2 receptors to G proteins (Sokoloff et al., 1992; Seabrook et al., 1992; Castro and Strange, 1993). It has been suggested that cell lines used to express recombinant D3 receptors have lacked the appropriate G protein. It has also been proposed that the D3 receptor is "locked" in a high affinity state and would, thus, be in a conformation that is not conducive to G protein coupling. The D3 receptor's much higher affinity for agonists DA and quinpirole lends support to this hypothesis. Additionally, our mutational studies of Asp 80-D2 point to a need for a particular conformation for G protein coupling. We had originally thought that studies of $D2_{444}$ and $D2_{415}$ would demonstrate specific differences in G protein coupling resulting from the 29 amino acid insertion in the third cytoplasmic loop. As it turns out, Okamato and Nishimoto (1992) have identified a sequence motif involved in the coupling of receptors to G proteins. The conserved motif includes regions between 10 and 26 residues in length, with at least 2 basic residues at the amino-terminal side, and the carboxy-terminal motif, B-B-X-B or B-B-X-X-B, where B is a basic residue (lysine, arginine, or histidine) and X is a non-basic residue. D2 receptors have two copies of this motif in the third cytoplasmic loop, amino acids (AA) 211-230 and AA292-316 of $D2_{444}$. D2 and D3 receptors have considerable sequence homology in the region 211-230, but D3 receptors lack the carboxy-terminal part of the motif (B-B-X-X-B) in that region and elsewhere in the third cytoplasmic loop. This could be another reason why D3 receptors seem to couple weakly to G proteins.

The D3 receptor has confounded many investigators because of its seeming "lack of function". In our hands, studies involving G protein coupling, such as GTP-induced shifts in affinity for agonists and inhibition of adenylyl cyclase, have also seemed tentative. We finally have concluded that there is some agonist-induced inhibition of isoproterenol-stimulated adenylyl cyclase activity, albeit modest, and consistent only when C6-D3 cells are grown to confluence.

We have also determined that quinpirole accelerates extracellular acidification in C6-D3 and C6-D2 cells (Neve et al., 1992). The accelerated extracellular acidification, which is thought to be due to stimulation of Na+/H+ exchange, was sensitive to the D2 and D3 antagonist epidepride and to inhibitors of Na+/H+ exchange, but was not sensitive to inactivation of pertussis toxin-sensitive G proteins.

Both C6-D2 receptors and C6-D3 receptors upregulate after prolonged treatment with agonists. That is, there is marked increase in receptor density when recombinant D2 and D3 receptor cell lines are treated for several hours by agonists. Surprisingly, D3 receptors have much higher increases than those reported for D2 receptors (Kozell et al., 1992; Zhang et al., 1992; Molinoff et al., 1992). There was no significant increase in mRNA in C6-D3 cells treated with the agonist NPA. Therefore, agonist-induced proliferation may be due to either a post-translational mechanism or due to a decreased rate of degradation of D3 receptors. Again, as treatment with pertussis toxin had no effect on agonist-induced proliferation, pertussis toxin-sensitive G proteins appear not to be involved.

It has been proposed (Ivins et al., 1991) that agonist-induced proliferation of D2 receptors occurs as a result of increased intracellular cAMP due to increased adenylyl cyclase activity which is observed after prolonged stimulation of D2 receptors (Ivins et al., 1991; Bates et al., 1991). In C6 cells we found that stimulation of D2 receptors with DA for 2 hours greatly enhanced adenylyl cyclase activity. However, this is probably not the mechanism for the proliferation of D3 receptors, because prolonged activation of D3 receptors by DA reduced the subsequent stimulation of adenylyl cyclase activity by isoproterenol. This heterologous desensitization of β–adrenergic receptors was inhibited by spiperone and was not observed in wild-type C6 cells.

In summary, D3 receptors are unique members of the D2-like family. They share a pharmacology that is similar to D2 receptors, but different in that certain agonists have much higher affinity for D3 receptors. Functional consequences of these differences are

still being studied. Compared to D2 receptors, D3 receptors couple weakly to pertussis toxin-sensitive G proteins. Differences in structure between D2 and D3 receptors in the putative third cytoplasmic loop may support structural arguments for the apparently weak coupling to G proteins. Both D2 and D3 receptors participate in agonist-induced extracellular acidification, arguably due to stimulation of Na+/H+ exchange, and prolonged agonist activation of both D2 and D3 receptors results in proliferation of receptors. D3 receptors exist in lower numbers in brain compared to D2 receptors, but are regionally specific for 'limbic' areas. This regional specificity combined with their somewhat differing pharmacology and function point to D3 receptors having a specific function in dopaminergic systems.

REFERENCES

Bates M.D., Senogles S.E., Bunzow J.R., Liggett S.B., Civelli O., and Caron M.G. (1991) Regulation of responsiveness at D_2 dopamine receptors by receptor desensitization and adenylyl cyclase sensitization. *Mol. Pharmacol.* **39**, 55-63.

Castro S.W. and Strange P.G. (1993) Coupling of D_2 and D_3 dopamine receptors to G-proteins. *FEBS Lett.* **315**, 223-226.

Dal Toso R., Sommer B., Ewert M., Herb A., Pritchett D.B., Bach A., Shivers B.D., and Seeburg P.H. (1989) The dopamine D_2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* **8**, 4025-4034.

Giros B., Sokoloff P., Martres M.-P., Riou J.-F., Emorine L.J., and Schwartz J.-C. (1989) Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature (Lond.)* **342**, 923-926.

Hayes G., Biden T.J., Selbie L.A., and Shine J. (1992) Structural subtypes of the dopamine D2 receptor are functionally distinct: Expression of the cloned $D2_A$ and $D2_B$ subtypes in a heterologous cell line. *Mol. Endocrinol.* **6**, 920-926.

Ivins K.J., Luedtke R.R., Artymyshyn R.P., and Molinoff P.B. (1991) Regulation of dopamine D2 receptors in a novel cell line (SUP1). *Mol. Pharmacol.* **39**, 531-539.

Kozell L.B., Starr S., Machida C.A., Neve R.L., and Neve K.A. (1992) Agonist-induced changes in density of D1, D2, and chimeric D1/D2 receptors. *Soc. Neurosci. Abstr.* **18**, 276.

Liu Y.F., Civelli O., Grandy D.K., and Albert P.R. (1992) Differential sensitivity of the short and long human dopamine D_2 receptor subtypes to protein kinase C. *J. Neurochem.* **59**, 2311-2317.

Machida C.A., Searles R.P., Nipper V., Brown J.A., Kozell L.B., and Neve K.A. (1992) Molecular cloning and expression of the rhesus macaque D1 dopamine receptor gene. *Mol. Pharmacol.* **41**, 652-659.

Moereels H. and Leysen J.E. (1993) Novel computational model for the interaction of dopamine with the D_2 receptor. *Receptors Channels* **1**, 89-9y.

Molinoff P.B., Filtz T.M., and Artymyshyn R.P. (1992) Regulation of D2 receptors in cultured cells. *Proc. 7th International CA Symp.* 218.

Monsma F.J., McVittie L.D., Gerfen C.R., Mahan L.C., and Sibley D.R. (1989) Multiple D_2 dopamine receptors produced by alternative RNA splicing. *Nature* **342**, 926-929.

Montmayeur J.-P. and Borrelli E. (1991) Transcription mediated by a cAMP-responsive promoter element is reduced upon activation of dopamine D_2 receptors. *Proc. Natl. Acad. Sci. USA* **88**, 3135-3139.

Montmayeur J.P., Guiramand J., and Borrelli E. (1993) Preferential coupling between dopamine D2 receptors and G-proteins. *Mol. Endocrinol.* **7**, 161-170.

Neve K.A., Cox B.A., Henningsen R.A., Spanoyannis A., and Neve R.L. (1991) Pivotal role for aspartate-80 in regulation of D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol. Pharmacol.* **39**, 733-739.

Neve K.A., Kozlowski M.R., and Rosser M.P. (1992) Dopamine D2 receptor stimulation of Na^+/H^+ exchange assessed by quantification of extracellular acidification. *J. Biol. Chem.* **267**, 25748-25753.

Neve, K.A., Henningsen, R.A., Kozell, L.B., Cox, B.A. and Neve, R.L. Structure-function analysis of dopamine-D_2 receptors by in vitro mutagenesis. In: *Dopamine receptor function and pharmacology*, edited by Niznik, H.B. New York: Marcel Dekker, Inc., 1993, p. 299-321.

Okamoto T. and Nishimoto I. (1992) Detection of G protein-activator regions in M_4 subtype muscarinic, cholinergic, and α_2-adrenergic receptors based upon characteristics in primary structure. *J. Biol. Chem.* **267**, 8342-8346.

Pollock,N.J., Manelli, A.M., Hutchins, C.W., Steffey, M.E., Mackenzie, R.G., and D.E. Frail (1992) Serine mutations in transmembrane V of the dopamine D1

receptor affect ligand interactions and receptor activation *J. Biol. Chem.* **267**,17780-17788.

Seabrook G.R., Patel S., Marwood R., Emms F., Knowles M.R., Freedman S.B., and Mcallister G. (1992) Stable expression of human D_3 dopamine receptors in GH_4C_1 pituitary cells. *FEBS Lett.* **312**, 123-126.

Seeman P. (1980) Brain dopamine receptors. *Pharmacol. Rev.* **32**, 229-313.

Selbie L.A., Hayes G., and Shine J. (1989) The major dopamine D2 receptor: Molecular analysis of the human $D2_A$ subtype. *DNA* **8**, 683-689.

Sokoloff P., Andrieux M., Besançon R., Pilon C., Martres M.-P., Giros B., and Schwartz J.-C. (1992) Pharmacology of human dopamine D_3 receptor expressed in a mammalian cell line: comparison with D_2 receptor. *Eur. J. Pharmacol-Molec. Pharm.* **225**, 331-337.

Strader C.D., Candelore M.R., Hill W.S., Sigal I.S., and Dixon R.A.F. (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.* **264**, 13572-13578.

Tester B.A., Henningsen R.A., Spanoyannis A., Neve R.L., and Neve K.A. (1991) Flag-$D2_{415}$ and Flag-$D2_{444}$, unique mutants of dopamine D2 receptors $D2_{415}$ and $D2_{444}$, offer a novel approach for rapid purification of D2 receptors. *Soc. Neurosci. Abstr.* **17**, 815.

Wang C.-D., Buck M.A., and Fraser C.M. (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol. Pharmacol.* **40**, 168-179.

Zhang L.-J., Smyk-Randall E.M., Monsma F.J., and Sibley D.R. (1992) Regulation of the D_{2L} receptor in stably transfected CHO cells. *Soc. Neurosci. Abstr.* **18**, 663.

Appendix 1

A Pivotal Role for Aspartate-80 in Regulation of Dopamine D-2 Receptor Affinity for Drugs and Inhibition of Adenylyl Cyclase

Kim A. Neve, Barbara A. Cox, Robert A. Henningsen,

Athena Spanoyannis, and Rachael L. Neve

Medical Research Service, VA Medical Center (K.A.N., B.A.C., R.A.H.), Departments of Pharmacology (K.A.N., R.A.H.) and Biochemistry and Molecular Biology (B.A.C.), Oregon Health Sciences University, Portland, Oregon; Department of Psychobiology, University of California, Irvine, California (A.S., R.L.N.)

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Running Title: Aspartate-80 and Dopamine D-2 Receptor Function

Summary

An aspartate residue corresponding to aspartate-80 of dopamine D-2 receptors is strictly conserved among G protein-coupled receptors. Mutation of this residue alters the function of several classes of neurotransmitter receptors. Dopamine D-2 receptors couple to the G protein, G_i, to inhibit adenylyl cyclase (ATP-pyrophosphate-lyase, cyclizing; EC 4.6.1.1). Like other G_i-coupled receptors, the binding of agonists and some antagonists to D-2 receptors is sensitive to pH and sodium. In the present report, we demonstrate that substitution of an alanine or glutamate residue for aspartate-80 severely impairs inhibition of adenylyl cyclase by D-2 receptors, and also abolishes or decreases the regulation of the affinity of D-2 receptors for agonists and substituted benzamide antagonists by sodium and pH. Our data support the hypothesis that the conformation of D-2 receptors is maintained by interactions of monovalent cations with aspartate-80. The regulation of D-2 receptors by this interaction has important consequences for the affinity of D-2 receptors for ligands, and for signal transduction by D-2 receptors.

Introduction

DA[1] D-2 receptors couple to G_i to attenuate the activity of adenylyl cyclase (1,2). Among the characteristics of G_i-coupled receptors that are shared by D-2 receptors is the ability to modulate several signaling pathways (3). In addition to inhibiting adenyly cyclase activity, activation of D-2 receptors has been reported to inhibit or stimulate hydrolysis of polyphosphoinositides (4,5), increase K^+ conductance (6), inhibit Ca^{2+} channels (7), and decrease Na^+/H^+ exchange (8).

G_i-coupled receptors also have in common the property of sensitivity to sodium. Regulation of DA D-2 receptors by sodium is reflected in several phenomena. First, sodium enhances inhibition of adenylyl cyclase activity by D-2 receptors (2). Second, in receptor binding assays carried out using membrane preparations, sodium decreases the affinity of D-2 receptors for agonists (9,10). Third, sodium greatly increases the affinity of D-2 receptors for substituted benzamide antagonists (11,12). The affinity of DA D-2 receptors for agonists and substituted benzamides is also altered by varying $[H^+]$ between pH 6.8 and 7.5 or 8 (13). These effects of pH and Na^+ on DA D-2 receptors may be related to the ability of D-2 receptors to inhibit Na^+/H^+ exchange (8).

We have proposed that this modulation of the affinity of D-2 receptors for ligands results from a Na^+- or H^+-dependent receptor isomerization. Thus, decreasing $[Na^+]$ or pH accelerates the dissociation of the substituted benzamide ligand [^{125}I]epidepride from D-2 receptors, as would be expected for an allosteric interaction in which the conformation of the receptor depends on the concentrations of Na^+ and H^+ (12, 13). Furthermore, inactivation of D-2 receptors by NEM is inhibited by high $[H^+]$ (pH 6.8) or Na^+, indicating that the accessibility of a target for alkylation by NEM is modified by a Na^+- or H^+-dependent conformational change (13-15).

For α_2-adrenergic receptors, increasing $[Na^+]$ or $[H^+]$ lowers the affinity of the receptors for agonists by acting directly on the receptors (16). The site of action of

sodium is intracellular, so that modulation of cytoplasmic [Na^+] alters the binding of agonists to α_2-adrenergic receptors on intact cells (17). The binding of agonists to D-2 receptors is regulated by sodium even after inactivation of G proteins by pertussis toxin (10) or alkylation (18), indicating that an interaction between G proteins and D-2 receptors is probably not involved in the regulation of ligand binding by sodium. We now report that replacement of aspartate-80 in D-2 receptors with an alanine residue results in the expression of a receptor that is insensitive to changes in [Na^+] and has reduced sensitivity to changes in [H^+]. Furthermore, the mutant receptor is no longer able to inhibit adenylyl cyclase activity, suggesting that signal transduction by D-2 receptors involves maintaining or adopting a particular receptor conformation as a result of the action of H^+ or Na^+ at Asp-80.

Materials and Methods

Site-Directed Mutagenesis of D-2 Receptors. Trans-PCR, which is used to join DNA fragments that contain a region of overlap, was used to perform site-directed mutagenesis by employing primers that contain the desired base substitutions. To create the alanine substitution for Asp-80, we made the oligonucleotide 5'-CTTGCTGTGGCTGCTCTTCTGGTG-3' and its reverse complement, which changes GAT (Asp) to GCT (Ala). To create the glutamate substitution for Asp-80, we made the oligonucleotide 5'-CTTGCTGTGGCTGAACTTCTGGTG-3' and its reverse complement, altering the GAT to GAA (Glu). Each mutation was carried out in two steps. In the first step, the fragments to be joined were amplified in separate reactions, in which the sense mutated primer was paired with a downstream unmutated antisense oligonucleotide primer, and the antisense mutated primer was paired with an upstream wild-type sense primer. Both reactions used 1 ng of $D\text{-}2_{415}$ cDNA as template. In the second step, PCR was performed using a small amount of each product from the first reactions, together with the outer primers. The PCR for both steps was done under

standard conditions (94°C for 1 min, 60°C for 2 min, 72°C for 3 min) for 20 cycles. The PCR product was extracted with phenol and chloroform, precipated using ethanol, and digested to completion with *Xho*I (within the 5' end of the PCR fragment) and *Bgl*II (within the 3' end of the fragment), yielding a 1.3 kb fragment including virtually all of the coding region of D-2_{415}. This fragment was gel-purified and ligated to pRSV-$D2_{415}$, which had been cleaved with the same enzymes and gel-purified away from its wild-type *XhoI-Bgl*II insert. Confirmation of the mutations was obtained by sequence analysis of the clones.

DNA-Mediated Expression of Mutant and Wild-Type D-2 Receptors in C_6 Glioma Cells. C_6 glioma cells were maintained as described previously (19), except that the medium was supplemented with 3% calf bovine serum and 2% fetal bovine serum. Transfection of C_6 cells was carried out by calcium phosphate precipitation (20). Exponentially growing cells were seeded in 10 ml of Dulbecco's modification of Eagle's medium (DMEM)/10 cm plate and incubated overnight. D-2_{415} and D-2_{444} were cloned into pRSV (21), modified by the addition of a polylinker containing several unique restriction enzyme sites. Plasmid DNA (15 μg), comprised of pRSV-D2 and pRSVneo (7:2), was mixed with 0.5 ml of 0.25 M $CaCl_2$. An equal volume of 2X BBS (50 mM *N*-,*N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added, and the mixture was incubated at room temperature for 20 min before adding by drops to the medium in the plate. The plate was incubated 20-24 hrs in an atmosphere of 3% CO_2/97% air, washed with phosphate-buffered saline, then re-fed and incubated in an atmosphere of 10% CO_2. On the second day after addition of DNA, the cells were split into 10 plates and incubated overnight before beginning selection with G418 (600 μg/ml, Sigma). Clonal G418-resistant cells were isolated after approximately 2 weeks of G418 treatment by trypsinization within a 10 ll cloning ring, transferred to 96-well plates, and expanded

into duplicate 60 mm plates. Cell lines positive for D-2 receptors, determined by binding of [^{125}I]epidepride, were expanded into 10 cm plates for further characterization.

Radioiodination of epidepride (NCQ 219). (S)-(-)-*N*-[(1-Ethyl-2-pyrrolidinyl)-methyl]-2,3-dimethoxy-5-(tri-*n*-butyltin)benzamide (20 μg/20 μl of absolute ethanol) was mixed with Na[^{125}I] (10 mCi/20 μl). The solution was acidified by adding 10 μl of 0.4 N HCl. Chloramine-T (20 μg/10 μl water) was added, and the reaction proceeded for 3 min. The reaction was stopped by addition of meta-sodium bisulfite (380 μg/20 μl of water), then alkalinized. After extraction into ether, the product was purified by HPLC, using a cyano-silica column (100 x 4.6 mm, Waters, Radial PAK 8NYCN4HP) in a mobile phase of 38% EtOH/62% 20 mM potassium phosphate, pH 6.8. Radiochemical yield was 80-90%.

Radioligand Binding Assays. Cells were lysed by replacing the growth 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 from the plate and centrifuged at 24,000 x *g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer at setting 6 for 10 sec in 5 mM K^+-HEPES (pH 7.4) and stored at -70° for receptor binding experiments. Aliquots of the membrane preparation were added to assay tubes containing (final concentrations): 50 mM Tris-HCl, pH 7.4, with 0.9% NaCl (Tris-buffered saline) except where indicated, 0.025% ascorbic acid, 0.001% bovine serum albumin, [^{125}I]epidepride (2000 Ci/mmol) or [^{3}H]spiperone, and appropriate drugs. In experiments to determine the effect of pH and Na^+ on binding affinity, Tris-buffered saline was not used. Reagents were added to each assay in water or 5 mM K^+-HEPES before the addition of 50 mM K^+-HEPES (final), pH 6.6 or 7.5, and 100 mM NaCl or 100 mM NMDG (22). (+)-Butaclamol (2 μM) or spiperone

(1 μM) was used to define nonspecific binding in assays of the binding of [^{3}H]spiperone or [^{125}I]epidepride, respectively. GTP (100 μM) was added to assays in experiments assessing the binding of agonists. Assays were carried out in duplicate except where indicated. Incubations were initiated by the addition of tissue, carried out at 30°C for 60 min, and stopped by the addition of 10 ml of ice-cold wash buffer (10 mM Tris, pH 7.4, and 0.9% NaCl) to each assay. The samples were filtered through glass-fiber filters (Schleicher & Schuell No. 30) and washed with an additional 10 ml of wash buffer. The radioactivity retained on the filter was counted using a Beckman LS 1701 scintillation counter or a gamma counter (LKB Clinigamma 1272).

Equilibrium binding assays. Saturation experiments were carried out in a volume of 5 ml ([^{3}H]spiperone), or 0.25 or 0.5 ml ([^{125}I]epidepride). Data were analyzed by nonlinear regression using the program GraphPAD. Competition experiments were carried out in a volume of 1 or 2 ml using [^{3}H]spiperone as the radioligand. IC_{50} values were determined by nonlinear regression analysis using GraphPAD. In all competition experiments the concentration of [^{3}H]spiperone ranged from approximately 120-180 pM. K_I values were calculated from experimentally determined IC_{50} values as described by Munson and Rodbard (23). Averages for K_I and K_D values are expressed as pK_I or $pK_D \pm$ SE. The standard Gibbs free energy change (ΔG^o) was calculated from the equation $\Delta G^o = RT \ln K_D$, where R is the gas constant and T is the temperature in degrees Kelvin. Protein was measured by the method of Peterson (24).

Treatment with NEM. Membrane aliquots were incubated with 3 mM NEM at pH 6.6 or 7.5 (20 mM K^+-HEPES), in the presence 50 mM NaCl or 50 mM NMDG. After 30 min at 37°C, the reaction was quenched by adding an equal volume 6 mM dithiothreitol to each preparation, and the density of binding sites was determined by saturation analysis of the binding of [^{125}I]epidepride in the presence of 100 mM

HEPES-buffered saline (pH 7.3). An aliquot (100 μl) of the membrane mixture was added to each 0.5 ml assay, for a final concentration of 0.3 mM NEM and 1.2 mM dithiothreitol. In preliminary experiments, these concentrations were determined to have no detectable effect on the binding of [^{125}I]epidepride.

Cyclic AMP Accumulation. The accumulation of cyclic AMP in intact cells was measured as described previously (19). Cells were seeded in 6-well cluster dishes at a density of 18,000/cm^2. On day 3 the growth medium was replaced by 1.5 ml HEPES-buffered L15 medium and the cells were incubated in air at 37^o for 2 hr. [^{3}H]Adenine (1 lCi/well) was added to the incubation medium 15 min before drug or vehicle. Incubation with drugs was carried out for 7 min, and terminated by rinsing 2 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 the percentage of [^{3}H]ATP converted to [^{3}H]cyclic AMP.

Results

Binding of [^{125}I]Epidepride to D-2$_{415}$ and D-2$_{444}$. DA D-2 receptor cDNAs encoding the D-2$_{415}$ (415 amino acid) and D-2$_{444}$ (444 amino acid) variants of D-2 receptors were cloned into pRSV (21) and expressed by stable transfection into C_6 glioma cells. The affinity of D-2$_{415}$ and D-2$_{444}$ for [^{125}I]epidepride, and the sensitivity of the two forms to changes in pH and [Na^+], was determined in membranes prepared from the cells. As shown in Table 1, the dependence of affinity values for binding of [^{125}I]epidepride to D-2$_{415}$ and D-2$_{444}$ on pH and [Na^+] was indistinguishable.

Binding of Radioligands to Wild-Type and Mutant D-2 Receptors. Two different point mutations were introduced into the D-2_{415} cDNA by the polymerase chain reaction (PCR) using a method termed trans-PCR (see "Materials and Methods"). Both mutations altered the DNA sequence encoding the amino acid residue, Asp-80. The first mutation resulted in the replacement of Asp-80 with Ala-80 (Ala80-D2); the second in its replacement with Glu-80 (Glu80-D2). Substitution of the aspartate residue with alanine was chosen because the small size of the side chain, and lack of reactive groups, make alanine less likely to cause nonspecific structural changes (25). The more conservative substitution of glutamate for aspartate might be expected to have less of a functional impact.

Cell lines were created that stably express mutant D-2 receptors by transfection of C_6 glioma cells with pRSV-D2_{415} into which a point mutation was introduced. Saturation analysis of the binding of [^{125}I]epidepride to membranes prepared from the cells expressing mutant receptors and cells expressing D-2_{415} (Fig. 1) indicated that the density of binding sites (B_{max}) was 100 $\pm$ 2.5 fmol/mg of protein for D-2_{415} (n = 4), 53 $\pm$ 5 fmol/mg of protein for Glu80-D2 (n = 3), and 496 $\pm$ 21 fmol/mg of protein for Ala80-D2 (n = 4). The respective affinity (pK_D) values for D-2_{415}, Glu80-D2, and Ala80-D2 were 10.36 $\pm$ 0.11, 9.73 $\pm$ 0.07, and 9.49 $\pm$ 0.14 pM. These experiments were carried out in the presence of 120 mM NaCl at pH 7.3. In contrast to the considerable decrease in D-2 receptor affinity for [^{125}I]epidepride resulting from the substitution of an alanine residue for Asp-80, [^{3}H]spiperone bound with similar affinity values to D-2_{415}, Glu80-D2, and Ala80-D2 (Table 2). Untransfected C_6 cells, or cells expressing pRSVneo, had no detectable binding of [^{125}I]epidepride (data not shown).

Regulation of the Affinity of Wild-Type and Mutant Receptors for Drugs by Na^+ and pH. One possible cause for the decreased affinity of Glu80-D2 and Ala80-D2 for

[^{125}I]epidepride, but not [^{3}H]spiperone, was that mutation of Asp-80 altered the regulation of D-2 receptors by Na^+ or H^+. This possibility was evaluated by determining the apparent affinity of several drugs for inhibiting the binding of [^{3}H]spiperone. [^{3}H]Spiperone was used in these experiments because of its relative insensitivity, compared to [^{125}I]epidepride, to changes in [Na^+] or pH between 6.5 and 8.0 (Table 2; ref. 13). The most dramatic effect of substitution of an alanine residue for Asp-80 was a complete loss of the sensitivity of D-2 receptors to sodium, as reflected in the lack of a sodium-induced shift in the apparent affinity of the substituted benzamide derivatives, epidepride and sulpiride (Fig. 2; Tables 2 and 3), and the agonists, NPA and DA (Fig. 3; Tables 2 and 3), for D-2 receptors. For example, K_I values for binding of epidepride to D-2_{415} in the presence and absence of sodium were 20 and 210 pM, respectively, whereas the corresponding values for binding to Ala80-D2 were 470 and 570 pM. The same mutation (Ala80-D2) reduced by half the sensitivity of the binding of all four compounds to lowering pH from 7.5 to 6.6. Thus, the K_I value for binding of epidepride to D-2_{415} was increased 31-fold from 20 to 620 pM as a result of decreasing pH to 6.6, but the K_I value for binding to Ala80-D2 was increased only 7-fold, from 0.47 to 3.4 nM. Lowering pH from 7.5 to 6.6 caused a 24-fold increase in the K_I value for binding of DA to D-2_{415}, from 0.8 to 19 uM, but the K_I value for binding of DA to Ala80-D2 was increased only 6-fold, from 1.8 to 11 lM.

Substitution of a glutamate residue for Asp-80 decreased but did not eliminate the regulation of affinity for epidepride, sulpiride, and NPA by sodium, and also decreased the effect of varying [H^+] on the binding of the substituted benzamides and agonists (Tables 2 and 3). The K_I value for binding of epidepride to Glu80-D2, for example, was increased only 2-fold in the absence of sodium, from 0.54 to 1.0 nM, and increased only 12-fold (6.7 nM) by lowered pH. With the exception of two conditions (comparison of the affinity of D-2_{415} and Glu80-D2 for NPA at pH 6.6 or

pH 6.6/NaCl) the apparent affinity of agonists for Glu80-D2 was reduced approximately 10-fold or more compared to D-2_{415} or Ala80-D2. In addition, the affinity of epidepride and sulpiride for Glu80-D2 tended to be lower than for D-2_{415} or Ala80-D2 (Table 2).

Inactivation of Wild-Type and Mutant D-2 Receptors by NEM. At pH 7.5, treatment with 3 mM NEM for 30 min reduced the density of D-2_{415} receptors by 76% (Fig. 4). Inactivation of D-2_{415} was inhibited by either NaCl (54% decrease) or lowered pH (25% decrease). The density of Ala80-D2 receptors was decreased only marginally at either pH 6.6 (17 %) or pH 7.5 (24%), indicating that the sensitivity of Ala80-D2 to NEM was not altered by varying $[H^+]$.

Inhibition of Adenylyl Cyclase Activity by Wild-Type and Mutant D-2 Receptors. In C_6 cells expressing D-2_{415}, but not in untransfected C_6 cells, DA attenuated isoproterenol-stimulated adenylyl cyclase activity in a concentration-dependent manner. Maximal inhibition was 75% of total enzyme activity, with an EC_{50} for DA of 56 nM (Fig. 5). The mutant Ala80-D2 was unable to mediate inhibition of adenylyl cyclase activity by DA. The ability of Glu80-D2 to mediate inhibition of adenylyl cyclase activity was greatly decreased. Maximal inhibition of activity in cells expressing Glu80-D2 was 25% of total activity, with an EC_{50} of 140 nM (Fig. 5).

Discussion

Asp-80, located in the second transmembrane domain of DA D-2 receptors, is strictly conserved among G protein-coupled receptors (26). The data presented here demonstrate that this negatively-charged amino acid residue is critical not only for regulation of the conformation of D-2 receptors by Na^+ and H^+, but also for coupling of the receptors to at least one signaling pathway.

Two molecular forms of D-2 receptors, generated by alternative splicing of a single gene product, have been identified (27-29). The two forms are identical except for a 29-amino acid insert present in the third cytoplasmic loop of one form ($D\text{-}2_{444}$) but lacking from the other ($D\text{-}2_{415}$). The present data demonstrate that [^{125}I]epidepride, like several other D-2 receptor ligands (28,30), did not differentiate between $D\text{-}2_{415}$ and $D\text{-}2_{444}$. Furthermore, the affinity of the alternatively spliced forms of D-2 receptors for [^{125}I]epidepride was regulated in a similar manner by monovalent cations. As reported previously (13), Na^+ and H^+ had opposing effects on the binding of the substituted benzamide derivative, [^{125}I]epidepride. Raising [Na^+] increased the affinity of $D\text{-}2_{415}$ and $D\text{-}2_{444}$ for [^{125}I]epidepride, whereas lowering pH to 6.6 decreased the affinity of both forms of D-2 receptors for [^{125}I]epidepride.

Because $D\text{-}2_{415}$ and $D\text{-}2_{444}$ did not differ in sensitivity to pH or Na^+, we chose to construct mutants of only one of the forms, $D\text{-}2_{415}$. Replacement of Asp-80 by an alanine residue (Ala80-D2) yielded receptors with unchanged affinity for [^{3}H]spiperone, but approximately 10-fold lower affinity for [^{125}I]epidepride, compared to wild-type receptors. Mutant receptors in which a glutamate residue was substituted for the aspartate residue (Glu80-D2) had moderately decreased affinity for [^{125}I]epidepride. The similar affinity of $D\text{-}2_{415}$, Ala80-D2, and Glu80-D2 for [^{3}H]spiperone suggests that substitution of alanine or glutamate for Asp-80 did not result in gross conformational changes. The decreased affinity of Ala80-D2 and Glu80-D2 for [^{125}I]epidepride was apparently related to a decreased sensitivity of the mutant receptors to Na^+ and H^+. Binding of epidepride, sulpiride, DA, and NPA to Ala80-D2 was completely insensitive to added NaCl, whereas the low pH-induced reduction in ΔG° with the binding of the drugs was decreased by 50%. The more conservative substitution of a glutamate residue for Asp-80 also greatly decreased the sensitivity of D-2 receptors to Na^+ and H^+, as reflected in the lower magnitude of the

ion-induced changes in ΔG^o, although the decreased sensitivity tended to be less than observed for Ala80-D2. One unexpected observation was that the affinity of Glu80-D2 for the agonists NPA and DA was lower than the affinity of either $D\text{-}2_{415}$ or Ala80-D2 for these agonists.

Substituted benzamide drugs are an unusual class of ligands. In rats, substituted benzamides antagonize some of the behavioral effects of stimulation of DA D-2 receptors by agonists, but are less likely to cause catalepsy than other D-2 receptor antagonists (31). Clinically, the compounds are antipsychotic drugs that induce less parkinsonism than classical neuroleptic drugs (32). Substituted benzamide D-2 receptor ligands are antagonists *in vitro*, as determined by their ability to prevent inhibition of adenylyl cyclase activity by D-2 receptor agonists (2). The extreme sensitivity to pH and Na^+ of the binding of substituted benzamide derivatives, however, distinguishes the drugs from classical antagonists at D-2 receptors, and from antagonists at other classes of receptors. For example, α_2-adrenergic receptor antagonists are approximately 10-fold less sensitive than agonists to changes in pH or $[Na^+]$ (16). Due to the unusual pharmacological profile of substituted benzamide D-2 receptor antagonists, it has been proposed that the compounds label a subclass of DA D-2 receptors (31), although *in vitro* data indicate that $[^3H]$spiperone and substituted benzamide ligands label the same population of sites (12,15,31,33). Our data demonstrate that the substituted benzamides differ from classical antagonists such as $[^3H]$spiperone in their mode of binding to D-2 receptors, as reflected in the selective effect of mutating Asp-80 on the binding of epidepride and sulpiride.

Experiments in which the rate of inactivation of DA D-2 receptors by NEM depends on $[Na^+]$ and $[H^+]$ have been interpreted as evidence that D-2 receptors undergo a Na^+- or H^+-induced conformational change that results in altered affinity of the receptors for substituted benzamide antagonists and agonists (13). If Na^+- and H^+-induced changes in affinity are due to allosteric regulation of the receptor rather

than to direct modification of amino acid residues involved in the binding of ligands, mutations that decrease the magnitude of ion-dependent changes in affinity should also decrease the effects of ions on the rate of inactivation of D-2 receptors by NEM. NEM-induced inactivation of wild-type D-2_{415} is inhibited by NaCl (14,15) and by lowering pH from 7.5 to 6.6 (present results; also, ref. 13). The most pronounced effect of substitution of alanine for Asp-80 was that, in the absence of NaCl, mutant receptors were inactivated by 30 min treatment with NEM as poorly at pH 7.5 as at pH 6.6. Thus, regardlesss of pH, Ala80-D2 appeared to be inactivated to an extent similar to that observed for D-2_{415} in the presence of H^+ at pH 6.6. Interestingly, at pH 7.5 the inactivation of Ala80-D2 was increased by NaCl, indicating that some conformational effects of pH and sodium persist after mutation of Asp-80.

As reported previously, D-2_{415} expressed in mammalian cells mediates inhibition of adenylyl cyclase activity by DA (10,34). The functional coupling of D-2_{415} to adenylyl cyclase activity was severely impaired by mutation of Asp-80. We were unable to detect reliable attenuation of enzyme activity by DA in cells expressing Ala80-D2, whereas DA inhibition of adenylyl cyclase activity in cells expressing Glu80-D2 was greatly reduced. The density of D-2 receptors in the three cell lines differed substantially. This would be unlikely to explain the lack of inhibition of adenylyl cyclase activity by Ala80-D2, since cells expressing this mutant had a receptor density five times that of the cells expressing D-2_{415}. Although the density of receptors on the cell line expressing Glu80-D2 (50 fmol/mg of protein) was half that of the cells expressing D-2_{415} that were used in the present study, this would not explain the low inhibition of adenylyl cyclase activity, because we have detected robust inhibition of enyzme activity in C_6 cells expressing wild-type D-2 receptors at a density of 40-60 fmol/mg of protein (unpublished observations). The diminished ability of the mutant receptors to inhibit adenylyl cyclase probably results from impaired coupling to G proteins, since our preliminary data indicate that GTP-sensitive binding of DA is

abolished or reduced by substitution of alanine or glutamate, respectively, for aspartate-80 (unpublished observations).

An aspartate residue corresponding to Asp-80 apparently serves a pivotal function in the regulation of drug binding and receptor coupling to signaling pathways for a number of neurotransmitter receptors. For β_2-adrenergic receptors, substitution of Asp-79 with an asparagine or alanine residue decreases the affinity of the mutant receptors for agonists, prevents GTP-sensitive interactions of the receptors with G proteins, and reduces isoproterenol-stimulated adenylyl cyclase activity (35,36). Mutation of Asp-71 of the m_1 muscarinic receptor increases affinity for the agonist carbachol and abolishes stimulation of phosphoinositide hydrolysis (37). Asp-79 of α_{2a}-adrenergic receptors is required for allosteric regulation of the receptors by Na^+ (26). It has been suggested that this conserved residue does not directly interact with ligands or G proteins; rather, the aspartic acid serves to maintain a particular receptor conformation needed for interaction with G proteins and signal transduction (35,37). Our results support and extend this hypothesis by indicating that the interaction of cations with the aspartate residue modulates receptor conformation.

These data link together, and specify a molecular mechanism for, many phenomena whose relationship to each other was uncertain. Virtually all the effects of sodium on D-2 receptors, including regulation of receptor conformation, regulation of the affinity of D-2 receptors for agonists and substituted benzamide antagonists, and perhaps including the effects of sodium on inhibition of adenylyl cyclase activity (2), may be due to an interaction of sodium with a single amino acid residue on D-2 receptors. In addition, some of the effects of varying pH on affinity of D-2 receptors for ligands, and on the conformation of D-2 receptors, are also mediated by Asp-80. Cations are presumably interacting with the carboxylate side chain of the aspartic acid, but the positioning of the carboxylate anion is apparently crucial in mediating the effects of cations, since substitution with glutamic acid was virtually as debilitating as

substitution with alanine. The role of this conserved amino acid residue in both the regulation of receptor conformation by ions and coupling to adenylyl cyclase and other signaling pathways suggests that the effects of these ions on G_i-coupled receptors in particular may be closely linked to mechanisms of signal transduction by the receptors. An interesting future line of investigation will be to determine the ability of Ala80-D2 amd Glu80-D2 to modulate other signaling pathways, including Na^+/H^+ exchange.

Acknowledgments

We thank Dr. F. Boyce for advice concerning expression vectors and for the trans-PCR protocol, Dr. O. Civelli for providing us with rat $D\text{-}2_{415}$ and $D\text{-}2_{444}$ cDNAs, and Dr. T. de Paulis for the [^{125}I]epidepride precursor and unlabeled epidepride.

References

1. Cote, T. E., E. A. Frey, C. W. Grewe, and J. W. Kebabian. Evidence that the dopamine receptor in the intermediate lobe of the rat pituitary gland is associated with an inhibitory guanyl nucleotide component. *J. Neural Transm. Suppl.* **18**:139-147 (1983).
2. Onali, P., M. C. Olianas, and G. L. Gessa. Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28**:138-145 (1985).
3. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2**:2686-2695 (1988).
4. Enjalbert, A., F. Sladeczek, G. Guillon, P. Bertrand, C. Shu, J. Epelbaum, A. Garcia-Sainz, S. Jard, C. Lombard, C. Kordon, and J. Bockaert. Angiotensin II and dopamine modulate both cAMP and inositol phosphate productions in anterior pituitary cells: Involvement in prolactin secretion. *J. Biol. Chem.* **261**:4071-4075 (1986).
5. Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli. Differential coupling of dopaminergic D_2 receptors expressed in different cell types: Stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in Ltk$^-$ fibroblasts, hyperpolarization, and cytosolic-free Ca^{2+} concentration decrease in GH_4C_1 cells. *J. Biol. Chem.* **265**:10320-10326 (1990).
6. Lacey, M. G., N. B. Mercuri, and R. A. North. Dopamine acts on D2 receptors to increase potassium conductance in neurones of rat substantia nigra zona compacta. *J. Physiol. (Lond.)* **392**:397-416 (1987).
7. Malgaroli, A. L., L. Vallar, F. R. Elahi, T. Pozzan, A. Spada, and J. Meldolesi. Dopamine inhibits cytosolic Ca^{2+} increases in rat lactotroph cells: Evidence of a dual mechanism of action. *J. Biol. Chem.* **262**:13920-13927 (1987).

8. Ganz, M. B., J. A. Pachter, and D. L. Barber. Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **265**:8989-8992 (1990).

9. Hamblin, M. W., and I. Creese. ^{3}H-Dopamine binding to rat striatal D-2 and D-3 sites: Enhancement by magnesium and inhibition by guanine nucleotides and sodium. *Life Sci.* **30**:1587-1595 (1982).

10. Neve, K. A., R. A. Henningsen, J. R. Bunzow, and O. Civelli. Functional characterization of a rat dopamine D-2 receptor cDNA expressed in a mammalian cell line. *Mol. Pharmacol.* **36**:446-451 (1989).

11. Imafuku, J. The characterization of [^{3}H]sulpiride binding sites in rat striatal membranes. *Brain Res.* **402**:331-338 (1987).

12. Neve, K. A., R. A. Henningsen, J. M. Kinzie, T. De Paulis, D. E. Schmidt, R. M. Kessler, and A. Janowsky. Sodium-dependent isomerization of dopamine D-2 receptors characterized using [^{125}I]epidepride, a high-affinity substituted benzamide ligand. *J. Pharmacol. Exper. Ther.* **252**:1108-1116 (1990).

13. Neve, K. A. Regulation of dopamine D-2 receptors by sodium and pH. *Mol. Pharmacol.* **39**:570-578 (1991).

14. Freedman, S. B., J. A. Poat, and G. N. Woodruff. Influence of sodium and sulphydryl groups on [^{3}H]sulpiride binding sites in rat striatal membranes. *J. Neurochem.* **38**:1459-1465 (1982).

15. Urwyler, S., and D. Coward. Binding of ^{3}H-spiperone and ^{3}H-(-)-sulpiride to dopamine D2 receptors in rat striatal membranes: methodological considerations and demonstration of the identical nature of the binding sites for the two ligands. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:115-122 (1987).

16. Nunnari, J. M., M. G. Repaske, S. Brandon, E. J. Cragoe, and L. E. Limbird. Regulation of porcine brain α_2-adrenergic receptors by Na^+, H^+, and inhibitors of Na^+/H^+ exchange. *J. Biol. Chem.* **262**:12387-12392 (1987).

17. Motulsky, H. J., and P. A. Insel. Influence of sodium on the α_2-adrenergic receptor system of human platelets: Role for intraplatelet sodium in receptor binding. *J. Biol. Chem.* **258**:3913-3919 (1983).

18. Urwyler, S. Mono- and divalent cations modulate the affinities of brain D1 and D2 receptors for dopamine by a mechanism independent of receptor coupling to guanyl nucleotide binding proteins. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**:374-382 (1989).

19. Neve, K. A., D. A. Barrett, and P. B. Molinoff. Selective regulation of *beta*-1 and *beta*-2 adrenergic receptors by atypical agonists. *J. Pharmacol. Exper. Ther.* **235**:657-664 (1985).

20. Chen, C. A., and H. Okayama. Calcium phosphate-mediated gene tranfer: A highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques* **6**:632-638 (1988).

21. Gorman, C., R. Padmanabhan, and B. H. Howard. High efficiency DNA-mediated transformation of primate cells. *Science* **221**:551-553 (1983).

22. Limbird, L. E., J. L. Speck, and S. K. Smith. Sodium ion modulates agonist and antagonist interactions with the human platelet *alpha*2-adrenergic receptor in membrane and solubilized preparations. *Mol. Pharmacol.* **21**:609-617 (1982).

23. Munson, P. J., and D. Rodbard. An exact correction to the "Cheng-Prusoff" correction. *J. Recept. Res.* **8**:533-546 (1988).

24. Peterson, G. L. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**:346-356 (1977).

25. Ward, W. H. J., D. Timms, and A. R. Fersht. Protein engineering and the study of structure-function relationships in receptors. *Trends Pharmacol. Sci.* **11**:280-284 (1990).

26. Horstman, D. A., S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, and L. E. Limbird. An aspartate conserved among G-protein receptors confers allosteric

regulation of α_2-adrenergic receptors by sodium. *J. Biol. Chem.* 265:21590-21595 (1990).

27. Dal Toso, R., B. Sommer, M. Ewert, A. Herb, D. B. Pritchett, A. Bach, B. D. Shivers, and P. H. Seeburg. The dopamine D_2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* **8**:4025-4034 (1989).
28. Giros, B., P. Sokoloff, M. -P. Martres, J. -F. Riou, L. J. Emorine, and J. -C. Schwartz. Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature (Lond.)* **342**:923-926 (1989).
29. Monsma, F. J., L. D. McVittie, C. R. Gerfen, L. C. Mahan, and D. R. Sibley. Multiple D_2 dopamine receptors produced by alternative RNA splicing. *Nature (Lond.)* **342**:926-929 (1989).
30. Grandy, D. K., M. A. Marchionni, H. Makam, R. E. Stofko, M. Alfano, L. Frothingham, J. B. Fischer, K. J. Burke-Howie, J. R. Bunzow, A. C. Server, and O. Civelli. Cloning of the cDNA and gene for a human D_2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **86**:9762-9766 (1989).
31. Ögren, S. V., H. Hall, C. Köhler, O. Magnusson, and S.-E. Sjöstrand. The selective dopamine D_2 receptor antagonist raclopride discriminates between dopamine-mediated motor functions. *Psychopharmacology* **90**:287-294 (1986).
32. Andersson, U., J.-E. Häggström, M.-I. Nilsson, and E. Widerlöv. Remoxipride, a selective dopamine D_2 receptor antagonist, in tardive dyskinesia. *Psychopharmacology* **94**:167-171 (1988).
33. Zahniser, N. R., and M. L. Dubocovich. Comparison of dopamine receptor sites labeled by [^{3}H]-*S*-sulpiride and [^{3}H]-spiperone in striatum. *J. Pharmacol. Exper. Ther.* **227**:592-599 (1983).
34. Albert, P. R., K. A. Neve, J. R. Bunzow, and O. Civelli. Coupling of a cloned rat dopamine-D_2 receptor to inhibition of adenylate cyclase and prolactin secretion. *J. Biol. Chem.* **265**:2098-2104 (1990).

35. Chung, F.-Z., C.-D. Wang, P. C. Potter, J. C. Venter, and C. M. Fraser. Site-directed mutagenesis and continuous expression of human ß-adrenergic receptors: Identification of a conserved aspartate residue involved in agonist binding and receptor activation. *J. Biol. Chem.* **263**:4052-4055 (1988).

36. Strader, C. D., I. S. Sigal, M. R. Candelore, E. Rands, W. S. Hill, and R. A. F. Dixon. Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263**:10267-10271 (1988).

37. Fraser, C. M., C.-D. Wang, D. A. Robinson, J. D. Gocayne, and J. C. Venter. Site-directed mutagenesis of m_1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**:840-847 (1989).

TABLE 1

Effect of Na^+ and H^+ on Affinity of D-2_{415} and D-2_{444} Receptors for [^{125}I]Epidepride

Affinity values, expressed as pK_D (the negative logarithm of the K_D value, in M), are given for the binding of [^{125}I]epidepride to membranes prepared from C6 glioma cells stably expressing either D-2_{415} or D-2_{444}. Values shown are the means ± SE from 3 or 4 independent experiments. Experiments in which pH was varied were carried out in the presence of 120 mM NaCl, and experiments in which [NaCl] was varied were carried out at pH 7.4.

Assay Condition	Affinity, pK_D ± SE	
	D-2_{415}	D-2_{444}
pH 7.5	10.61 ± 0.09	10.42 ± 0.13
pH 7.0	10.22 ± 0.11	10.18 ± 0.09
pH 6.8	9.86 ± 0.09	9.88 ± 0.04
50 mM NaCl	10.23 ± 0.01	10.30 ± 0.01
1 mM NaCl	9.83 ± 0.04	9.82 ± 0.07
0 NaCl	9.57 ± 0.06	9.61 ± 0.06

TABLE 2

Effect of H^+ and Na^+ on Drug Affinity for Wild-Type and Mutant D-2 receptors

The apparent affinity (K_I) of the indicated drugs was determined by inhibition of the binding of [^{3}H]spiperone. Affinity values for [^{3}H]spiperone are K_D values, and were used to calculate K_I values for the other drugs. Data are expressed as pK_D or pK_I (the negative logarithm of the equilibrium dissociation constant, in M) $\pm$ SE from 3-5 independent experiments. Experiments with the agonists DA and NPA were carried out in the presence of 100 μM GTP. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or in the presence of 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

Drug	Affinity			
	pH 6.6/NaCl	pH 6.6	pH 7.5/NaCl	pH 7.5
[^{3}H]Spiperone				
D2-415	10.55 ± 0.01	10.41 ± 0.07	11.10 ± 0.05	10.97 ± 0.08
Ala80-D2	10.70 ± 0.02	10.68 ± 0.08	11.08 ± 0.07	11.03 ± 0.10
Glu80-D2	10.35 ± 0.07	10.43 ± 0.10	10.72 ± 0.12	10.78 ± 0.13
Epidepride				
D2-415	9.21 ± 0.12	8.51 ± 0.07	10.69 ± 0.05	9.67 ± 0.10
Ala80-D2	8.47 ± 0.03	8.32 ± 0.03	9.33 ± 0.04	9.25 ± 0.03
Glu80-D2	8.17 ± 0.11	7.97 ± 0.06	9.26 ± 0.12	9.00 ± 0.10
Sulpiride				
D2-415	6.31 ± 0.12	5.37 ± 0.03	7.96 ± 0.08	7.02 ± 0.02
Ala80-D2	5.70 ± 0.04	5.65 ± 0.02	6.55 ± 0.05	6.45 ± 0.02
Glu80-D2	5.45 ± 0.05	5.39 ± 0.03	6.54 ± 0.04	6.30 ± 0.02
NPA				
D2-415	7.73 ± 0.08	7.91 ± 0.07	8.37 ± 0.04	8.84 ± 0.13
Ala80-D2	8.30 ± 0.14	8.31 ± 0.07	8.92 ± 0.10	8.78 ± 0.05
Glu80-D2	7.33 ± 0.18	7.39 ± 0.40	7.52 ± 0.05	7.75 ± 0.04
Dopamine				
D2-415	4.73 ± 0.02	4.72 ± 0.03	5.80 ± 0.06	6.09 ± 0.07
Ala80-D2	5.08 ± 0.04	4.98 ± 0.04	5.78 ± 0.04	5.75 ± 0.05
Glu80-D2	3.55 ± 0.03	3.42 ± 0.13	4.53 ± 0.04	4.22 ± 0.04

TABLE 3

Shift in Wild-Type and Mutant Receptor Affinity Induced by H^+ or Na^+

The magnitude of the shift in affinity resulting from varying pH or [Na^+] was determined from the data in Table 2. The free energy change of binding (ΔG^o) was calculated for each assay condition, and the magnitude of the change in ΔG^o ($\Delta\Delta G^o$) was determined from the absolute value of the difference between two values for ΔG^o. Values are expressed as kcal/mol. The data in Table 2 yield two values for $\Delta\Delta G^o$ resulting from changing pH ($\Delta\Delta G^o_{pH}$), one in the presence and one in the absence of NaCl, and two values for $\Delta\Delta G^o$ resulting from changing [NaCl] ($\Delta\Delta G^o_{NaCl}$), one each at pH 6.6 and 7.5. To simplify, values are shown for the conditions that resulted in the greatest shift in affinity of D-2_{415} for each drug. Thus, $\Delta\Delta G^o_{pH}$ shown for binding of epidepride and sulpiride to D-2_{415} and each mutant was calculated using affinity values determined in the presence of NaCl, and $\Delta\Delta G^o_{NaCl}$ was calculated from values determined at pH 7.5. For both agonists, the values shown are $\Delta\Delta G^o_{pH}$ in the absence of NaCl and $\Delta\Delta G^o_{NaCl}$ at pH 7.5. Absence of a shift in affinity in the direction observed for D-2_{415} is indicated by --.

Drug	Variable	Receptor		
		D2-415	Ala80-D2	Glu80-D2
Epidepride	$\Delta\Delta G^o_{pH}$	2.07	1.19	1.52
	$\Delta\Delta G^o_{NaCl}$	1.42	0.12	0.38
Sulpiride	$\Delta\Delta G^o_{pH}$	2.29	1.19	1.48
	$\Delta\Delta G^o_{NaCl}$	1.30	0.15	0.33
NPA	$\Delta\Delta G^o_{pH}$	1.29	0.67	0.50
	$\Delta\Delta G^o_{NaCl}$	0.68	--	0.31
Dopamine	$\Delta\Delta G^o_{pH}$	1.91	1.09	1.11
	$\Delta\Delta G^o_{NaCl}$	0.42	--	--

Figure Legends

Fig. 1. Altered binding of [^{125}I]epidepride to D-2 receptors resulting from mutation of Asp-80. C_6 cell lines expressing either D-2_{415} or one of the mutant D-2 receptors were isolated. Saturation isotherms of the binding of [^{125}I]epidepride to membranes prepared from the cells are shown from a representative experiment. Assays were carried out at pH 7.4 in the presence of 120 mM NaCl. Results are plotted as radioligand bound, expressed as a percentage of B_{max} for each tissue, *vs.* the corrected free concentration of [^{125}I]epidepride (total radioligand added minus amount bound). In this experiment, affinity (K_D) and B_{max} values, respectively, for each tissue were 45 pM and 151 fmol/mg of protein (D-2_{415}), 185 pM and 101 fmol/mg of protein (Glu80-D2), and 367 pM and 626 fmol/mg of protein (Ala80-D2). These data are shown here for graphic comparison of radioligand binding to wild-type and mutant D-2 receptors, but were not included in the mean pK_D and B_{max} values presented in the text. In those experiments, higher radioligand concentrations were used to characterize binding to Glu80-D2 and Ala80-D2, to obtain more accurate estimates for K_D and B_{max}. Inset: The same data, expressed as actual rather than normalized values, are transformed and plotted as radioligand specifically bound (fmol/mg of protein)/free radioligand (pM) *vs.* radioligand specifically bound (fmol/mg of protein).

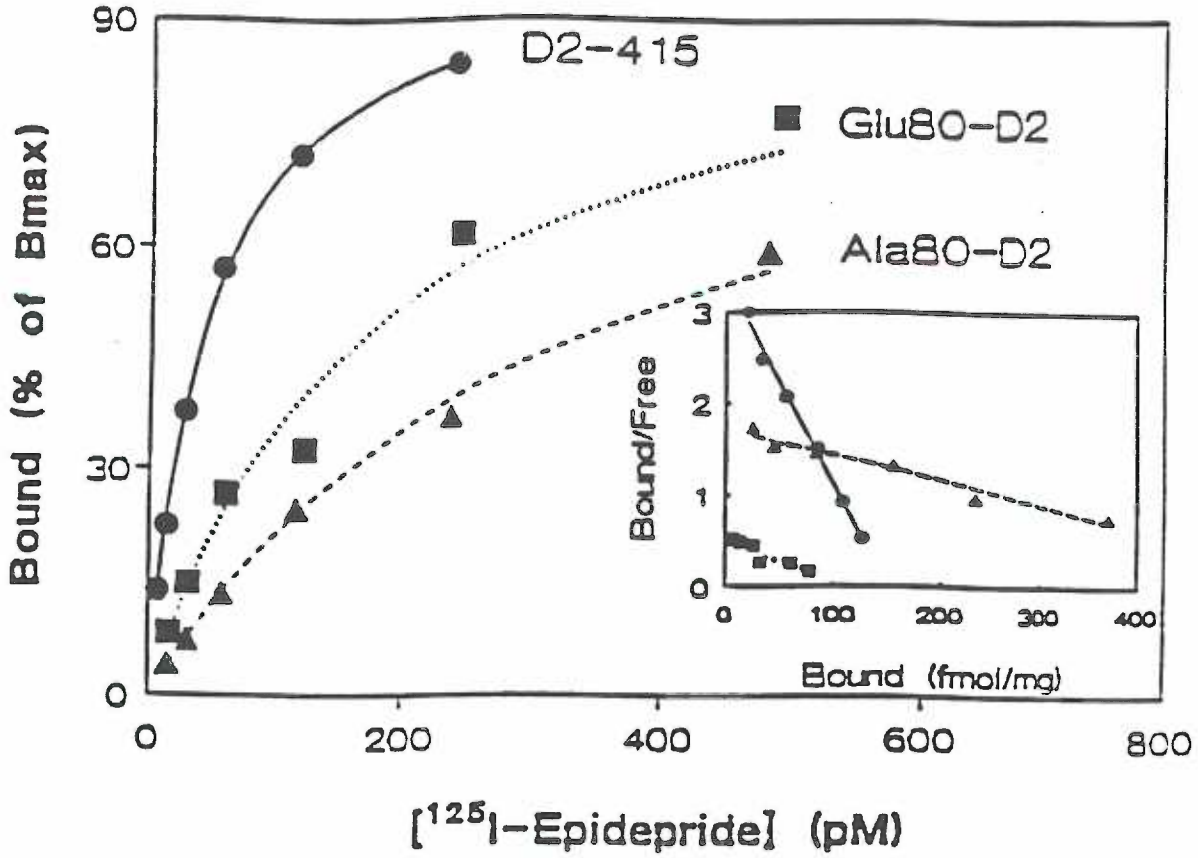
Fig. 2. Inhibition by epidepride of the binding of [^{3}H]spiperone to D-2_{415} and Ala80-D2. Data are shown from a representative experiment in which the binding of 120 pM [^{3}H]spiperone to (A) D-2_{415} or (B) Ala80-D2 was inhibited by increasing concentrations of epidepride. Results are plotted as radioligand bound, expressed as a percentage of total binding in the absence of inhibitor, *vs.* the log of the concentration of epidepride. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

Fig. 3. Inhibition by dopamine of the binding of [^{3}H]spiperone to D-2_{415} and Ala80-D2. Data are shown from a representative experiment in which the binding of 140 pM

[^{3}H]spiperone to (A) D-2_{415} or (B) Ala80-D2 was inhibited by increasing concentrations of dopamine. Results are plotted as radioligand bound, expressed as a percentage of total binding in the absence of inhibitor, *vs.* the log of the concentration of dopamine. Assays were carried out at the indicated pH in the presence of 100 mM NMDG-Cl (pH 6.6 and 7.5) or 100 mM NaCl (pH 6.6/NaCl and pH 7.5/NaCl).

Fig. 4. Inactivation of mutant and wild-type D-2 receptors by NEM. Each bar represents the mean ± SE of B_{max} values from 6 (D-2_{415}) or 7 (Ala80-D2) experiments in which membranes prepared from C_6 cells expressing the receptors were incubated with 3 mM NEM for 30 min. The density of receptors was then quantified by saturation analysis of the binding of [^{125}I]epidepride. Data are expressed as a percentage of the control (untreated) density of receptors.

Fig. 5. Inhibition of adenylyl cyclase activity by D-2_{415}, Glu80-D2, and Ala80-D2. Adenylyl cyclase activity was assessed by measuring the conversion of [^{3}H]ATP to [^{3}H]cyclic AMP in C_6 cells expressing wild-type or mutant D-2 receptors. Results shown are mean ± SE of 3 (Glu80-D2, Ala80-D2) or 4 (D-2_{415}) independent experiments, expressed as a percentage of total enzyme activity stimulated by 1 μM isoproterenol in the absence of dopamine. Total activity was 6.6 ± 0.6% (D-2_{415}), 6.3 ± 0.7% (Glu80-D2), and 6.5 ± 0.7% (Ala80-D2). Basal conversion of [^{3}H]ATP to [^{3}H]cyclic AMP was 0.07%, or approximately 1% of isoproterenol-stimulated enzyme activity.



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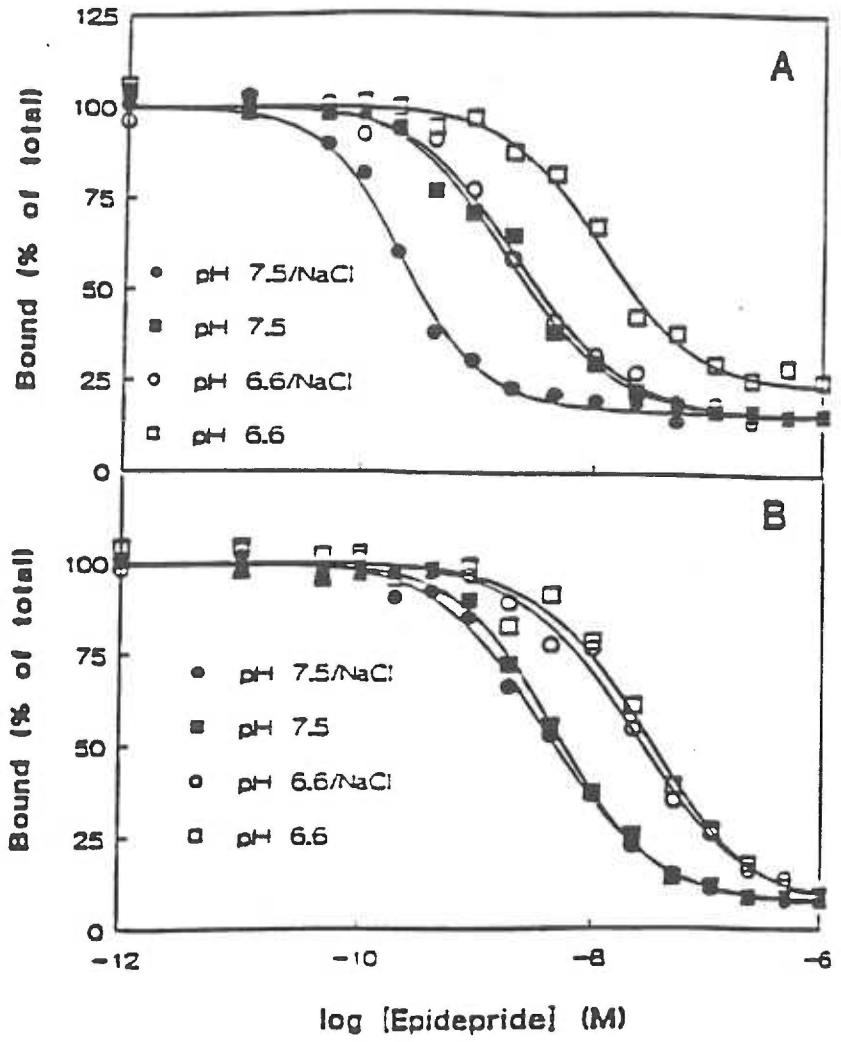

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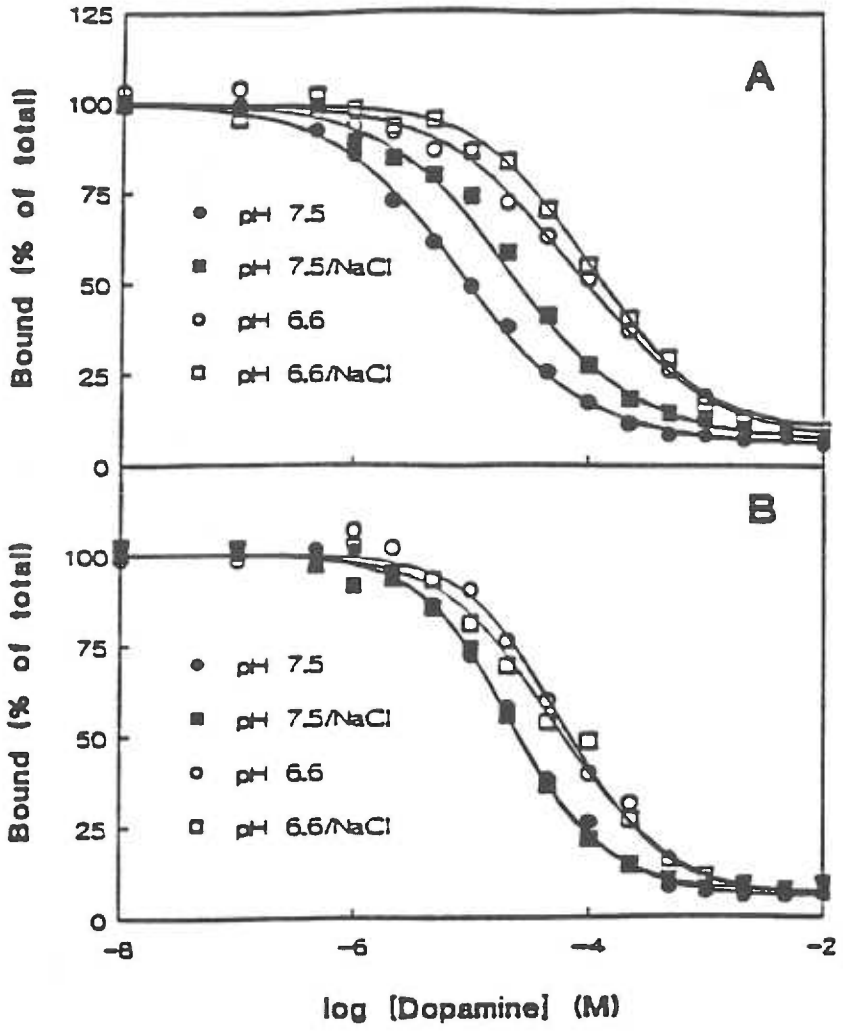

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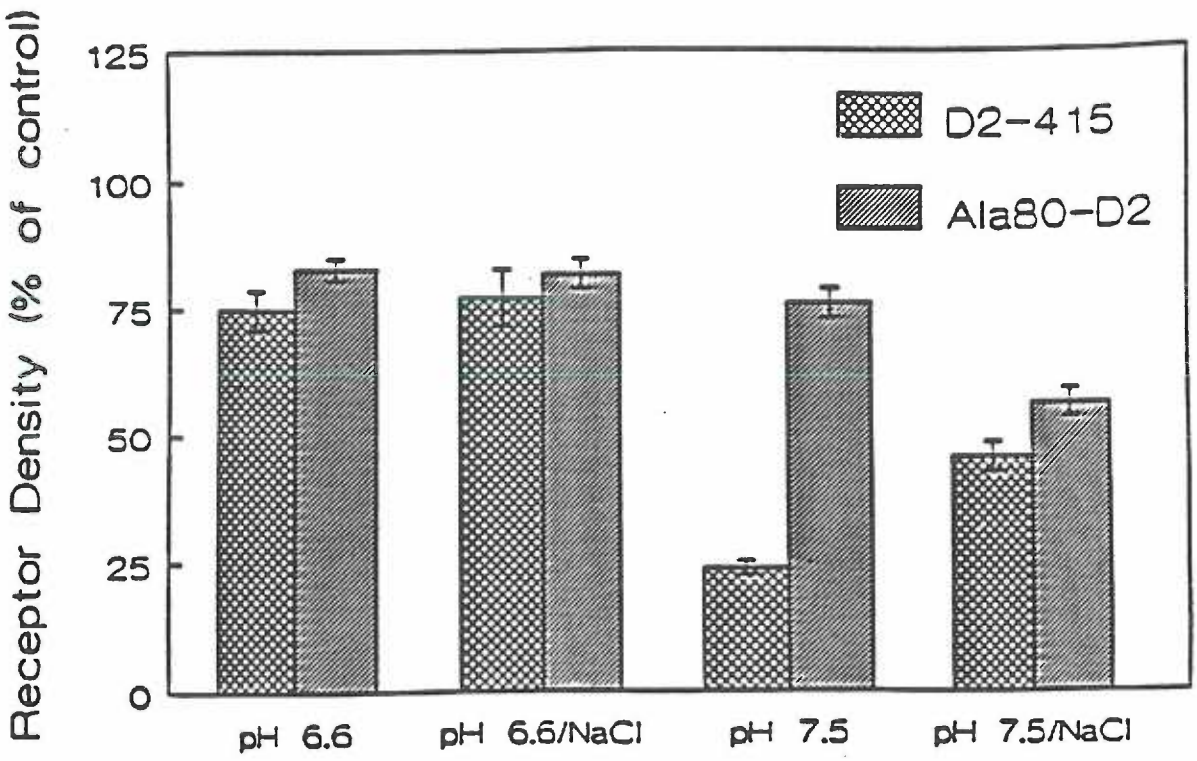

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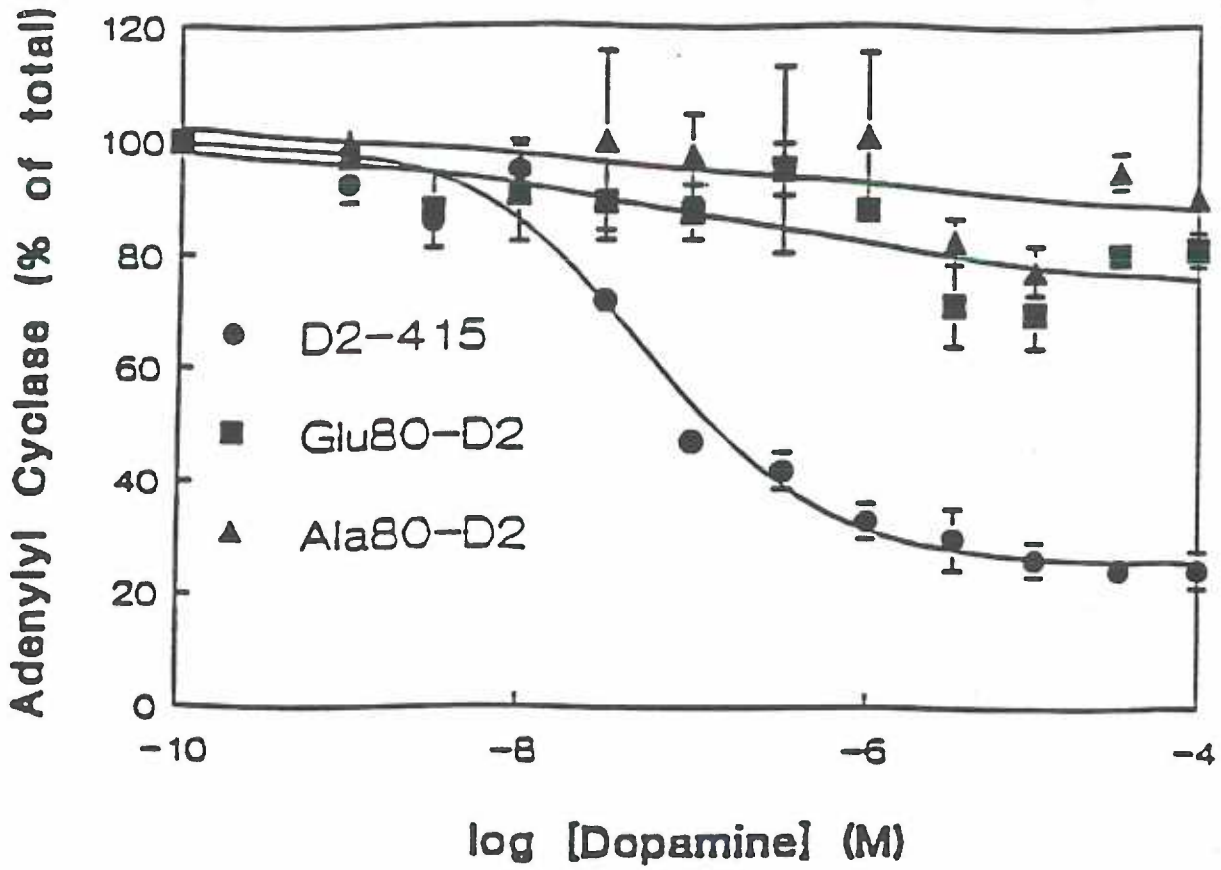

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