pH Dependence of Ligand Binding to D_2 Dopamine Receptors†

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ABSTRACT: The binding of a range of ligands to D_2 dopamine receptors in bovine caudate nucleus and recombinant CHO cells expressing the receptor has been determined at different pH values between 4.5 and 8.5. The maximum number of D_2 dopamine receptor binding sites in each tissue was not affected by the change in pH, but the affinity of ligands for binding to the receptors was decreased as the pH was decreased. For classical dopamine antagonists, e.g. spiperone and haloperidol, the data on pH dependence of the dissociation constant for receptor binding indicated that the protonation of a single ionizing group on the receptor (pK_a ~6) influenced the binding process. For antagonists of the substituted benzamide class, the data indicated that the protonation of two ionizing groups (pK_a between 6 and 7) influenced the ligand binding process. These ionizing residues may correspond to Asp 114 for the classical antagonists and Asp 114 and Asp 80 for the substituted benzamide antagonists. Further evidence for the participation of carboxyl residues in the ligand binding process was obtained from the inhibition by *N,N'*-dicyclohexylcarbodiimide of the binding of [^{3}H]spiperone and [^{3}H]YM 09151-2 to D_2 receptors in the recombinant CHO cells.

The mechanism of ligand binding for G-protein-linked receptors is beginning to be understood. It is thought that, for receptors for small molecule ligands, e.g. acetylcholine, noradrenaline, and dopamine, the ligand binding site is formed from the bundling of the seven putative transmembrane spanning α-helical regions that have been postulated to be present in these kinds of receptors [see for example Baldwin (1993)]. The ligands then bind within this cavity, about one-third of the way in from the membrane, with the free energy of ligand binding being realized from the electrostatic, hydrogen bond, and hydrophobic interactions between the ligand and amino acid side chains of the receptor. This can be visualized in some of the models that have been proposed for these receptors [see for example Hibert et al. (1991), Livingstone et al. (1992), Trumpp-Kallmeyer et al. (1992), Donnelly et al. (1993), and Teeter et al. (1994)], but it is now important to define these interactions experimentally in order to understand the ligand binding process in detail.

For the D_2 dopamine receptor, chemical modification with reagents specific for particular amino acid side chains has provided some evidence for the participation of carboxyl groups in the binding of antagonists (Williamson & Strange, 1990). It seems likely that this may reflect the disruption by the modifying agent of an electrostatic interaction between the ligands and an aspartic acid residue (Asp 114) in the putative third transmembrane spanning region (TM III). Site specific mutagenesis has been used to define more clearly the roles certain amino acids play in the ligand binding process and has implicated Asp 80 (TM II) [which may regulate the conformation of the receptor (Neve et al., 1991)], Asp 114 (TM III) [probably important for electrostatic interaction as above (Mansour et al., (1993)], Ser 193, 194, and 197 (TM V) [which may form hydrogen bonds to ligands, particularly catechol agonists (Cox et al., 1993; Mansour et al., 1993; Naylor et al., 1995)], and His 394 (TM VI) [which may interact with certain substituted benzamide antagonists (Woodward et al., (1994)]. A further way to examine the role of ionizing residues is to analyze the pH dependence of ligand binding, and this technique has been used to help understand the interactions of agonists and antagonists with muscarinic acetylcholine receptors (Asselin et al., 1983; Ehlert & Delen, 1990; Birdsall et al., 1989) and the interaction of insulin with its receptor (Waelbroeck, 1982). Using this approach, some evidence was provided that classical and substituted benzamide antagonists bound to D_2 dopamine receptors in a different manner (Williamson & Strange, 1990; Presland & Strange, 1991), and in the present report, we describe more detailed studies on the pH dependence of ligand binding for D_2 dopamine receptors in bovine brain and at recombinant D_2 receptors expressed in CHO cells.

MATERIALS AND METHODS

Preparation of Membranes from Bovine Caudate Nucleus and CHO6 Cells. A mixed mitochondrial/microsomal membrane preparation of bovine caudate nucleus was made largely as described in Leonard et al. (1987). Briefly, bovine caudate nucleus was homogenized in 0.3 M sucrose solution containing 20 mM HEPES, 10 mM EDTA, 1 mM EGTA (pH 7.4), and freshly added phenylmethanesulfonyl fluoride (to 0.1 mM) with 12−15 strokes of a Teflon/glass homogenizer at 4 °C. The homogenate was centrifuged (1000*g*, 10 min), and the supernatant was kept at 4 °C while the pellet was rehomogenized. After centrifugation (1000*g*, 10 min), the supernatants were combined and recentrifuged (47000*g*, 60 min). The supernatant was discarded, and the pellet was resuspended (2 mL/g of tissue) in buffer [20 mM HEPES, 1 mM EDTA, and 1 mM EGTA containing protease inhibitors (antipain, aprotinin, chymostatin, leupeptin, and pepstatin, each at 5 μg/mL final concentration)]. Protein was

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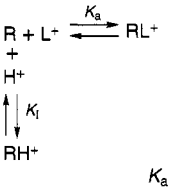
determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Tissue homogenates were stored in aliquots at −80 °C.

CHO6 cells [CHO cells stably transfected with the rat $D_{2(long)}$ receptor gene (Castro & Strange, 1993)] were grown in RPMI medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and 200 μg/mL active geneticin in an atmosphere of 5% CO_2 at 37 °C. Confluent cells were scraped from culture flasks in a buffer containing 20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 0.25 M sucrose (pH 7.4) at 4 °C and homogenized using 30 strokes of a Dounce homogenizer after the addition of a freshly prepared solution of phenylmethanesulfonyl fluoride (to 0.1 mM). The homogenate was centrifuged (260*g*, 10 min) the pellet discarded, and the resulting supernatant centrifuged at 47000*g* for 60 min. The pellet from this centrifugation was resuspended in buffer (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA (pH 7.4) containing protease inhibitors) at ~1 mg/mL and stored at −80 °C.

pH Dependence of the Binding of Ligands to D_2 Dopamine Receptors. Membranes from either bovine caudate nucleus or CHO6 cells were centrifuged (12000*g*, 4 min) and resuspended in buffer (28.5 mM citric acid, 28.5 mM diethylbarbituric acid, 28.5 mM potassium dihydrogen orthophosphate, 28.5 mM boric acid, 0.8 mM magnesium sulfate, and 110 mM sodium chloride, at the pH indicated). Membrane preparations from either bovine caudate nucleus (50−100 μg of protein) or CHO6 cells (10−50 μg) were then incubated with [^{3}H]spiperone (28 Ci/mmol; ~0.25 nM for competition experiments, 25 pM to 5nM for saturation experiments) in triplicate in a final volume of 1 mL of buffer together with other drugs as indicated for 45 min at 25 °C. Assays were terminated by rapid filtration through GF/B glass fiber filters on a Brandel cell harvester with a 15 mL wash of buffer. Radioactivity trapped on the filters was determined by liquid scintillation counting. Specific [^{3}H]-spiperone binding was defined in saturation experiments as the difference in binding in parallel assays containing 3 μM (+)- and (−)-butaclamol. In competition experiments, specific [^{3}H]spiperone binding was defined as the binding inhibited by 3 μM (+)-butaclamol. Mianserin (0.3 μM) was included in all assays with bovine caudate nucleus in order to inhibit binding of [^{3}H]spiperone to $5HT_2$ serotonin receptors (Withy et al., 1981). Some assays were performed using the radioligand [^{3}H]YM 09151-2 (84 Ci/mmol) using the same protocol.

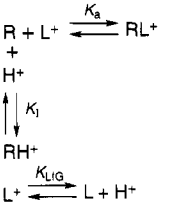
Radioligand binding data were analyzed using the computer program LIGAND in order to obtain the K_d values from saturation experiments and the K_i values from competition experiments at the different pH values. All competition and saturation data fit well to a one-binding site model. The data for the dependence of ligand dissociation constants on pH were then fitted using the program ENZFITTER to different models after conversion of the K_d/K_i values to $K_{a(apparent)}$ values.

(1) The first is a model where the binding of the ligand depends on the ionization of a single ionizing group on the receptor. Protonation of this residue alters the affinity of the receptor for the ligand sufficiently so that under the conditions used here there is essentially no binding remaining. $K_{a(apparent)}$ is the association constant for ligand (L^+)/receptor interaction at a given pH. K_a is the association constant when the ionizing group is completely ionized. K_I is the dissociation constant for ionization of the receptor.



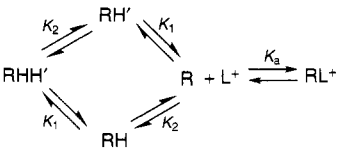
$$K_{a(apparent)} = \frac{K_a}{(1 + H^+/K_I)}$$

(2) Another is a model as in 1 but where the ionization of the ligand itself is also taken into account. K_{LIG} is the dissociation constant for ionization of the ligand.



$$K_{a(apparent)} = \frac{K_a}{(1 + H^+/K_I)(1 + K_{LIG}/H^+)}$$

(3) Another is a model where the binding of the ligand depends on the ionization of two groups on the receptor whose dissociation constants are K_1 and K_2. Protonation of either group alters the affinity of the receptor sufficiently so that under the conditions used for the assays there is no ligand binding.



$$K_{a(apparent)} = \frac{K_a}{(1 + H^+/K_1)(1 + H^+/K_2)}$$

(4) Another is a model as in 3 but where the ionization of the ligand itself is also taken into account.

$$K_{a(apparent)} = \frac{K_a}{(1 + H^+/K_1)(1 + H^+/K_2)(1 + K_{LIG}/H^+)}$$

The fits to data using different models were compared using the extra sum of squares principle and an F-test (Wetherill, 1981) in order to determine the best fit. Data from individual experiments were analyzed simultaneously in order to obtain the best fit parameters.

Chemical Modification of D_2 Dopamine Receptors Using N,N'-Dicyclohexylcarbodiimide. Cell membranes (1 mg) were incubated in buffer [20 mM sodium phosphate, 110 mM sodium chloride, and 0.8 mM magnesium chloride (pH 6.0)] with a range of concentrations of *N,N'*-dicyclohexylcarbodiimide (3−100 μM) for 3 h at 15 °C in a total volume of 1 mL. The membranes were then washed twice by centrifugation (12000*g*, 10 min) and resuspension of the pellet in buffer at pH 7.5. [^{3}H]Spiperone or [^{3}H]YM 09151-2 binding was then determined in the membranes as described above.

RESULTS

pH Dependence of Ligand Binding to D_2 Dopamine Receptors in Bovine Caudate Nucleus. [^{3}H]Spiperone was

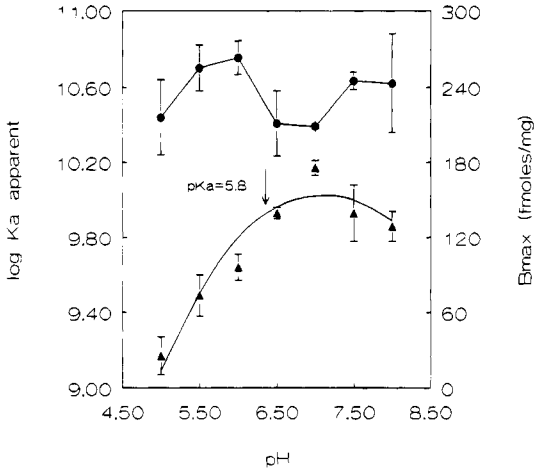


FIGURE 1: pH dependence of the binding of [^{3}H]spiperone to D_2 dopamine receptors in bovine caudate nucleus. Saturation analyses of [^{3}H]spiperone binding to D_2 dopamine receptors in bovine caudate nucleus were performed at different pH's as described in the Materials and Methods, and the B_{max} and K_d values were derived. The data for B_{max} and K_d are the means ± range for two experiments with points determined in triplicate. The data for pH dependence are fitted to model 2 with a pK_a of 5.8 for the ionizable group on the receptor and a pK_a for the ligand of 8.3. The data for K_d at each pH were transformed into $K_{a(apparent)}$ values as in model 2 and are shown as (●) (B_{max}) and (▲) [log $K_{a(apparent)}$].

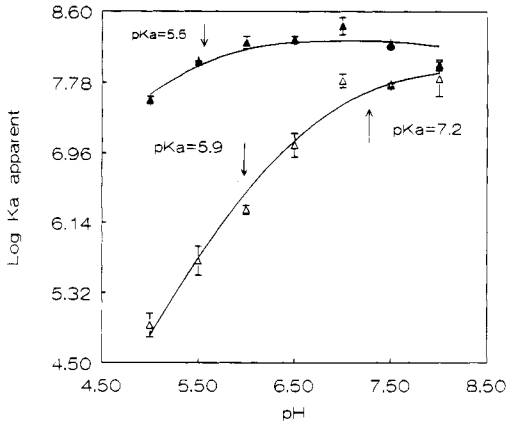


FIGURE 2: pH dependence of the binding of haloperidol (▲) and clebopride (△) to D_2 dopamine receptors in bovine caudate nucleus. Competition experiments were performed at different pH's as described in the Materials and Methods, and K_i values were derived and transformed into $K_{a(apparent)}$ values for calculation purposes. The data are given as means ± range for two experiments with points determined in triplicate. The data for haloperidol have been fitted to model 2 with a pK_a for the ionizable group on the receptor of 5.5 and a pK_a for the ligand of 8.7, whereas the data for clebopride have been fitted to model 3 with pK_a's for the ionizing groups on the receptor of 5.9 and 7.2.

used to detect receptors in this tissue, and 0.3 μM mianserin was included in the assays to prevent the binding of the radioligand to $5HT_2$ serotonin receptors (Withy et al., 1981). Saturation analyses were performed at different pH values over the range 5 to 8 using a citrate/barbiturate/borate/phosphate buffer that maintains a constant ionic strength at the different pH values. The data fit well to single binding site models at all pH values; the number of receptors detected by the radioligand (B_{max}) was unaffected by alteration of pH, but the dissociation constant was increased at both high and low pH (Figure 1). The data for the variation of K_d fit well to a model (model 2) which assumes that the radioligand binds to the receptor in its protonated state; a single ionizing group (pK_a = 5.8 ± 0.1, mean ± standard error (SE), two experiments) on the receptor influences the binding of the radioligand, and the radioligand itself undergoes deprotonation at high pH with a pK_a of 8.3 (Kilpatrick et al., 1986). These effects of pH on the bovine receptor were reversible (Williamson & Strange, 1990).

The pH dependence of the binding of other ligands to the bovine receptor was determined in competition experiments versus [^{3}H]spiperone (Figure 2), and the data (which all fit well to one binding site models) are summarized in Table 1. For haloperidol and (+)-butaclamol, the data for the pH dependence of the K_i values fit well to the model (model 2) where a single ionizing group on the receptor influences ligand binding and there is deprotonation of the ligand at high pH (pK_a values for the receptor and ligand are given in Table 1). For the substituted benzamide ligands (Figure 2), more complex models (models 3 and 4) were required to describe the data which included a second ionizing group on the receptor. The pK_a values for the receptor and ligands derived from this analysis are given in Table 1.

pH Dependence of Ligand Binding to Rat D_2 Dopamine Receptors Expressed in CHO6 Cells. D_2 dopamine receptors in CHO6 cells were examined in saturation and competition experiments with [^{3}H]spiperone which were performed as described above except that mianserin was omitted. Saturation analyses were performed over a range of pH values, and the data fit well to one-binding site models in all cases; the B_{max} values obtained were unaffected by the variation of pH, but the K_d values increased as the pH was decreased (Figure 3). These changes were reversible as shown in experiments where the receptor preparation was incubated at a given pH and the pH adjusted to 7.4 and the preparation assayed for [^{3}H]spiperone binding; no loss of [^{3}H]spiperone

Table 1: Binding of Antagonists to D_2 Dopamine Receptors in Bovine Caudate Nucleus and Recombinant CHO Cells Expressing the Rat Receptor[a]

	bovine caudate nucleus		CHO6 cells (rat receptor)	
	K_i (nM)	pK_a (receptor)	K_i (nM)	pK_a (receptor)
(+)-butaclamol	2.9 ± 0.7 (2)	5.6 ± 0.1	5.7 ± 0.5 (2)	5.5 ± 0.1
haloperidol	6.1 ± 0.4 (2)	5.5 ± 0.1	1.5 ± 0.1 (2)	5.9 ± 0.1
spiperone	0.12 ± 0.01 (2)	5.8 ± 0.1	0.078 ± 0.01 (2)	6.1 ± 0.1
clebopride	18 ± 1 (2)	5.9 ± 0.3	2.2 ± 0.3 (3)	6.0 ± 0.6
		7.2 ± 0.3		7.2 ± 0.6
DO 710	110 ± 20 (2)	6.3 ± 0.9	7.8 ± 1.2 (3)	6.8 ± 0.3
		6.9 ± 0.9		6.8 ± 0.3
(−)-sulpiride	93 ± 16 (3)	5.6 ± 0.5	27 ± 6 (3)	6.8 ± 0.2
		7.1 ± 0.3		6.9 ± 0.2
raclopride	16.1 ± 1.1 (2)	6.7 ± 0.4	4.8 ± 0.4 (3)	6.7 ± 0.3
		6.7 ± 0.4		6.7 ± 0.3
YM 09151-2	0.28 ± 0.06 (2)	6.0 ± 0.2	0.41 ± 0.1 (3)	4.8 ± 1.7
		6.7 ± 0.2		7.1 ± 1.7

[a] The binding of antagonists to D_2 dopamine receptors was determined in saturation and competition experiments with [^{3}H]spiperone at different pH values as described in the Materials and Methods. K_i/K_d values measured at pH 7.5 are expressed as mean ± SE (n experiments). The pH dependence of K_i/K_d values was analyzed as described in the text, and pK_a values (mean ± SE) are given for the best fit model. In each case, in a single experiment, the binding of ligand was determined at seven pH values. The experiments were repeated as indicated, and all the data were analyzed together using ENZFITTER as described in the text. Ligand protonation/deprotonation was accounted for by using the following pK_a values: (+)-butaclamol, 7.2; clebopride, 8.2; haloperidol, 8.7; raclopride, 6.7; spiperone, 8.3; sulpiride, 9.1; YM 09151-2, 7.8 (Kilpatrick et al., 1986; El Tayar et al., 1988).

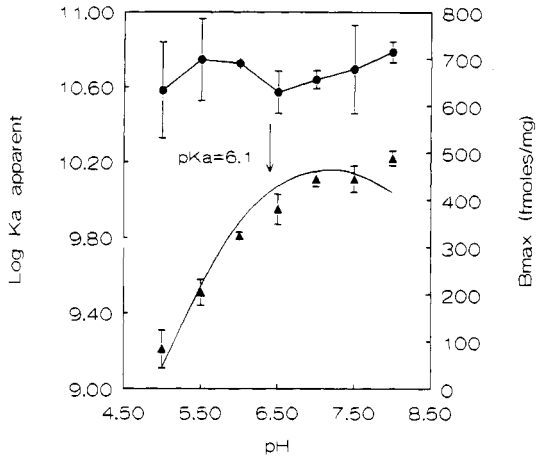


FIGURE 3: pH dependence of the binding of [^{3}H]spiperone to D_2 dopamine receptors in recombinant CHO cells. Saturation analyses of the binding of [^{3}H]spiperone to D_2 dopamine receptors in recombinant CHO cells were performed at different pH's as described in the Materials and Methods, and B_{max} and K_d values were derived. The data for B_{max} and K_d are the means ± range for two experiments with points determined in triplicate. The data for pH dependence are fitted to model 2 with a pK_a for the ionizable residue on the receptor of 6.1 and a pK_a for the ligand of 8.3. The data for K_d at each pH were transformed into $K_{a(apparent)}$ values as in model 2 and are shown as (●) (B_{max}) and (▲) [log $K_{a(apparent)}$].

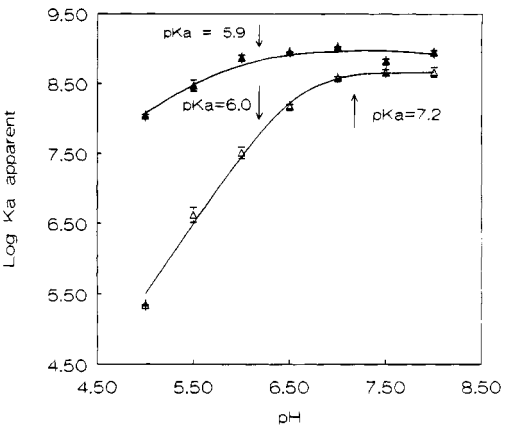


FIGURE 4: pH dependence of the binding of haloperidol (▲) and clebopride (△) to D_2 dopamine receptors in recombinant CHO cells. Competition experiments were performed at different pH's as described in the Materials and Methods, and K_i values were derived and transformed into $K_{a(apparent)}$ values for calculation purposes. The data are given as means ± range for two experiments with points determined in triplicate. The data for haloperidol have been fitted to model 2 with a pK_a for the ionizable group on the receptor of 5.9 and a pK_a for the ligand of 8.7, whereas the data for clebopride have been fitted to model 3 with pK_a values for the two ionizable groups on the receptor of 6.0 and 7.2.

binding was observed. The data for the variation in K_d fit well to a model (model 2) where a single ionizing group (pK_a = 6.1 ± 0.1, mean ± SE, two experiments) on the receptor influences ligand binding and the ligand undergoes deprotonation with a pK_a of 8.3.

The pH dependence of the binding of other ligands to the receptor was studied in competition experiments versus [^{3}H]-spiperone. The pattern of data was very similar to that seen for the bovine receptor described above; the binding of (+)-butaclamol and haloperidol depended on the protonation of a single group on the receptor, whereas the binding of antagonists of the substituted benzamide class depended on the protonation of two groups on the receptor. The data and derived pK_a values are given in Figure 4 and Table 1.

Chemical Modification of D_2 Dopamine Receptors in CHO6 Cells Using N,N′-Dicyclohexylcarbodiimide. The effect of *N,N′*-dicyclohexylcarbodiimide on D_2 dopamine receptors expressed in CHO6 cells was determined in experiments using [^{3}H]spiperone and the substituted benzamide [^{3}H]YM 09151-2 to detect the receptors. For both radioligands, dose dependent inhibition of ligand binding was observed with increasing concentrations of *N,N′*-dicyclohexylcarbodiimide (Figure 5) and the concentrations of the reagent that inhibited radioligand binding were similar (IC_{50} = 13 ± 1 μM and 5.3 ± 1.2 μM, respectively, mean ± SE, three experiments). The inhibition of radioligand binding was due to a reduction in B_{max} in each case with little effect on the K_d ([^{3}H]spiperone binding: control, K_d = 69 ± 1 pM, B_{max} = 765 ± 42 fmol/mg; after 50 μM *N,N′*-dicyclohexylcarbodiimide treatment, K_d = 76 ± 1 pM, B_{max} = 86 ± 8 fmol/mg. [^{3}H]YM 09151-2 binding: control, K_d = 67 ± 4 pM, B_{max} = 1091 ± 55 fmol/mg; after 25 μM *N,N′*-dicyclohexylcarbodiimide treatment, K_d = 61 ± 9 pM, B_{max} = 311 ± 26 fmol/mg, mean ± range, two experiments). The inhibition of radioligand binding could be prevented by prior occupancy of the receptors by the selective antagonists haloperidol and (−)-sulpiride; e.g. 100 μM (−)-sulpiride afforded 85 and 63% protection, respectively, against the effects of *N,N′*-dicyclohexylcarbodiimide on the binding of [^{3}H]spiperone, and [^{3}H]YM 09151-2 and 1 μM haloperidol afforded 74 and 100% protection, respectively.

DISCUSSION

In the present report, we have analyzed in detail the pH dependence of the binding of ligands to D_2 dopamine receptors in bovine caudate nucleus and in a recombinant CHO cell line (CHO6, Castro & Strange, 1993) expressing the rat receptor from its cloned gene. D_2 dopamine receptors in the two systems have been well-characterized in previous work (Withy et al., 1981; Leonard et al., 1987; Castro & Strange, 1993). The aim of the present study was to learn about the interaction of ligands with these receptors. The results of the study have shown that in both systems classical dopamine antagonists bind to the receptor in a different manner than antagonists of the substituted benzamide class.

Saturation analyses performed for both the rat and bovine receptors with the radioligand [^{3}H]spiperone over a range of pH's showed that in each tissue the receptor population detected did not change as the pH was altered. The dissociation constants of this ligand for binding to the receptors were, however, affected by the pH change, and these effects will be considered below together with data on the pH dependence of the binding of other ligands obtained from competition studies versus [^{3}H]spiperone.

For the compounds (+)-butaclamol, haloperidol, and spiperone (classical dopamine antagonists), pH dependence studies in both tissues indicated that a single ionizing group on the receptor influenced ligand binding. The pK_a values for this group were in the range 5.5−6.1 for both tissues. In contrast, the binding of several antagonists of the substituted benzamide class was influenced by two ionizing residues whose pK_a values were generally in the range 6−7 and were often, but not always, different. In several cases, the ligand underwent deprotonation at high pH, and this has been taken into account in the analysis. These data show clearly that

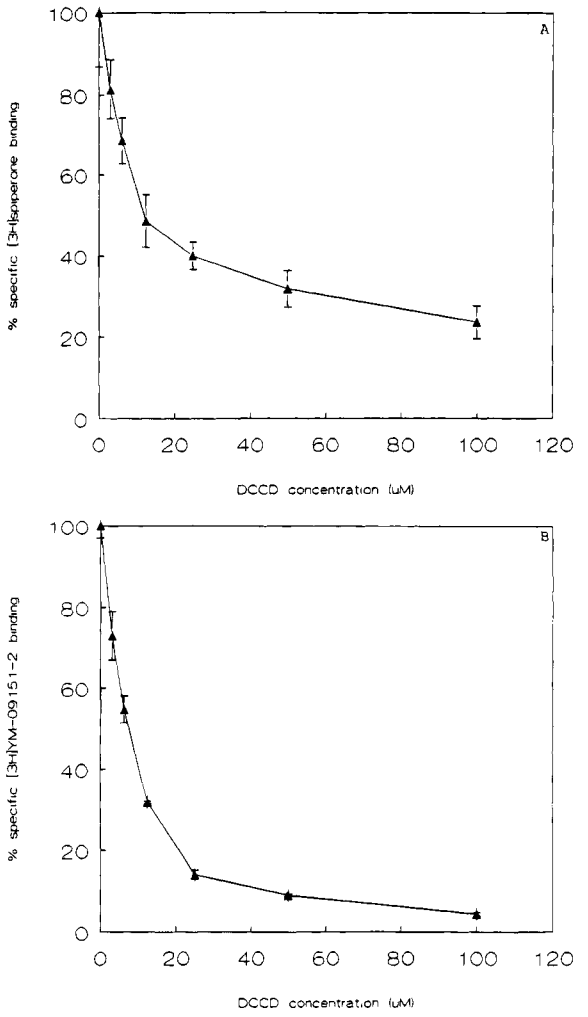


FIGURE 5: Effects of *N,N'*-dicyclohexylcarbodiimide on the binding of [^{3}H]spiperone (A) and [^{3}H]YM 09151-2 (B) to D_2 dopamine receptors in recombinant CHO cells. The data are from a representative experiment which has been replicated three times with similar results, and the data points are the means ± standard error of triplicate determinations.

these two classes of ligand bind in a different manner to the D_2 dopamine receptor. Other studies have shown that the substituted benzamides behave differently in their interaction with D_2 dopamine receptors when compared with other ligands. The binding of the substituted benzamides is sensitive to Na^+ ions (Theodorou et al., 1980) and the mutation of certain amino acid residues (Asp 80, His 394) (Neve et al., 1991; Woodward et al., 1994) and is affected by the insertion that differentiates the long and short forms of the D_2 dopamine receptor (Castro & Strange, 1993). The binding of the classical antagonists is not affected by these same influences. Some of these effects seem to be related to the sensitivity of the substituted benzamides to the conformation of the receptor.

The behavior of the receptors from the two sources is very similar, but the binding of ligands to the rat receptor seems to be of higher affinity than to the bovine receptor. This is most pronounced for the substituted benzamide drugs. We have already noted this difference in studies on bovine and rat brain (Leonard et al., 1988). The rat and bovine receptors are 96% homologous within the transmembrane spanning regions (Chio et al., 1990), and there are no major changes in amino acids that might directly alter the binding of ligands. The difference therefore presumably relates to differences in the conformation of the receptor which have a particular effect on the binding of the substituted benzamides.

From the present data on the pH dependence of ligand binding, it is not possible to assign amino acid residues to the ionizing groups seen, but it is possible to provide some speculations. As the effects of pH can be modeled in terms of one or two ionizing groups, then it seems unlikely that the observations can be accounted for by general effects of pH on multiple residues on the receptor protein. It is also unlikely that the effects reflect denaturation of the receptor as the effects are reversible. Therefore, it is likely that the effects observed reflect alteration of the ionization state of specific residues which in turn either affects the conformation of the receptor, and the affinity of the receptor for ligands, or disrupts an electrostatic interaction between ligand and receptor. Similar behavior was seen in both the bovine and rat receptors, consistent with a high degree of sequence conservation between the receptors from the two species (Bunzow et al., 1988; Chio et al., 1990). Within the putative ligand-binding site of the D_2 dopamine receptor, there are three ionizing residues that could contribute to the pH dependence of ligand binding: Asp 80 (TM II), Asp 114 (TM III), and His 394 (TM VI). Chemical modification studies (Williamson & Strange, 1990; U. D'Souza and P. G. Strange, unpublished data) indicated that sulfydryl and tyrosyl residues were unlikely to be involved in direct interactions with ligands and so are unlikely to contribute to the pH dependence of ligand binding.

A plausible candidate residue for the single ionizing group ($pK_a = \sim 6$) that affects the binding of classical dopamine antagonists is Asp 114. An analogous aspartic acid residue is found in other receptors that bind cationic amines, e.g. the β_2-adrenergic receptor, and it has been proposed that this residue forms the counterion for the cationic amino group of ligands when they form an electrostatic bond to the receptor (Strader et al., 1994). Mutagenesis of this residue strongly attenuates binding of ligands to the β_2-adrenergic receptor (Strader et al., 1994) and the D_2 dopamine receptor (Mansour et al., 1992). Also, modification of carboxyl groups attenuates binding of ligands to D_2 dopamine receptors in bovine brain (Williamson & Strange, 1990) and in the present study for the rat receptor (see below for further discussion of these data). A pK_a of ~6 is rather high for a carboxyl group, but the corresponding residue in the muscarinic acetylcholine receptor has also been reported to have a pK_a of ~6 (Birdsall et al., 1989; Ehelrt & Delen, 1990). Possibly, the microenvironment of the ligand binding site of these receptors is unusual so that the pK_a is raised; the ligand binding site is generally hydrophobic, but there are three ionizing groups (Asp 80, Asp 114, and His 394) that may be close enough to affect one another's ionization (see below). The data analysis employed here assumes that the protonation of the ionizable group eliminates ligand binding in the assay conditions employed. If this group were Asp 114, then the elimination of an electrostatic interaction caused by the protonation of the carboxyl group should reduce the ligand binding affinities substantially. For the β_2-adrenergic receptor, mutation of the corresponding amino acid residue reduces the affinities of ligands by a factor of about 10^4 (Strader et al., 1988) which under the experimental conditions used here would lead to the elimination of ligand binding. It is unlikely that changes in the ionization state of Asp 80

or His 394 contribute to this pH dependence directly, as when these residues have been mutated, the binding of antagonists such as spiperone is essentially unchanged (Neve et al., 1991; Woodward et al., 1994; U. D'Souza and P. G. Strange, unpublished results). The actual values for the pK_a of the receptor form a range between 5.5 and 6.1 when effects on the binding of different ligands are tested. There is a tendency for spiperone to show higher values in both systems and for (+)-butaclamol to show lower values, and this may reflect slightly different conformations of the receptor which bind the different drugs.

The binding of the substituted benzamide antagonists is dependent on the protonation of two ionizing groups, and although there is some variation in the values seen for different compounds and in the two species, the two groups tend to have pK_a values in the range 6−7. Where the two groups have different pK_a's, these tend to lie at the upper and lower ends of this range. In line with the results from chemical modification and the discussion above, it seems likely that one of the groups is Asp 114 which participates in an electrostatic interaction with the cationic amino group of these drugs. Also, mutagenesis of Asp 114 eliminates the binding of the substituted benzamide [^{3}H]raclopride to D_2 dopamine receptors (Mansour et al., 1992).

The nature of the second ionizing group that affects the binding of the substituted benzamide drugs is less clear. The pK_a value of the group would be consistent with a histidine residue, but mutagenesis of His 394 renders the binding of sulpiride more sensitive to pH rather than reducing the pH sensitivity (Woodward et al., 1994). The other candidate within the ligand binding site is Asp 80, although this would have to have an unusually high pK_a for a carboxyl group. This could reflect the hydrophobic environment of the binding site and the interactions among the three ionizing groups (Asp 80, Asp 114, and His 394) at the binding site. Mutation of Asp 80 reduces but does not eliminate the sensitivity of the binding of sulpiride to a change of pH from 8 to 6 (Neve et al., 1991). In our own preliminary experiments on the Asn 80 mutant, we have found that, whereas the affinity of sulpiride for binding to the native receptor is reduced by a factor of 21 by a change of pH from 7.5 to 6, for the Asn 80 mutant, the reduction in affinity is only 6-fold. These data suggest that Asp 80 may contribute to the pH sensitivity of the binding of the substituted benzamides, but the effects of the mutation do not seem great. The change of pH from 8 or 7.5 to 6 will, however, alter the ionization state of both of the ionizable groups that influence the binding of the substituted benzamides so that the mutation of Asp 80 could only produce a partial effect. Second, the effects of Asp 80 on the binding of the substituted benzamide drugs may be modulatory; it is unlikely that Asp 80 interacts directly with the substituted benzamide drugs. This conclusion is based on the high degree of conservation of this residue in G-protein-coupled receptors which make it unlikely to be involved in specific interactions in receptors and the position of the residue about two-thirds of the distance in from the membrane surface, the other residues involved in direct interactions with ligands being found about one-third of the distance from the membrane surface. Neve et al. (1991) have shown that D_2 receptors are allosterically regulated by Na^+ and H^+ ions and that in the Asn 80 mutant regulation by Na^+ is eliminated and as indicated above regulation by H^+ is reduced. It seems that interaction of Na^+ and H^+ with Asp 80 is important for regulation of the conformation of the receptor so that it binds the substituted benzamide drugs with a high affinity. The equations used to define the effects of the ionizable groups (models 3 and 4) assume that the protonation of either of the ionizable groups will effectively eliminate the binding of the ligand under the assay conditions used here. It is likely, however, that, owing to the modulatory nature of the interactions, the protonation of Asp 80 will not eliminate the binding of the substituted benzamides; for example, the affinity of sulpiride is reduced by a factor of about 50 by the Asn 80 mutation (Neve et al., 1991; U. D'Souza and P. G. Strange, unpublished results), and the effect of the removal of Na^+ ions from the assay buffers is reduction of the affinity of sulpiride by a factor of ~6-fold (Neve, 1991; Woodward et al., 1994). Again this supports the idea that the Asn 80 mutation would reduce but not eliminate the pH dependence of sulpiride binding, and the data available at present are consistent with the idea that Asp 80 is the second ionizable residue affecting the pH dependence of the binding of the substituted benzamide drugs.

The data obtained with the carboxyl specific reagent dicyclohexylcarbodiimide here for the receptor expressed in CHO6 cells and previously in bovine brain (Williamson & Strange, 1990) showed that modification of carboxyl groups strongly attenuates antagonist binding, supporting the idea that carboxyl groups are important for the binding of these ligands. It is interesting to note that in the present study with the rat receptor and the previous study with the bovine receptor about 20% of the receptors remained unmodified even at high concentrations of the reagent when the receptors were detected with [^{3}H]spiperone, whereas in the present study when the substituted benzamide [^{3}H]YM 09151-2 was used to detect the receptors, all the receptors are modified by the same concentrations of the reagent. The two radioligands label the same maximum number of binding sites so this cannot be a reflection of two different populations of receptors before modification. One possible explanation for the data entails modification of more than one carboxyl group at the ligand binding site. For example, both Asp 80 and Asp 114 are likely to be modified by dicyclohexylcarbodiimide. Modification of Asp 114 should eliminate the binding of a ligand, but it may be that modification of Asp 80 alters the conformation of the receptor so that modification of Asp 114 occurs more slowly. Under the particular conditions of the experiments, this would result in 80% modification of Asp 114 and 80% inhibition of [^{3}H]-spiperone binding. Since the binding of substituted benzamide drugs is reduced by mutation of Asp 80, the modification of Asp 80 and Asp 114 will lead to complete inhibition of [^{3}H]YM 09151-2 binding.

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