Dopamine D\textsubscript{2} Receptor Dimer Formation

EVIDENCE FROM LIGAND BINDING*

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We have examined the binding of two radioligands ([^3]H)spiperone and [^3]H)raclopride) to D\textsubscript{2} dopamine receptors expressed in Chinese hamster ovary cells. In saturation binding experiments in the presence of sodium ions, both radioligands labeled a similar number of sites, whereas in the absence of sodium ions [^3]H)raclopride labeled about half the number of sites labeled by [^3]H)spiperone. In competition experiments in the absence of sodium ions, however, raclopride was able to inhibit [^3]H)spiperone binding fully. In saturation analyses with [^3]H)spiperone in the absence of sodium ions raclopride exerted noncompetitive effects, decreasing the number of sites labeled by the radioligand. These data are interpreted in terms of a model where the receptor exists as a dimer, and in the absence of sodium ions, raclopride exerts negative cooperativity across the dimer both for its own binding and the binding of spiperone. A model of the receptor has been produced that provides a good description of the experimental phenomena described here.

The G-protein-coupled receptors (GPCRs)\textsuperscript{1} constitute a large family of proteins responsible for the transduction of a wide range of signals (e.g. hormones, neurotransmitters, odorants, light, etc.) via G-proteins (1). GPCRs possess a common structural motif of seven \textalpha-helical membrane-spanning domains, and it is often assumed that the functional unit (i.e. the ligand binding and G-protein interaction domains) of the GPCR is wholly contained in a single polypeptide. Indeed, most models of GPCR function assume a monomeric receptor interacting with the G-protein (see, for example, Ref. 2). Several lines of evidence, however, suggest that the some GPCRs may exist in dimeric or oligomeric forms.

Immunoblotting has in several cases revealed species corresponding not only to the predicted molecular weight of the receptor but also to multiples of the molecular weight. Bands corresponding to approximately twice the predicted molecular weight of the receptor have been interpreted as homodimers for several receptors including D\textsubscript{2} dopamine (3, 4), D\textsubscript{3} dopamine (5), \beta\textsubscript{2}-adrenergic (6), substance P (7), opiate (8) and M\textsubscript{1} and M\textsubscript{2} muscarinic receptors. Expression of either chimera alone did not result in any detectable binding of typical radiolabeled muscarinic or adrenergic ligands. However, cotransfection of COS7 cells with both chimeras resulted in the appearance of binding activity corresponding to both native receptors. This has lead to the proposal that some GPCRs might form domain-swapped dimers (16). Evidence for GPCR interaction in cells has been obtained by expressing GPCRs fused to different chromophores. Transfer of energy between the chromophores has been shown for the \beta\textsubscript{2}-adrenergic receptor (17) and somatostatin SSTR5 receptor (10) and provides good evidence for the close proximity of the two molecules of GPCRs.

Some radioligand binding studies suggest differences in the number of binding sites labeled by different radioligands. At M\textsubscript{2} muscarinic receptors, the antagonist [^3]H)QNB labeled twice as many sites as did [^3]H)AF-DX 384 or N-[^3]H)methylscopolamine under certain conditions (18). These data were interpreted in terms of a model where the receptor exists as a tetramer. The D\textsubscript{2} dopamine receptor is of interest in this regard. Several studies suggest that the substituted benzamide radioligand [^3]H)nemonapride can label more D\textsubscript{2} receptor sites in radioligand binding studies than the butyrophenone [^3]H)spiperone (3, 19–21), although this was not seen in all reports (22, 23). For D\textsubscript{3} dopamine receptors expressed in recombinant cells, Seeman et al. (21) reported that [^3]H)raclopride labeled more D\textsubscript{2} dopamine receptor sites than did [^3]H)spipereone, although Malmberg et al. (23) were unable to replicate these findings. Interestingly, Hall et al. (24) found that the number of sites labeled by [^3]H)raclopride in rat striatal membranes was dependent on the conditions used. [^3]H)Raclopride labeled more sites in the presence of sodium ions than in their absence. The number of sites labeled by [^3]H)spiperone was, however, unaffected by sodium ions and was similar to the number of sites labeled by [^3]H)raclopride in the presence of sodium ions. Theodorou et al. (25) found that another substituted benzamide, [^3]H)sulpiride, also detected more D\textsubscript{2} receptor binding sites in rat striatum in the presence of sodium ions than it did in their absence, and similar observations have been made for [^3]H)raclopride binding to the related D\textsubscript{4} dopamine receptor expressed in recombinant cells (26).

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\textsuperscript{1} The abbreviations used are: GPCR, G-protein-coupled receptor; NMDG, N-methyl-D-glucamine; ANOVA, analysis of variance; CHO, Chinese hamster ovary.

This paper is available on line at http://www.jbc.org
These observations are not consistent with the labeling by these radioligands of single populations of independent D2 dopamine receptors. They are more consistent with the labeling of oligomeric arrays with different degrees of cooperativity between the monomeric units, depending on assay conditions (18, 27). Because of the importance of the D2 receptor in the actions of the antipsychotic drugs, we have examined this phenomenon in more detail. In this paper, therefore, we have studied the binding of two radioligands ([3H]spiperone and [3H]raclopride) to D2 dopamine receptors expressed in CHO cells and provide evidence for the formation of homodimers for this receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Spiperone (15–30 Ci/mmol) was purchased from Amersham Pharmacia Biotech, and [3H]raclopride (60–86 Ci/mmol) was purchased from PerkinElmer Life Sciences. S-(−)-Sulpiride, haloperi- dol, and butaclamol were purchased from RBI (Natick, MA). All other materials were obtained from commercial sources and were of the highest available purity.

**Cell Growth and Membrane Preparation**—CHO cells expressing the human D2<sub>short</sub> dopamine receptor (28) were grown as monolayers in RPMI medium supplemented with 2 mM glutamine, 200 μg/ml active penicillin, 10% fetal calf serum (37 °C in a moist, 5% CO2 atmosphere). Cells were washed with 5 ml of ice-cold buffer A (20 mM HEPES, 1 mM EDTA (free acid) 1 mM EGTA, pH 7.4, with KOH), removed from the flask by gentle shaking with 2-mm diameter glass beads in 5 ml of buffer A, and homogenized with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 280,000 × g for 10 min, and the resulting supernatant centrifuged at 48,000 × g for 1 h at 4 °C. The pellet was resuspended in ice-cold buffer A to ~5–10 mg/ml, and aliquots were stored at −70 °C. Protein concentration was determined by the method of Lowry et al. (29).

**Saturation Radioligand Binding Experiments**—Control experiments were performed in buffer A, while 100 mM NaCl or 100 mM N-methyl-D-glucamine (NMDG) was included in the buffer to determine the effects of sodium ions or ionic strength. Total and nonspecific binding were defined in the presence of 3 μM (-)butaclamol and 3 μM (+)-butaclamol, respectively. [3H]Raclopride saturation binding experiments were performed in a total volume of 0.5 ml using 3–15 μg of membrane protein per tube and 10 concentrations of [3H]raclopride typically ranging between 20 pM and 10 nM. [3H]Spiperone saturation binding experiments were performed in total volumes of either 1 or 10 ml, both using 10–30 μg of membrane protein/tube and 10 concentrations of the radioligand, typically between 10 pM and 5 nM for 1 ml saturations, or 16 concentrations between 1 PM and 1 nM for 10 ml experiments. In experiments that included raclopride or haloperidol, the range of [3H]spiperone concentrations was varied according to the apparent Kd values obtained in initial experiments. Each experiment was performed in triplicate and incubated at 25 °C for 3 h (for [3H]raclopride and 1 ml [3H]spiperone experiments) or 7 h (10 ml [3H]spiperone experiments), by which time the radioligands had reached equilibrium. Experiments were terminated by rapid filtration through Whatman GF/C glass fiber filters using a Brandel cell harvester followed with four washes of 3 ml of ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4). Filter discs were soaked in 2 ml each of Optiphase HiSafe 3 (Wallac) for at least 6 h before radioactivity was determined by liquid scintillation spectroscopy.

**Inhibition of Equilibrium [3H]Spiperone Binding**—A range of concentrations of the competing ligand were incubated with 10–30 μg of membranes and a fixed concentration of [3H]spiperone in triplicate for 3 h at 25 °C before harvesting as described above. Total and nonspecific binding were defined in the presence of 3 μM (-)butaclamol and 3 μM (+)-butaclamol, respectively.

**Data Analysis**—Data were analyzed using Prism (GraphPad, San Diego CA). In saturation experiments, specifically and nonspecifically bound [3H]spiperone were calculated from saturation data using the method of Golds et al. (30), which makes a correction for the depletion of the radioligand. Data were fitted to equations describing one- or two-site binding models, and the best fit was determined using an F-test. Competition experiments were fitted to four-parameter logistic equations, and the best fit between a variable Hill coefficient and a Hill coefficient fixed to unity was determined using an F-test. In the analysis of the competition data, the free radioligand concentration was taken as the added minus total bound in the absence of competitor. The amount bound will be in fact be different at the top and bottom of the competition curve. The total bound was, however, ~12 and ~1% of the added radioligand in the absence and presence, respectively, of saturating concentrations of competitor ([3H]spiperone, ~0.25 nM). The total bound radioligand in the absence of competitor was <10% for the higher radioligand concentrations used. The effect of this correction on estimates of Kd is slight (~5% at 0.25 nM radioligand).

The statistical significance of difference between data was determined at the 0.05 level, using ANOVA or Student's t test, as appropriate. Kd and Kd<sub>max</sub> values were first converted to the respective normally distributed negative logarithm (pKd<sub>B</sub> or pKd<sub>B</sub><sub>max</sub>). Mean values are quoted with the respective S.E.

**RESULTS**

[3H]Raclopride Labels Two Different Receptor Populations, Depending on the Presence of Sodium Ions—Saturation binding studies were performed on human D2<sub>short</sub> dopamine receptors expressed in membrane preparations from recombinant CHO cells. [3H]Raclopride binding in the absence of sodium ions (“control” conditions) revealed a single population of binding sites with a Kd of 1.1 nM and B<sub>max</sub> of 0.84 pmol/mg of membrane protein (Fig. 1 and Table I). In the presence of 100 mM NaCl, the data were also best described by a single population of binding sites in which the Kd for [3H]raclopride was decreased significantly to 0.23 nM and B<sub>max</sub> significantly increased to 1.63 pmol/mg. 100 mM NMDG was used as a control for changes in ionic strength, and experiments performed under these conditions gave values for Kd of 1.2 nM and B<sub>max</sub> of 0.89 pmol/mg, which did not differ significantly from the control values (ANOVA, p > 0.05).

Saturation binding experiments with [3H]spiperone were performed under two conditions: large volume (10-ml) assays to minimize the extent of ligand depletion and, more routinely, smaller volume (1-ml) assays. In control conditions, in both assay volumes a single population of binding sites was found, with B<sub>max</sub> values of 1.2 pmol/mg and 1.6 pmol/mg for 10-ml and 1-ml volume assays, respectively (Table I). These values are not significantly different (ANOVA, p > 0.05). The dissociation constant of [3H]spiperone, 15 μM, was significantly lower when determined in 10-ml assays, compared with the value, 63 μM, found in 1-ml assays (Table I).

In [3H]spiperone saturation assays performed in a 10-ml volume, the B<sub>max</sub> value found in the presence of 100 mM NaCl, 2 pmol/mg, was not significantly different from that found in
The binding of \(^{3}H\)spiperone and \(^{3}H\)raclopride to \(D_2\) dopamine receptors expressed in CHO cells was determined as described under “Experimental Procedures.” \(K_d\) and \(B_{\text{max}}\) values were determined for both radioligands, and \(pK_d\) and \(B_{\text{max}}\) values are given as the mean ± S.E. with the number of experiments in parentheses. The corresponding \(K_d\) value is given as nM. \(^{3}H\)Spiperone saturation assays were performed in a total volume of 10 ml in the presence of 100 mM NaCl or 100 mM NMDG or in the absence of either ion and were also performed in a total volume of 1 ml in the absence of ions. \(^{3}H\)Raclopride saturation assays were performed in 0.5 ml volumes under the same ionic conditions as \(^{3}H\)spiperone. \(B_{\text{max}}\) determinations were all performed on the same preparation of membranes in order to allow comparisons, whereas \(K_d\) values are from different preparations. The concentration of receptor binding sites in the assays is given in pm.

### Table I

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Control</th>
<th>Na(^+)</th>
<th>NMDG</th>
<th>Control (1 ml spiperone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiperone (10 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pK_d)</td>
<td>10.84 ± 0.11 (7)</td>
<td>10.87 ± 0.05 (4)</td>
<td>10.76 ± 0.13 (4)</td>
<td>10.20 ± 0.04 (52)</td>
</tr>
<tr>
<td>(K_d) (nM)</td>
<td>1.21 ± 0.11 (3)</td>
<td>0.013</td>
<td>1.99 ± 0.13 (4)</td>
<td>0.017</td>
</tr>
<tr>
<td>(B_{\text{max}}) (pmol/mg)</td>
<td>3.6</td>
<td>6.0</td>
<td>1.61 ± 0.06 (4)</td>
<td>1.56 ± 0.15 (3)</td>
</tr>
<tr>
<td>[Receptor] (pm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raclopride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pK_d)</td>
<td>8.97 ± 0.11 (7)</td>
<td>9.63 ± 0.06 (7)</td>
<td>9.82 ± 0.09 (6)</td>
<td></td>
</tr>
<tr>
<td>(K_d) (nM)</td>
<td>1.1</td>
<td>0.23</td>
<td>0.89 ± 0.18 (3)</td>
<td></td>
</tr>
<tr>
<td>(B_{\text{max}}) (pmol/mg)</td>
<td>0.84 ± 0.16 (4)</td>
<td>1.63 ± 0.27 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Receptor] (pm)</td>
<td>25.2</td>
<td>48.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

In this study, we have examined the binding of two ligands (raclopride and spiperone) to human \(D_2\) dopamine receptors expressed in CHO cells. Saturating and competition experiments have been used to show that the \(D_2\) receptor functions as an oligomer and that the properties of this oligomeric receptor may be modulated by the ionic conditions.

In saturation binding assays, sodium ions were found to exert allosteric effects on \(^{3}H\)raclopride binding to recombinant human \(D_2\) dopamine receptors. The presence of sodium ions was found to increase the affinity of \(^{3}H\)raclopride for the \(D_2\) receptor, and this phenomenon has been described extensively before for drugs of the substituted benzamide class (25, 32–38). In the present study, the dissociation constant decreased from 1.1 nM approximately 5-fold to 0.23 nM. This effect was specific to sodium ions, since the presence of an equal concentration (100 mM) of NMDG as a control for changes in ionic strength had no appreciable effect on the \(K_d\) of \(^{3}H\)raclopride.

Sodium ions also exerted a second effect on \(^{3}H\)raclopride binding to \(D_2\) receptors by changing the number of binding sites that were labeled (\(B_{\text{max}}\)). A 2-fold increase in \(B_{\text{max}}\) was found in the presence of sodium ions as compared with the absence of sodium ions. Again, there was no appreciable effect of NMDG compared with the absence of ions. This effect of sodium ions on the number of sites labeled by \(^{3}H\)raclopride has been noticed before in studies of \(D_2\) dopamine receptors in the brain and in recombinant cells (24, 39) but not analyzed further.

In contrast to \(^{3}H\)raclopride, the \(K_d\) of \(^{3}H\)spiperone for binding to \(D_2\) receptors was unaffected by the presence or absence of sodium ions, in agreement with many other reports (19, 24, 35, 39). It should be noted that experimental design can be important in determining the affinity of some high affinity radioligands. Here an assay volume of 1 ml gave a value for the \(K_d\) of \(^{3}H\)spiperone of ~63 pm. When assays were performed to

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minimize ligand depletion via binding to receptor and nonspecifically to tissue (i.e. use of a 10-ml total volume, in which there is 10-fold more radioligand present but the same amount of receptor), this experimental protocol provided an estimate of $K_d$ for $[^3H]$spiperone of 15 pm. Even under these conditions, there is some depletion at the lower concentrations of radioligand, but the estimate of $K_d$ agrees well with determinations of $K_d$ for spiperone designed to eliminate depletion artifacts (23). The effects of such artifacts on determination of $K_d$ values have been discussed in detail elsewhere (23–43).

The effect of sodium ions on the number of binding sites labeled by $[^3H]$spiperone was less than for $[^3H]$raclopride. $B_{\text{max}}$ values for $[^3H]$spiperone were similar in the presence of sodium ions, in the presence of NMDG, and in the absence of monovalent cations (in 1-ml assays) and similar to the $B_{\text{max}}$ seen for $[^3H]$raclopride in the presence of sodium ions. In 10-ml assays in the absence of monovalent cations, some reduction in $B_{\text{max}}$ was seen for $[^3H]$spiperone. These data suggest that the $B_{\text{max}}$ of $[^3H]$spiperone is less sensitive to monovalent cations than the $B_{\text{max}}$ of $[^3H]$raclopride, but from the data of Table I it appears that the binding of $[^3H]$spiperone is not completely insensitive to the effects of sodium ions. An important comparison can be made between the $B_{\text{max}}$ values for the two radioligands in the presence of sodium ions and in the presence of NMDG, since this comparison takes account of the effects of ionic strength. In this comparison, $[^3H]$spiperone labels similar numbers of sites under both conditions and similar to the $B_{\text{max}}$ for $[^3H]$raclopride in the presence of sodium ions. In the presence of NMDG, $[^3H]$raclopride labels about half the number of sites. Previous reports would suggest that the binding of both $[^3H]$spiperone and $N$-$[^3H]$methylspiperone are largely insensitive to sodium ions (19, 24, 25, 35). Discrepancies between the number of $D_2$
receptors labeled by different radioligands in vitro have been reported extensively and for several ligands, e.g. [3H]spiperone (or N-[[3H]methylspiperone] compared with either [3H]raclopride (21, 24), [3H]sulpiride (42), or [3H]nomepiperone (3, 19, 21), although this was not seen in all reports (22, 23).

In the present study, therefore, in the presence of sodium ions, [3H]raclopride and [3H]spiperone label similar numbers of sites, whereas in the absence of sodium ions [3H]raclopride labels fewer sites than are labeled by [3H]spiperone (66% under control conditions and 55% in the presence of NMDG). If all of these sites are independent and the interaction of spiperone and raclopride is competitive, then it would be expected that in the absence of sodium ions raclopride would not fully compete with a high concentration of [3H]spiperone for binding to the receptors. It was found, however, that raclopride inhibited all of the specific [3H]spiperone binding (to within 5% of the level of nonspecific binding defined by 3 μM (+)-butaclamol) over a range of [3H]spiperone concentrations between 0.2 and 4.3 nM, suggesting that raclopride may exhibit some negative cooperativity with [3H]spiperone at the D2 dopamine receptor.

In order to examine this further, we performed saturation binding assays with [3H]spiperone in the presence of different concentrations of raclopride. First, however, this experimental design was employed with a ligand that has been shown to act in a purely competitive manner at D2 receptors, haloperidol (31). Inclusion of haloperidol in [3H]spiperone saturation binding assays reduced the apparent affinity of the radioligand in a concentration-dependent manner, as seen in Fig. 3B. The Kd value derived from this analysis was in excellent agreement with values derived using assay conditions defined to avoid artifacts (23, 31, 41). The Bmax of the radioligand was unaffected by haloperidol, as seen in Fig. 3A. These data are in agreement with a simple competitive model of two ligands competing for a single population of identical binding sites.

In a similar experimental design, raclopride (in the absence of sodium ions) also decreased the apparent affinity of [3H]spiperone, but the observed decrease in affinity was smaller than predicted assuming competitive inhibition and using the Kd of raclopride derived from saturation analyses. Similarly, in competition experiments versus [3H]spiperone in the absence of sodium ions, the Ki for raclopride implied a lower affinity than suggested from saturation analyses. These differences in estimates of ligand affinity suggest that there is negative cooperativity between the two ligands. In addition, however, raclopride lowered the apparent Bmax of [3H]spiperone in the saturation experiments. None of these observations is predicted by a simple competitive model. The observations are similar to those of Hall et al. (24), who tested a single concentration of raclopride (30 nM) in the absence of sodium ions and found a 40% decrease in the Bmax of [3H]spiperone and a 4-fold decrease in apparent affinity of this radioligand for binding to rat striatal membranes.

When the present experiments were performed in the presence of sodium ions, raclopride decreased the apparent affinity of [3H]spiperone in a manner closer, but not identical, to that predicted from the competitive model. The Bmax of [3H]spiperone was not affected by raclopride in the presence of sodium ions in these studies. Similarly, in competition studies with

![Figure 4](http://www.jbc.org/)

**Fig. 4.** A, the Bmax of [3H]spiperone is unaffected by raclopride in the presence of sodium ions. [3H]Spiperone saturation binding assays were performed in a total volume of 1 ml in the presence of raclopride and 100 mM NaCl as described under “Experimental Procedures.” Data were fitted to a one-binding site model, and Bmax values were determined.

B, Schild plot of raclopride effect on [3H]spiperone affinity in the presence of sodium ions. [3H]spiperone saturation binding assays were performed in a 1-ml volume in the presence of 100 mM NaCl and in the presence of a range of concentrations of raclopride as described under “Experimental Procedures.” Data were fitted to a one-binding site model and the Kd obtained. Data from each of four experiments were transformed according to the method of Schild (44), and the mean was determined to be 19.5 nM (7.71 ± 0.06, pKd ± S.E., n = 14).

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Raclopride inhibition of [3H]spiperone binding. Raclopride/[3H]spiperone competition experiments were performed with 0.22 (■), 0.66 (▲), or 3.46 nM (●) [3H]spiperone as described under “Experimental Procedures.” Specific [3H]spiperone binding was determined as that inhibited by 3 μM (+)-butaclamol. The curves shown are representative curves from single experiments with data points determined in triplicate and are best described by a one-binding site model. The IC50 values from all experiments at all [3H]spiperone concentrations were converted to Ki values by the method of Cheng and Prusoff (49), and the mean was determined to be 19.5 nM (7.71 ± 0.06, pKd ± S.E., n = 14).
Dopamine $D_2$ Receptor Dimers

raclopride versus $[^3H]$spiperone in the presence of sodium ions, the $K_i$ was very similar to the $K_d$ derived from saturation analyses. These data show that sodium ions affect the interaction between $[^3H]$spiperone and raclopride at the $D_2$ dopamine receptor and that the interaction is largely competitive in the presence of sodium ions.

It should be noted that we analyzed some of the data obtained from saturation analyses in the present study using the method of Schild (44). This method is strictly applicable only when there is no change in the $B_{max}$ of the radioligand. It is, therefore suitable for analyses of the effects of haloperidol (without Na$^+$) and raclopride (with Na$^+$) on saturation analyses of $[^3H]$spiperone binding. Saturation analyses of $[^3H]$spiperone binding in the presence of raclopride (no Na$^+$) do show a change in $B_{max}$. The application of this technique in this case does, however, allow us to compare the $pA_2$ value of raclopride, based on its effects on saturation analyses of $[^3H]$spiperone, with the value of log $K_d$ for raclopride determined in saturation analyses.

The data described above, particularly those in the absence of sodium ions, cannot be described by a simple competitive model, so other models must be considered. A model in which the $D_2$ dopamine receptor is able to form a dimeric unit is described under “Appendix,” and simulations of the experiments according to the model have been compared with the data. In this model, two receptor monomers are able to interact, providing two identical ligand binding sites per dimer, which allows the binding of one equivalent of a ligand to affect the binding of a second equivalent of the same ligand or of a different ligand in a cooperative manner. In this model, in the absence of sodium ions, the binding of the first and second equivalents of either $[^3H]$raclopride or $[^3H]$spiperone exert little cooperativity with each other, so both halves of each dimeric receptor are occupied by each radioligand, and the affinities of the first and second equivalents of ligand are similar.

In the absence of sodium ions, the binding of one equivalent of $[^3H]$raclopride to one half of the dimeric receptor exerts a strong negative cooperativity on the binding of the second equivalent such that the affinity of the second site of the dimer for $[^3H]$raclopride is greatly reduced, and little binding at this site is detected for the range of radioligand concentrations used in the present study. The number of sites labeled under the conditions used in Fig. 1 (highest $[^3H]$raclopride concentration $\sim 7$ nM) is then about 60% of that found in the presence of sodium ions. The observed $B_{max}$ of $[^3H]$spiperone is, however, much less affected by the absence of sodium ions, and the total number of sites occupied is similar to that occupied by $[^3H]$raclopride in the presence of sodium ions (i.e. both halves of the dimer are occupied over the range of radioligand concentrations used in the present study). The model suggests that the first and second equivalents of $[^3H]$spiperone exert little cooperativity across the dimer in the absence or presence of sodium ions.

In saturation binding assays with $[^3H]$spiperone in the presence of raclopride and the absence of sodium ions, there is an apparent reduction in the $B_{max}$ of the radioligand, and the concentrations of raclopride required to affect $[^3H]$spiperone binding are higher than predicted from saturation analyses. These observations may be explained in the model if raclopride exerts homotropic negative cooperativity with itself and heterotropic negative cooperativity with $[^3H]$spiperone across the dimer.

The negative cooperativity between raclopride and $[^3H]$spiperone means that for moderate concentrations of $[^3H]$spiperone not all of the binding sites are occupied by $[^3H]$spiperone. This leads to an apparent reduction in $B_{max}$ dependent on the conditions of the experiment (range of radioligand concentrations) and the analysis of the data according to a single saturation. If it were possible to use an extended range of concentrations of radioligand, then all of the sites would be occupied. The combination of homotropic negative cooperativity between two molecules of raclopride and the heterotropic negative cooperativity between raclopride and spiperone leads to the need for higher concentrations of raclopride (than predicted from simple competition) to prevent $[^3H]$spiperone binding. In the presence of sodium ions, the strength of cooperativity is reduced, and the ligands behave more competitively. These effects are also seen where the same experiment is performed but in a standard competition format where a range of concentrations of raclopride is used to inhibit the binding of $[^3H]$spiperone. In the absence of sodium ions, the $K_i$ for raclopride derived from these experiments is higher than the $K_d$ derived from saturation analyses, whereas in the presence of sodium ions the $K_i$ for raclopride is very similar to the $K_d$ derived from saturation analyses.

The simulations of experiments using the model described under “Appendix” are for the most part in good agreement with the data obtained. This indicates that the model (Scheme 1) proposed here provides a first approximation to describing the experimental phenomena described here. An alternative possible model that could explain some of the behavior seen in the present study would be one where raclopride binds to two sets of independent sites of higher and lower affinity in the absence of sodium ions. This might explain some of the experimental observations such as the effects of raclopride on the $B_{max}$ of $[^3H]$spiperone. For such a model, however, if spiperone has equal affinities for the two putative sets of sites, it would be expected that raclopride inhibition of $[^3H]$spiperone binding would be described by inhibition curves with Hill coefficients substantially less than 1. In the present study, raclopride/$[^3H]$spiperone inhibition curves do not exhibit such behavior, and this observation seems to rule out the “two-site” model.

The data described in the present report can, therefore, be approximated in terms of a scheme in which there are two interacting sites in a dimer for the $D_2$ dopamine receptor. Some of the observations reported here have been reported for $D_2$ receptors in the brain (24) so that this phenomenon is not confined to receptors expressed in recombinant cells. The present data complement data obtained on this receptor using protein chemical methods under denaturing conditions, where the existence of dimers was inferred (3). The model may also explain some other phenomena that have been observed for the $D_2$ dopamine receptor such as the observation of two rates of radioligand dissociation (31, 45), the complex pH dependence of the binding of some ligands (46), and the pseudocompetition ofamiloride analogues versus $[^3H]$spiperone binding with Hill coefficients close to 2 (31, 45).

The dimeric model may be an oversimplification, since there are reports that other radioligands (e.g. $[^3H]$nemonapride) are able to label more receptors than $[^3H]$spiperone (19, 21), suggesting that higher order species than dimers may exist. The present data are, however, consistent with homodimers. There are also some similarities between the present observations with the $D_2$ dopamine receptor and the model proposed by Wreggett and Wells (18) for the muscarinic acetylcholine receptor, suggesting that the observations may have some generality.

APPENDIX

A Model Describing the Interaction of Two Competing Ligands with a Dimeric Receptor

In Scheme 1, R is a divalent receptor oligomer with binding sites for competitive ligands A and B for which equilibrium
association constants are $K_a$ and $K_b$, respectively. The allostERIC constants $\alpha$ and $\beta$ govern the effect of the presence of a first equivalent of ligand on the formation of a homo-bi-ligated species for ligands A and B, respectively. The constant $\gamma$ governs formation of the hetero-bi-ligated species.

$$K_a = \frac{[RA]}{[R][A]} \alpha K_a = \frac{[RAB]}{[RB][A]} \gamma K_a = \frac{[RAB]}{[RA][B]}$$

$$K_B = \frac{[RB]}{[R][B]} \beta K_B = \frac{[RBB]}{[RB][B]} \gamma K_B = \frac{[RBB]}{[RA][B]}$$

**SCHEME 1**

Stoichiometrically equivalent species (e.g. monoligated species where the ligand is bound to either half of the dimer) are assumed to be functionally indistinguishable so the equilibrium constant is taken as the same. Association constants describing the scheme above are listed in Scheme 2.

$$2[R]_{total} = 2[R] + 2[RA] + 2[RB] + 2[RAB] + [RAB] + [RBB] \quad \text{(Eq. 1)}$$

$$2[R]_{total} = 2[R][1 + 2K_A[A] + 2K_B[B] + 2\gamma K_A[A][B] + \alpha K_A[A]^2 + \beta K_B[B]^2 \quad \text{(Eq. 2)}$$

The amount of ligand B which is bound is defined as follows.

$$[B]_{bound} = 2[R]_{bound} = 2[R][K_B[B] + \gamma K_A K_B[A][B] + \beta K_B[B]^2 \quad \text{(Eq. 3)}$$

Thus fractional occupancy of ligand binding sites by ligand B is described as follows.

$$\frac{[B]_{bound}}{2[R]_{total}} = \frac{K_B[B] + \gamma K_A K_B[A][B] + \beta K_B[B]^2}{1 + 2K_A[A] + 2K_B[B] + 2\gamma K_A K_B[A][B] + \alpha K_A[A]^2 + \beta K_B[B]^2 \quad \text{(Eq. 5)}$$

Similarly, for ligand A, fractional occupancy of binding sites is described as follows.

$$\frac{[A]_{bound}}{2[R]_{total}} = \frac{K_A[A] + \alpha K_A K_B[A][B] + \beta K_B[B]^2}{1 + 2K_A[A] + 2K_B[B] + 2\gamma K_A K_B[A][B] + \alpha K_A[A]^2 + \beta K_B[B]^2 \quad \text{(Eq. 6)}$$

Equations 5 and 6 were then used to simulate the experimental data reported above. It was important that the model described three experimental observations presented above: first, the reduction in $[\text{H}]$raclopride $B_{max}$ in the absence of sodium ions; second, the reduction of $[\text{H}]$spiperone $B_{max}$ and affinity by raclopride; and third, full competition for $[\text{H}]$spiperone binding by raclopride in competition experiments. Values of the parameters $\alpha$, $\beta$, and $\gamma$ (0.015, 1, and 0.2, respectively) were found that when used in the model provided simulations that described each of these experimental observations well.

First, considering the change in the $B_{max}$ for $[\text{H}]$raclopride in the presence and absence of sodium ions, $[\text{H}]$raclopride was represented by ligand A, and it was assumed that the apparent differential binding capacities for the radioligand were due to the occupancy of approximately one-half of the sites in the dimer in the absence of sodium ions and both sites in the presence of sodium ions for the range of radioligand concentrations used in the experiments described above. In the presence of sodium ions, where the radioligand occupies both sites with a single apparent affinity, the microscopic association constant ($K_a$) can be equated to the reciprocal of the macroscopic dissociation constant $K_d$ (0.23 nM, Table I). This conclusion may be derived using Equation 6 in the absence of ligand B and with $\alpha = 1$.

In the absence of sodium ions, the microscopic and macroscopic dissociation constants ($K_{micro}$ and $K_{macro}$, respectively) for occupancy of the $i$th site on a protein with $n$ sites (47, 48) are given by Equation 7.

$$K_{micro} = K_{macro}((n + 1 - i)/i) \quad \text{(Eq. 7)}$$

For the first site of a dimer and substituting $K_{macro}$ as the dissociation constant for the radioligand in a ligand binding assay ($K_d$ values in Table I) and $K_{micro}$ as $1/K_A$ in Equation 5 or 6, then $K_d = 2K_A$. Hence, under these conditions $K_A = 4.17 \times 10^6 \text{M}^{-1}$ in the presence of sodium ions or NMDG.

Fig. 6 shows simulated $[\text{H}]$raclopride saturation data together with the actual data points as in Fig. 1. The simulations shown have been generated with no competing ligand and with values of $\beta = 1$, $\gamma = 0.2$, and $\alpha$ set to either 1 or 0.015. When $\alpha = 1$ (with Na+), the radioligand labels both sites of the dimer, and the maximum number of binding sites are labeled with a single affinity. When $\alpha = 0.015$ (without Na+), the radioligand will label both sites of the dimer at high concentrations, but there is some flattening of the saturation curve so that if the range of $[\text{H}]$raclopride concentrations is limited to that used in Figs. 1 and 6 then it appears as if only about half of the sites are being labeled.

Sodium ions had little effect on the $B_{max}$ of $[\text{H}]$spiperone, which can be represented in the model by ligand B. If $K_B$ is taken to be the reciprocal of the $K_d$ of $[\text{H}]$spiperone and the allosteric constant, $\beta$, assumed to be 1, then $[\text{H}]$spiperone labels the maximum number of binding sites in a single saturation (Fig. 2).

The second consideration is that in the absence of sodium ions, inclusion of 10 $\mu$M raclopride in $[\text{H}]$spiperone saturation experiments reduced the experimentally measured $B_{max}$ to approximately three-quarters of that determined in the absence of competing ligand (Fig. 2) and increased the apparent $K_d$ to $\approx 3.5 \text{nM}$. Intermediate concentrations of raclopride exerted smaller effects. These experimental data can be simulated in the model (Fig. 7) by taking $K_A$ as $1/2K_d$ for $[\text{H}]$spiperone and $K_B$ as $1/2K_d$ for $[\text{H}]$spiperone as outlined above and values of $\alpha = 0.015$, $\beta = 1$, $\gamma = 0.2$, and $[A] = 1$, 3, or 10 $\mu$M. If very high concentrations of $[\text{H}]$spiperone are used, then both sites of the dimer will be occupied, although the presence of raclopride leads to a flattening of the saturation curve. If the range of concentrations of $[\text{H}]$spiperone (ligand B) is limited to a maximum of 10 nM as used in the experiments described in Fig. 2, then the presence of raclopride leads to an apparent reduction in the number of sites labeled by $[\text{H}]$spiperone.

In the presence of sodium ions, the highest concentration of raclopride tested, 36 nM, had no significant effect on the exper-
Dopamine D₂ Receptor Dimers

The examples above indicate that the model is capable of describing the interactions of spiperone and raclopride reported above and that D₂ dopamine receptors may therefore exist as dimers. It is notable that the model presented here is essentially an abbreviation of the tetrameric receptor model described by Wreggett and Wells (18), in which radioligand binding to the M₂ muscarinic acetylcholine receptor was found to be consistent with the receptor forming tetrameric oligomers. The radioligand [³H]QNB labeled 1.5–2-fold more sites than [³H]NMS or [³H]AFDX. The data from experiments

![Graph 1](image1.png)

**Fig. 6.** Comparison of simulated and actual raclopride saturation analysis of a dimeric receptor. Equation 6 was used to simulate data representing the occupancy of a hypothetical receptor dimer by ligand A (raclopride) over a range of concentrations from 1 fM to 10 nM, and the simulated curves are shown together with the real data (from Fig. 1) in the presence of Na⁺ (▲) or NMDG (▼) or in the absence of either ion (●). In the simulations, the following parameter values were used: ▲, $K_A = 4.35 \times 10^5 \text{M}^{-1}$ (the reciprocal of the $K_d$ for [³H]raclopride determined in the presence of Na⁺), $\alpha = 1$, $[B] = 0$. ▼, $K_A = 4.55 \times 10^5 \text{M}^{-1}$ (the reciprocal of the $K_d$ for [³H]raclopride determined in the absence of Na⁺ or NMDG), $\alpha = 0.015$, $[B] = 0$.

![Graph 2](image2.png)

**Fig. 7.** Comparison of simulated and actual spiperone saturation analysis of a dimeric receptor in the presence and absence of raclopride. Equation 5 was used to simulate data representing the occupancy of a hypothetical receptor dimer by ligand B (spiperone) over a range of concentrations from 1 fM to 10 nM in the presence or absence of ligand A (raclopride). The simulated curves are shown together with the real data (taken from Fig. 2A). In the simulations, the following parameter values were used: $K_A = 4.55 \times 10^5 \text{M}^{-1}$ (the reciprocal of the $K_d$ for [³H]raclopride determined in the absence of sodium ions), $K_B = 6.67 \times 10^{10} \text{M}^{-1}$ (1/$K_d$ for [³H]spiperone determined in the absence of sodium ions), $\alpha = 0.015$, $\beta = 1$, $\gamma = 0.2$, and $[A] = 0$ (▲), 1 μM (▽), 3 μM (●), and 10 μM (●).

![Graph 3](image3.png)

**Fig. 8.** Comparison of simulated and actual data for inhibition of spiperone binding by raclopride at a dimeric receptor. Equation 5 was used to simulate data representing the inhibition of a fixed concentration of ligand B (spiperone) by a range of concentrations of ligand A (raclopride) between 1 pM and 10 nM. The simulated curves are shown together with the real data (taken from Fig. 5). In the simulations the following parameter values were used: $K_A = 4.55 \times 10^5 \text{M}^{-1}$ (the reciprocal of the $K_d$ for [³H]raclopride determined in the absence of sodium ions), $K_B = 6.67 \times 10^{10} \text{M}^{-1}$ (1/$K_d$ for [³H]spiperone determined in the absence of sodium ions), $\alpha = 0.015$, $\beta = 1$, $\gamma = 0.2$, and $[B] = 0.22 \text{nM}$ (▲), 0.66 nM (▽), and 3.46 nM (●).
with [3H]AFDX could be described by a dimeric model, while data with [3H]NMS required at least a trimer, but data with all three radioligands required the assumption of at least a tetrameric arrangement. It is possible then that the D2 dopamine receptor could exist as higher order oligomers, but a dimer is sufficient to describe the data presented here. Use of other radioligands and immunological techniques would be required to demonstrate the existence of dopamine receptor oligomers.

REFERENCES
