Dopamine Displaces $[^3H]$Domperidone From High-Affinity Sites of the Dopamine D2 Receptor, But Not $[^3H]$Raclopride or $[^3H]$Spiperone in Isotonic Medium: Implications for Human Positron Emission Tomography

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ABSTRACT Because the high-affinity state of the dopamine D2 receptor, D2High, is the functional state of the receptor, has a role in demarcating typical from atypical antipsychotics, and is markedly elevated in amphetamine-sensitized rats, it is important to have a method for the convenient detection of this state by a ligand. The present data show that, in contrast to $[^3H]$spiperone or $[^3H]$raclopride, $[^3H]$domperidone labels D2High sites in the presence of isotonic NaCl in either striatum or cloned D2Long receptors, yielding a dopamine dissociation constant (1.75 nM) in agreement with that found with $[^3H]$dopamine. Increased labeling of D2High sites occurred with $[^3H]$domperidone after severe disruption of the cells, suggesting that $[^3H]$domperidone has better access to the D2 receptor from the cytoplasmic aspect of the cell membrane. The density of the $[^3H]$domperidone-labeled D2 receptors was the same as that of the $[^3H]$raclopride-labeled D2 receptors, but twice the density of $[^3H]$spiperone sites for human cloned D2Long receptors, compatible with the monomer-dimer concept of the D2 receptor. $[^3H]$domperidone readily labels the D2High sites in postmortem human brain homogenates. Although $[^3H]$spiperone or $[^3H]$raclopride can occupy D2High sites, the inability of 1–10 nM dopamine to displace these ligands under isotonic conditions suggests that these ligands may not be suitable for monitoring the physiological high-affinity state of the dopamine D2 receptor by means of $[^11C]$methylspiperone or $[^11C]$raclopride in humans. Synapse 49:209–215, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

The dopamine D2 receptor exists in two states. The high-affinity state of the D2 receptor is sensitive to dopamine concentrations in the 1–10 nM range (although values of up to 100 nM have been reported). The low-affinity state of the D2 receptor is sensitive to high dopamine concentrations between 100–2,000 nM (Hamblyn et al., 1984; Watanabe et al., 1985; Grigoriadis and Seeman, 1986).

Although the high-affinity state of the dopamine D2 receptor (or D2High) is the functional state of the receptor in the anterior pituitary (George et al., 1985), D2High has not been widely studied in nervous tissue. This is because dopamine competition with the commonly used ligands ($[^3H]$spiperone or $[^3H]$raclopride) on homogenized tissues reveal that D2 receptors are predominantly (~85–90% of the total population) in the low-affinity state (Seeman et al., 1985; Huff and Molinoff, 1982). However, using striatal slices and $[^3H]$spiperone, Richfield et al. (1986) found that 90% of...
the D2-labeled sites were in the high-affinity state, with a dopamine dissociation constant of 8.2 nM.

The clinical relevance of D2High in the nervous system has recently become apparent in several different types of studies. First, the striata from rats sensitized to amphetamine had a normal density of D2 receptors, but revealed a 360% increase in the density of the D2High sites, compatible with a marked supersensitivity to dopamine-mimetics (Seeman et al., 2002).

Second, the absolute value of the dopamine dissociation constant at D2High, 1.75 nM, separates antipsy- chotic drugs into traditional ones (which typically elicit parkinsonism) and atypical ones (which elicit little or no parkinsonism) (Seeman, 2002). While this value of 1.75 nM was obtained on human cloned D2 receptors, the dopamine dissociation constant reported in the literature for D2High ranges from 2 nM (using [3H]raco- lopride; Malmberg et al., 1998) to 4.5–229 nM (using [3H]spiperone; Grünewald et al., 1996; Chio et al., 1994; Wiens et al., 1998; Payne et al., 2002) to 22–56 nM (using [125I]iodosulpride; Sokoloff et al., 1992; Perachon et al., 1999).

Third, although D2High is the physiologically functional state, it has not been possible to measure the density or proportion of D2High states by either [11C]raclopride or [11C]methylspiperone by means of positron emission tomography in humans (Aalto et al., 2002).

One of the reasons for different dopamine dissociation constants at D2High is that the competition curves between dopamine and [3H]spiperone, [3H]raclopride, or [125I]iodosulpride do not reveal a clear, unambiguous high-affinity component which is distinct or separate from the low-affinity component. That is, the high- and low-affinity components blend into one another and one must use computer-assisted separation of the components, with attendant assumptions and limitations, to determine the dopamine dissociation constant at D2High (Watanabe et al., 1985). Another reason is that the dissociation constant can depend on the ligand used and on the final concentration of NaCl, despite all the appropriate corrections (Watanabe et al., 1985; Seeman and Van Tol, 1995).

In addition to the difficulty in determining the true dopamine dissociation constant at D2High, there is lack of agreement on the density of dopamine D2 receptors as labeled by the various dopaminergic ligands. In particular, the density of [3H]spiperone-labeled D2 sites has been reported as equal to the density of [3H]domperidone-labeled D2 sites (Huff and Molinoff, 1982; Lazareno and Nahorski, 1982) or higher than the density of [3H]domperidone-labeled D2 sites (Hall and Wedel, 1986).

Therefore, in order to obtain a reliable method for measuring the dopamine dissociation constant at D2High and to compare the densities of the various ligands on cloned human D2 receptors, we compared the binding of [3H]spiperone, [3H]raclopride, and [3H]domperidone (Martres et al., 1978; Baudry et al., 1979; Grigoriadis and Seeman, 1986) to cloned dopamine D2 receptors.

MATERIALS AND METHODS

Frozen rat brains were purchased (Pel-Freez Biologicals, Rogers, AR) and stored at ~70° until used. The brain was partly thawed and the striata removed. The striata were homogenized in buffer (4 mg frozen tissue per ml buffer) using a Teflon-glass homogenizer with the piston rotating at 500 rpm and 10 up-and-down strokes of the glass container. The buffer contained 50 mM Tris-HCl (pH 7.4 at 20°), 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl2, 4 mM MgCl2, and either 10 mM or 120 mM NaCl (see Results). The homogenate was not washed, centrifuged, or preincubated because previous work found that more than 30% of the D2 receptors were lost by these procedures (Seeman et al., 1984).

Postmortem human frozen tissues were prepared in the same manner as that for the rat tissues. The frozen postmortem human striata were obtained from the National Neurological Research Specimen Bank (Los Angeles, CA). The diagnosis of schizophrenia was taken from the case notes as recorded by the physicians in charge. The frozen human tissues were dissected free of gross myelin tracts, homogenized, and further processed as described above for the rat striata.

We used the human cloned dopamine D2Long receptor (in Chinese hamster ovary cells; Liu et al., 2000). The cells were harvested by gently scraping the cells off the bottom of the Petri dish, centrifuged, resuspended in phosphate-buffered saline (0.9% NaCl), recentrifuged, and the pellet frozen at ~70°. When used, the frozen pellet was thawed and the cells suspended at 200 μg protein/ml. The suspension was homogenized for 5 sec (Polytron, setting 5), without any further washing or centrifugation.

[3H]Raclopride (60–80 Ci/mmol; final concentration of 2 nM in the incubation tube), [3H]spiperone (101 Ci/mmole; final concentration of 250 pM), were purchased from PerkinElmer Life Sciences (Boston, MA). [3H]Domperidone was custom-synthesized as [phenyl-3H(N)]domperidone (42 Ci/mmol) by PerkinElmer Life Sciences and used at a final concentration of 1.2 nM.

The competition between a drug and a [3H]ligand for binding at the receptors was done as follows. Each incubation tube (12 × 75 mm, glass) received, in the following order, 0.5 ml. buffer, 0.25 ml [3H]ligand, and 0.25 ml of tissue homogenate. The tubes, containing a total volume of 1 ml, were incubated for 2 h at room temperature (20°C), after which the incubates were filtered using a 12-well cell harvester (Titertek, Skatron, Lier, Norway) and buffer-presoaked glass fiber filter mats (No. 7034, Skatron, Sterling, VA). After filtering the incubate the filter mat was rinsed with buffer for 15 sec (7.5 ml buffer). The filters were pushed
out and placed in scintillation minivials (Packard Instruments, Chicago, IL). The minivials received 4 ml each of scintillant (Ready Solve, Beckman, Palo Alto, CA) and were monitored 6 h later for tritium in a Packard 4660 scintillation spectrometer at 55% efficiency. The competition data were analyzed as previously described (Seeman et al., 1985); the program provided two statistical criteria to judge whether a two-site fit was better than a one-site fit, or whether a three-site fit was better than a two-site fit.

RESULTS

Dopamine dissociation constant at D2High in striatum

In order to determine the dopamine dissociation constant at D2High, it was necessary to have a procedure which revealed a substantial number of receptors to be in the high-affinity state. Isotonic levels of NaCl (100–120 mM) induce D2High receptors into their D2Low state, especially at 37°C (Watanabe et al., 1985), obscuring the dissociation constant of dopamine at D2High and increasing the error in the computer-assisted resolution of the dissociation constant at the high-affinity state. In fact, earlier studies deliberately omitted NaCl in order to determine the affinities of dopamine agonists at the high- and low-affinity states of D2 (Seeman et al., 1985). However, because the binding of antipsychotic benzamides (raclopride, sulpiride) requires sodium ions (Seeman et al., 1985; Jarvie et al., 1987), the present study used a low concentration of NaCl (10 mM) to permit a clear demarcation of the D2High state. This is shown in Figure 1, where [3H]spiperone and [3H]raclopride, although revealing less than 4% of the D2 receptors in the high-affinity state in the presence of 120 mM NaCl, detected 30 ± 5% (SE, n = 10) of the receptors in the high-affinity state in the presence of 10 mM NaCl. The addition of 200 µM guanilylimidodiphosphate (Gpp[NH]p) eliminated the high-affinity component, but had no effect on the low-affinity site (data not shown).

Dopamine dissociation constant at D2High for cloned D2 receptors

While the proportion of D2High sites was less than 4% (0–4%, n = 8) in the rat striatum, using [3H]spiperone or [3H]raclopride (Fig. 1), the proportion of D2High sites was hardly detectable with these ligands when using human cloned D2 receptors in isotonic NaCl (Fig. 2A,B). [3H]Domperidone, however, in contrast to [3H]spiperone or [3H]raclopride, readily revealed 40 ± 5% (SE, n = 8) of the D2 receptors to be in the high-affinity state in isotonic NaCl (Fig. 2C) or in hypotonic NaCl (data not shown). In a series of such dopamine/[3H]domperidone experiments, the dopamine dissociation constant, Ki, was 1.75 ± 0.2 nM (SE, n = 8). This agreed with a direct determination of 1.5 ± 0.2 nM (SE, n = 6) for the dopamine dissociation constant, Kd, using [3H]dopamine on the cloned D2 receptor (Fig. 3). The observation that the Kd for dopamine is lower than the Ki for dopamine is consistent with earlier work indicating that the apparent dissociation constant, Ki, of a compound varies in practice with the affinity of the competing radioligand; in general, for any particular drug it has been repeatedly noted in the literature that the Kd is consistently lower than the Ki (Seeman and Van Tol, 1995).

Characteristics of [3H]domperidone binding to D2 receptors

Using a range of [3H]domperidone concentrations (0.08–4.5 nM), the Kd value was 0.42 ± 0.05 nM (SE, n = 4) on the cloned D2 receptor and 0.47 ± 0.05 nM (SE, n = 3) on the rat striatal homogenate, in agree-
ment with earlier values (Grigoriadis and Seeman, 1986).

Using the same homogenate of cloned D2 receptors in CHO cells, the density of D2 receptors for \([3H]\)domperidone was 50 fmol/filter, compared to 57 fmol/filter for \([3H]\)raclopride and 22.6 fmol/filter for \([3H]\)spiperone, using 50 nM 5262 g protein per filter.

The inhibition constants of antipsychotics, when competed vs. \([3H]\)domperidone on cloned D2 receptors, were similar to those found when competing vs. \([3H]\)raclopride (Seeman, 2002), as shown in Table I. These data indicate that the nature of the dopamine D2 receptors labeled by \([3H]\)domperidone is similar to that labeled by \([3H]\)raclopride, except that \([3H]\)domperidone is far more sensitive than \([3H]\)raclopride to being displaced by dopamine.

**Role of Na\(^+\) ions for D2High**

Thus, raising the NaCl concentration from 10 to 120 mM prevented the ready detection of D2High by dopamine/\([3H]\)spiperone or dopamine/\([3H]\)raclopride competition experiments (Figs. 1, 2). In order to see whether raising the NaCl could also eliminate the detection of D2High by \([3H]\)domperidone, dopamine/\([3H]\)domperidone competition experiments were done in 200 mM NaCl. Figure 4 shows that 200 mM NaCl either abolished the D2High state or masked the labeling of D2High by \([3H]\)domperidone. These data suggest that sodium ions may either alter the conformation of the dopamine D2 differentially altering the binding of the three ligands, or that the three ligands may have different potencies in competing with sodium ions for binding to the D2 receptor, as discussed in Jarvie et al. (1987).

**Access of D2High to \([3H]\)domperidone**

Because \([3H]\)domperidone is a positively charged molecule at biological pH, it has low permeability across biological membranes. Therefore, the effect of membrane disruption was tested. We found that the highest proportion of D2High sites were detected by \([3H]\)domperidone when the thawed CHO cells were

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**TABLE I. Antipsychotic Ki dissociation constants**

\((\text{D2Long in CHO cells})\)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Using ([3H])domperidone</th>
<th>Using ([3H])raclopride</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>((0.9 \text{nM; } K_d = 0.41 \text{nM})) ((\text{average of } n = 2))</td>
<td>((1.5 \text{nM; } K_d = 1.7 \text{nM})) ((\text{average of } n = 3-25))</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>101</td>
<td>122</td>
</tr>
<tr>
<td>Clozapine</td>
<td>58</td>
<td>62</td>
</tr>
<tr>
<td>Amoxapine</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Loxapine</td>
<td>10</td>
<td>9.6</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>6.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Prochlorperazine</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Pimozide</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Raclopride</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Risperidone</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>0.8</td>
<td>0.99</td>
</tr>
<tr>
<td>Spiperone</td>
<td>0.07</td>
<td>0.04</td>
</tr>
</tbody>
</table>
broken by Polytron homogenization (setting 5; 5 sec), compared to just vortexing the cells for 5 sec (Fig. 5).

**D2High in human striatum**

The present use of [$^3$H]domperidone to detect D2High in rat striatum or cloned D2 receptors in CHO cells in isotonic NaCl is readily applicable to human tissues. For example, a series of human control striata (putamen) revealed that D2High was $35 \pm 5\%$ (SE, n = 5) of the D2 population, while that for schizophrenia tissues was $19 \pm 5\%$ (SE, n = 3), as illustrated in the examples in Figure 6. However, because of the large number of uncontrolled premortem variables in such postmortem human tissues, these results only serve to report that a more extensive set of such experiments to measure D2High is feasible. Such experiments should also be done in hypotonic medium.

**DISCUSSION**

The main finding is that, in contrast to [$^3$H]spiperone or [$^3$H]raclopride, [$^3$H]domperidone labels D2High sites in the presence of isotonic NaCl (Figs. 1, 2, 4). Second, dopamine/[$^3$H]domperidone competition experiments yielded a dopamine dissociation constant, $K_d$, of 1.75 $\pm$ 0.2 nM, in reasonable agreement with the dissociation constant, $K_d$, found directly with [$^3$H]dopamine for D2High on cloned D2 receptors (Fig. 3).

These two findings, therefore, justify using [$^3$H]domperidone to measure D2High sites in human postmortem tissue, as exemplified in Figure 6. In brain tissues, however, the endogenous dopamine, especially in nonwashed brain tissues, can be expected to occupy a proportion of D2High sites and could thereby alter the dopamine dissociation constant detected by [$^3$H]domperidone. For example, in the case of postmortem schizophrenia tissues (Fig. 6), the endogenous dopamine can be higher than normal, thus reducing the apparent density of the D2High sites. Furthermore, endogenous dopamine could account for the high $K_i$ value of 8.2 nM dopamine found for the high-affinity component of dopamine D2 receptors labeled by [$^3$H]spiperone in brain slices (Richfield et al., 1986).

The increased labeling of D2High sites by [$^3$H]domperidone after severe disruption of the cells (Fig. 5)
suggests that [3H]domperidone may actually have better access to the D2 receptor from the cytoplasmic aspect of the cell membrane. Although it is known that the aspartic acid binding site on the dopamine D2 receptor is in the middle of the hydrophobic transmembrane region, the mode of access of the antipsychotic to this site has not yet been elucidated.

Using the same homogenate of cloned D2 receptors in CHO cells, the density of D2 receptors for [3H]domperidone was 1 fmol/µg protein, compared to 1.1 fmol/µg protein for [3H]raclopride and 0.45 fmol/µg protein for [3H]spiperone. These values are consistent with previous data showing that the density of [3H]benzamides sites (i.e., [3H]nemonapride) was twice the density of [3H]spiperone sites for human cloned D2Long receptors (Seeman et al., 1992), and compatible with the idea that butyrophenones label the monomer of D2, while benzamides label the D2 dimer (Zawarynski et al., 1998).

Equally important, the density of the [3H]domperidone-labeled D2 receptors was essentially the same as that of the [3H]raclopride-labeled D2 receptors, indicating that the higher density of D2High sites detected by [3H]domperidone cannot be attributed to a higher density of D2 receptors labeled by [3H]raclopride.

The present data only hold for the labeling of dopamine D2High receptors by [3H]domperidone in vitro, because [3H]domperidone does not permeate the blood–brain barrier in vivo.

The present data are relevant to physiological processes in the dopamine system examined by means of human PET. Although [11C]raclopride is commonly used to measure the release of endogenous dopamine in the brain (Volkow et al., 1994; Wong et al., 1997; Breier et al., 1999; Laruelle, 2000; Kapur, 2003), this displacement of [11C]raclopride occurs at high concentrations of dopamine exceeding 100 nM (Seeman et al., 1989). This high concentration of 100 nM dopamine primarily corresponds to dopamine inhibiting the binding of [11C]raclopride to the low-affinity state of the dopamine D2 receptors and is associated with the internalization of dopamine D2 receptors into the postsynaptic neurons (Ko et al., 2002). This action of 100 nM dopamine (or higher) does not reflect the binding of [11C]raclopride to the high-affinity states of D2 or the physiological action of dopamine, which occurs between 1.6–10 nM dopamine (George et al., 1985; Kawagoe et al., 1992; Park and Park, 2000). For example, it is possible that the density of D2 high-affinity sites may well be an important aspect in causing psychotic symptoms, as suggested by the work on amphetamine-induced sensitization, a model for psychosis (Seeman et al., 2002). Therefore, in order to measure these D2 high-affinity sites in psychotic patients by means of PET, it may be useful to design ligands other than [11C]raclopride and [11C]spiperone (see also Aalto et al., 2002), because these ligands are insensitive to 1–10 nM dopamine in isotonic medium.

Furthermore, although [3H]domperidone detected 40% of the dopamine D2 receptors to be in the high-affinity state in homogenized striata, an even higher proportion of 90% for the high-affinity component of the D2 receptors occurs in brain slices (Richfield et al., 1986). It is possible that factors which promote the high-affinity state have been disrupted or removed in the homogenized tissue. Considering that the high-affinity state of the dopamine D2 receptor is the functional physiological state (George et al., 1985), and considering that the proportion of the high-affinity component is higher in the more intact tissue slice, it is possible or likely that more than 90% of the D2 receptors in vivo are in the functional high-affinity state. These considerations provide further justification for using radioligands which are more reflective of the D2 high-affinity state in health and disease.

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