Agonist and Inverse Agonist Activity at the Dopamine D₃ Receptor Measured by Guanosine 5’-[γ-Thio]Triphosphate-[³⁵S] Binding

ÅSA MALMBERG, ÅSA MIKAELS and NINA MOHELL

Department of Molecular Pharmacology, Preclinical R & D, Astra Arcus AB, S-151 85 Södertälje, Sweden

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ABSTRACT

In this study, the ligand-receptor-G protein interactions of the dopamine D₃ receptor expressed in Chinese hamster ovary cells were investigated using guanosine 5’-[γ-thio]triphosphate-[³⁵S] (³⁵S)GTPγS and receptor binding experiments. Dopamine stimulated the [³⁵S]GTPγS binding in a guanine nucleotide, magnesium and sodium-dependent manner. Dopamine and quinpirole produced maximal stimulation of the [³⁵S]GTPγS binding whereas (+)-7-OH-DPAT and (-)-3-PPP were partial agonists. Interestingly, several compounds previously classified as D₂ receptor antagonists behaved as inverse agonists at the D₃ receptor, i.e., they inhibited the basal [³⁵S]GTPγS binding in a dose dependent fashion. Haloperidol, (+)-UH-232, (+)-AJ-76 and raclopride were full inverse agonists but clozapine was a partial inverse agonist. Pertussis toxin treatment abolished the D₃ receptor-mediated agonist as well as inverse agonist responses, indicating the involvement of G/Gₙ proteins in both processes. According to the ternary complex model, agonists should bind with higher affinity to the G protein coupled receptor (RG) and thereby shift the equilibrium from free receptor (R) toward RG, which produces a biological response. However, an inverse agonist should bind with higher affinity to R than to RG and thereby inhibit the basal activity of the cell. We found that the high affinity agonist binding site (RG) was abolished by pertussis toxin treatment of the cells. However, the inverse agonists bound with the same affinity to untreated and pertussis toxin treated D₃ receptor membranes. Thus, we found no evidence for the hypothesis that inverse agonists would shift the equilibrium from RG toward R by binding with higher affinity to R than to RG.

Dopamine is known to play a central role in the central nervous system neurotransmission. Dysfunctions of the dopaminergic pathways are implicated with several disorders in the brain including Parkinson’s disease and schizophrenia. Parkinson’s disease involves degeneration of the nigrostriatal dopaminergic pathway, whereas schizophrenia has been suggested to be due to an elevated dopaminergic activity in the limbic areas of the brain (Seeman, 1987; Schwartz et al., 1993). Various neuroleptics are believed to elicit their antipsychotic effect through the blockade of the D₂ receptors in the limbic system, although the blockade of the same receptors in the basal ganglia is thought to be associated with the so-called extrapyramidal side effects, e.g., rigidity, tremor, dyskinesia and akathisia (Reynolds, 1992; Creese, 1983). The dopamine hypothesis of schizophrenia is supported by the very good correlation between the clinical potency of various neuroleptics and their affinity for the D₂ receptors (Seeman, 1987; Seeman, 1992). However, many neuroleptics display similar affinities for the new “D₂-like receptors” i.e., D₃ and D₄ receptors, which implies that they may mediate some of the antipsychotic effects. Although the novel D₂-like receptors are much less abundant (about 1% of the density of D₂ receptors) their restricted distribution to the limbic brain areas makes them interesting as potential targets for new antipsychotic drugs with less side effects (Schwartz et al., 1993).

The D₃ receptor has been shown to couple to several signaling pathways including cyclic AMP production, calcium currents, rate of acidification, mitogenesis and c-fos expression (Lajiness et al., 1995; Seabrook et al., 1994; Sautel et al., 1995; Potenza et al., 1994; Freedman et al., 1994; Chio et al., 1994). However, relatively weak intracellular responses have been observed as compared to the corresponding D₂ receptor-mediated effects. Furthermore, the agonist binding to the D₃ receptor seems to be less sensitive to guanine nucleotides, which may indicate a less efficient coupling of the D₃ receptor to the G proteins (Sokoloff et al., 1990; Sokoloff et al., 1992; Chio et al., 1994; Castro and Strange, 1993).

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; [³⁵S]GTPγS, guanosine 5’-[γ-thio]triphosphate-[³⁵S]; (-)-3-PPP, (-)-(S)-3-(3-hydroxyphenyl)-N-propylpiperidine; (+)-7-OH-DPAT, (+)-(R)-7-hydroxy-2-(dipropylamino)tetralin; (+)-UH-232, (+)-(1S,2R)-5-methoxy-1-methyl-2-(dipropylamino)tetralin; (+)-AJ-76, (+)-(1S,2R)-5-methoxy-1-methyl-2-(propylamino)tetralin; CHO, Chinese hamster ovary.
Receptor-mediated activation of G proteins affects the GDP/GTP exchange and involves a high affinity binding of GTP to the α subunit of the G protein. GTPYs is a non-hydrolyzable analogue of GTP and it binds to all types of G proteins with high affinity (Wieland and Jakobs, 1994). In this study the binding characteristics of [35S]GTPYs were studied in order to investigate the first step in the D3 receptor-mediated signaling pathway.

Methods

Materials. Mouse fibroblast (Ltk−) cells expressing the human D3A (long isoform, 443 amino acids) receptor were obtained from Dr. O. Civei (Vollum Institute, Portland, OR). CHO cells expressing the human D3 receptor were purchased from Institut National de la Santé et de la Recherche Médicale Institute (Paris, France). All tissue culture reagents were purchased from GIBCO Ltd. (Paisley, Scotland, UK). [3H]Raclopride (specific activity, 74-81 Ci/mmol), [3H]quinuclidine (specific activity 40 Ci/mmol) and [35S]GTPYs (specific activity 1000-1274 Ci/mmol) were obtained from Du Pont NEN (Du Pont New England Nuclear, Boston, MA). Pertussis toxin, (+)-butaclamol and (−)-(S)-3-(3-hydroxyphenyl)-N-propylpiperidine ((−)-3-PPP) were purchased from Research Biochemicals International (Natick, MA). Dopamine, haloperidol and quinpirole were obtained from Sigma Chemical Co. (St. Louis, MO). Raclopride was synthesized at the Department of Chemistry (Astra Arcus AB, Södertälje, Sweden). (+)-7-hydroxy-2-(dipropylamino)tetralin ((+)-7-OH-DPAT), (−)-1S,2R-5-methoxy-1-methyl-2-(dipropylamino)tetralin ((−)-Uh-232) and (±)-1S,2R-5-methoxy-1-methyl-2-(propylamin)-tetralin ((±)-Aj-76) were gifts from Dr. A. M. Johansson (Department of Organic Pharmaceutical Chemistry, Uppsala University, Uppsala, Sweden).

Membrane preparations. The cells expressing cloned human dopamine receptors were grown and the membranes prepared as described previously (Malmberg et al., 1993). The membranes were stored in aliquots at −70°C. Pertussis toxin treatments were performed 18 hr before harvesting by addition of 100 ng pertussis toxin per ml growth medium. Untreated cells were grown in parallel. On the day of the experiment, the frozen cell membranes were thawed, homogenized with an Ultra-Turrax, and suspended in appropriate binding buffer.

[35S]GTPYs binding. The [35S]GTPYs binding assays were performed as described previously by (Lazareno et al., 1993), with some modifications. The [35S]GTPYs binding assays were carried out in triplicates in a total volume of 0.5 ml. The assay buffer contained 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, pH 7.6 and 1 µM GDP. This composition of the buffer was chosen based on the experiments described below. Omission of sodium ions (100 mM N-methyl-D-glucamine) was added to retain ionic strength led to an increased basal [35S]GTPYs binding but a decreased dopamine-induced stimulation and haloperidol-induced inhibition of the [35S]GTPYs binding. Omission of magnesium ions (2 mM EDTA) were added to bind any remaining magnesium ions) dramatically decreased the basal [35S]GTPYs binding and dopamine and haloperidol were without effect (both in the presence and absence of sodium ions).

Addition of GDP keeps the G protein in a GDP-ligated form which is necessary for the measurement of dopamine- and haloperidol-induced responses. The basal [35S]GTPYs binding was significantly reduced by GDP (0.1, 1, 10 µM). Maximal effect of dopamine and haloperidol on [35S]GTPYs binding was found at 0.1 to 1 µM GDP for both D3 and D3A receptors. A GDP concentration of 1 µM gave the best signal to noise ratio. The [35S]GTPYs binding was shown to increase with increasing protein concentrations in a linear manner. A protein concentration of 20 to 35 µg/tube was used.

To assure equilibrium before the addition of radiolabel, membranes, GDP and appropriate drugs were preincubated for 30 min at 30°C. [35S]GTPYs (80-150 pM) was added and the incubation was continued for 30 min at 30°C. The incubation time was based on preliminary experiments which showed that agonist stimulated [35S]GTPYs binding was linear up to 20 to 30 min at 30°C. The reaction was terminated by rapid filtration through Whatman GF/B filters and subsequent washing with cold buffer (50 mM Tris-HCl, 5 mM MgCl₂, 1 mM EDTA and 120 mM NaCl, pH 7.4, at 22°C) using a Brandel cell harvester. The radioactivity was determined in a Packard 2200TR liquid scintillation counter at about 100% efficiency.

[3H]Raclopride binding. The binding assays were carried out in duplicate at 30°C for 60 min. The assay buffer contained 50 mM Tris-HCl, 5 mM KCl, 4 mM MgCl₂, 1 mM EDTA and 120 mM NaCl, pH 7.6, if not otherwise stated. The membranes were suspended in binding buffer to a final concentration of 80 to 90 µg protein/ml for untreated and 40 to 50 µg protein/ml for pertussis toxin treated D3A-Ltk− cells and 20 µg/ml for untreated and 80 µg/ml for pertussis toxin treated D3-CHO cells (the receptor concentration was 80-100 pM). The total incubation volume was 0.5 ml except for the competition studies in the absence of sodium where the volume was 1 ml. In competition experiments 2 nM (in the presence of sodium) or 4 nM (in the absence of sodium) of [3H]raclopride was incubated with 10 to 12 concentrations (2 points/log unit) of the competing ligand. All ligands were dissolved in ascorbic acid (final concentration 0.1%). Nonspecific binding was defined with 1 µM (+)-butaclamol. The incubations were terminated by rapid filtration through Whatman GF/B filters (coated with 0.3% polyethyleneimine to minimize nonspecific binding) and subsequent washing with cold buffer (50 mM Tris-HCl, pH 7.4) using a Brandel cell harvester. Scintillation cocktail (Packard Ultima Gold, 4 ml) was added and the radioactivity was determined in a Packard 2200TR liquid scintillation analyzer at about 50% efficiency. Protein concentration was determined by the method of (Markwell et al., 1978) or (Lowry et al., 1951), with bovine serum albumin as standard.

[3H]Quinpirole binding. The [3H]quinpirole binding studies were performed as described for the [3H]raclopride binding assays with the following modifications. The membranes were suspended in binding buffer containing 50 mM Tris-HCl, 5 mM KCl, 4 mM MgCl₂, 1 mM EDTA and 120 mM N-methyl-D-glucamine, pH 7.6, to a final concentration of 190 µg protein/ml for untreated and 110 µg protein/ml for pertussis toxin-treated D3A-Ltk− cells, and 15 µg protein/ml for untreated and 100 µg protein/ml for pertussis toxin treated D3-CHO cells (the receptor concentration, as determined with [3H]raclopride, was 200-250 pM). The total incubation volume was 1 ml.

Data analysis. The receptor binding data were analyzed by nonlinear regression using the LIGAND program (Munson and Rodbard, 1980). One- and two-site curve fitting was tested in all experiments and the two-site model was accepted when it significantly improved the curve-fit (P < .05; F test). The [35S]GTPYs binding data were analyzed by nonlinear regression and sigmoidal dose-response curve-fitting using PRISM 2.0 (GraphPad Software, San Diego, CA). Student’s paired t test and analysis of variance were used for statistical comparisons.

Results

Effect of dopaminergic agonists on [35S]GTPYs binding. Figure 1 shows representative dose-response curves for dopamine agonists of the D3 receptor-mediated stimulation of [35S]GTPYs binding. Dopamine and quinpirole were full agonists producing maximal stimulation of the [35S]GTPYs binding, while (+)-7-OH-DPAT and (-)-3-PPP behaved as partial agonists.

Table 1 summarizes the effects of various agonists on the [35S]GTPYs binding mediated by D3 and D3A receptors. The rank order of potency at these receptors was (+)-7-OH-DPAT > quinpirole > (-)-3-PPP > dopamine. The EC50...
slightly different rank order of potency at the D2A receptor, (about 35% of the dopamine stimulation). The agonists had a stimulation of the \[^{35}\text{S}\]GTP\(\alpha\) statistically significant difference compared with dopamine (\(\text{EC}_{50}\) for the D3 receptor, (−)-3-PPP had the lowest intrinsic activity OH-DPAT and (−)-3-PPP were partial agonists (table 1). As to 280 fmol/mg of protein for D3 and D2A receptors, respectively. The receptor densities (\(B_{\text{max}}\)) were 10700 (D3) and 990 (D2A) pmol/g of protein. Footnote indicates a statistically significant difference compared with dopamine \((P < .05, ^{\text{a}} P < .001)\).

values were fairly similar for quinpirole, (−)-3-PPP and dopamine ranging from 5.4 to 12.6 nM, although (−)-7-OH-DPAT was clearly more potent with an \(\text{EC}_{50}\) value of 0.44 nM.

At the D2A receptor only dopamine produced maximal stimulation of the \[^{35}\text{S}\]GTP\(\alpha\) binding. Quinpirole, (−)-7-OH-DPAT and (−)-3-PPP were partial agonists (table 1). As for the D3 receptor, (−)-3-PPP had the lowest intrinsic activity (about 35% of the dopamine stimulation). The agonists had a slightly different rank order of potency at the D2A receptor, namely (+)-7-OH-DPAT ≥ (−)-3-PPP > quinpirole ≥ dopamine. (−)-3-PPP was more potent (>10-fold) than quinpirole and dopamine, and had about the same \(\text{EC}_{50}\) value as (+)-7-OH-DPAT (table 1). It should be noted that a direct comparison of \(\text{EC}_{50}/\text{E}_{\text{max}}\) values between D3 and D2A receptors is not relevant because the receptor levels are different. The density of the receptors has been shown to affect the potency and efficacy of the agonists (see below and Tiberi and Caron, 1994). In addition, the receptors are expressed in different cell lines which also might affect the \(\text{EC}_{50}\) and \(\text{E}_{\text{max}}\) values.

The expression level of the D3 receptors varied considerably between the different cell cultures, in contrast to the D2A receptor level which remained stable. The D3 receptor expression levels used in this study were 3410 and 10700 pmol/g protein. Dopamine was found to be clearly more potent in cells with the higher receptor density (\(\text{EC}_{50}\) was 11.7 ± 4.4 \(\mu\)M, \(n = 6\), and 12.1 ± 3.1 \(\mu\)M, \(n = 4\), respectively).

Effect of dopaminergic antagonists on \[^{35}\text{S}\]GTP\(\alpha\) binding. Representative dose-response curves for haloperidol, raclopride and clozapine of the D3 receptor-mediated inhibition of \[^{35}\text{S}\]GTP\(\alpha\) binding are shown in figure 2. Table 2 summarizes the effects of various dopamine antagonists on the \[^{35}\text{S}\]GTP\(\alpha\) binding mediated by D3 receptors. Haloperidol produced maximal inhibition of the basal \[^{35}\text{S}\]GTP\(\alpha\) binding and (+)-UH-232, raclopride and (+)-AJ-76 were almost as effective (table 2). Thus, these compounds can be classified as full inverse agonists whereas clozapine was a

Table 1
The effect of various agonist on the \[^{35}\text{S}\]GTP\(\alpha\) binding

<table>
<thead>
<tr>
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<th>D3 Receptors</th>
<th>D2A Receptors</th>
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<tbody>
<tr>
<td></td>
<td>(\text{EC}_{50}) (nM)</td>
<td>(\text{E}_{\text{max}}) (%)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>12.6 ± 1.4</td>
<td>100</td>
</tr>
<tr>
<td>Quinpirole</td>
<td>5.4 ± 1.1</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>(−)-7-OH-DPAT</td>
<td>0.44 ± 0.02</td>
<td>75 ± 8(^{\text{b}})</td>
</tr>
<tr>
<td>(−)-3-PPP</td>
<td>9.6 ± 1.8</td>
<td>41 ± 4(^{\text{b}})</td>
</tr>
</tbody>
</table>

The experiments were performed as described in "Methods." The results are means ± S.E.s of three to nine experiments. The stimulation \((\text{E}_{\text{max}})\) is expressed as percent of the maximal stimulation observed with dopamine (100%) which was included in all experiments. The maximal stimulations were 58.9 ± 8.6 fmol/mg of protein (32 ± 2% over basal \[^{35}\text{S}\]GTP\(\alpha\) binding) for D3 (n = 9) and 35.6 ± 4.1 fmol/mg of protein (15 ± 1% over basal \[^{35}\text{S}\]GTP\(\alpha\) binding) for D2A (n = 9) receptors. The basal \[^{35}\text{S}\]GTP\(\alpha\) binding varied between 120 to 330 and 150 to 280 fmol/mg of protein for D3 and D2A receptors, respectively. The receptor densities (\(B_{\text{max}}\)) were 10700 (D3) and 990 (D2A) pmol/g of protein. Footnote indicates a statistically significant difference compared with dopamine \((P < .05, ^{\text{a}} P < .001)\).
The D3 and D2A receptor densities were 3410 and 990 pmol/g of protein, respectively. Thus, the D3 receptor density (Bmax) was 10700 pmol/g of protein in this series of experiments. Footnote indicates statistically significant difference compared with haloperidol (P < .05).

In contrast to the EC50 value of dopamine, the IC50 value for haloperidol was not affected by the different expression levels of D3 receptors (360 ± 80 nM, n = 5, and 360 ± 90 nM, n = 3, for Bmax 3400 and 10700 pmol/g, respectively).

The various compounds were also tested at the D2A receptor-mediated [35S]GTPγS binding. Only a slight inhibitory effect of haloperidol on the basal [35S]GTPγS binding was observed. The other compounds tested were silent antagonists.

**Effect of pertussis toxin treatment on D3 and D2A receptor-mediated [35S]GTPγS binding.** Figure 3 shows the effect of pertussis toxin treatment on D3 and D2A receptor-mediated [35S]GTPγS binding (fig 3, A and B, respectively). The pertussis toxin treatment abolished both the stimulation by dopamine and the inhibition by haloperidol of the [35S]GTPγS binding. This indicates that both D3 and D2A receptors couple functionally to Gi/Go proteins. Figure 3 also shows that coincubation of dopamine and haloperidol with D3 (A) or D2A (B) receptor membranes canceled the stimulatory and inhibitory effects, respectively.

**Effect of pertussis toxin on [3H]raclopride and [3H]quinpirole binding to dopamine D3 and D2A receptors.** In agreement with our previous results [3H]raclopride bound with high affinity, with a Kd of about 1 nM, to D3 and D2A receptors (Malmberg and Mohell, 1995; Malmberg et al., 1993). The D3 and D2A receptor densities were 3410 and 990 pmol/g protein, respectively (table 3). The pertussis toxin treatment of the cells decreased the receptor densities to 1150 and 790 pmol/g protein, respectively. Thus, the D3 receptor density was decreased about 3-fold (table 3). The affinity of [3H]raclopride for the remaining D3 or D2A receptors was, however, not affected by the pertussis toxin treatment (P > .05).

Also in agreement with our previous results the [3H]quinpirole binding to D3 receptors was best described with a two-site model (Malmberg and Mohell, 1995). The affinity difference between the high and low affinity binding sites was about 10-fold (table 3). The biphasic binding is clearly indicated by the curved Scatchard plot shown in figure 4B. The Bmax values obtained with [3H]raclopride and [3H]quinpirole for D3 receptors were not significantly different (P > .05).
The 

Kd

d value of 2.74 nM corresponds to its affinity for the low affinity binding site for untreated D3 receptors (4.40 nM, P < 0.05) (table 3). The Bmax value of 1230 pmol/g protein for the pertussis toxin-treated D3 receptor membranes was not significantly different (P > 0.05) from the Bmax value of 1150 pmol/g determined with [3H]raclopride (table 3).

Competition of [3H]raclopride binding by dopamine and haloperidol. To investigate the effect of G protein coupling on the affinities of the agonist dopamine and the inverse agonist haloperidol, competition studies with [3H]raclopride were performed. The experiments were done in the presence and in the absence of sodium with both untreated and pertussis toxin-treated D3 and D2A receptor membranes. The results are summarized in table 4A (D3) and 4B (D2A).

As expected, the analysis of dopamine competition curves for [3H]raclopride binding revealed high and low affinity

TABLE 3

The effect of pertussis toxin treatment on [3H]quinpirole and [3H]raclopride binding to dopamine D2A and D3 receptors

<table>
<thead>
<tr>
<th></th>
<th>[3H]Quinpirole</th>
<th>[3H]Raclopride</th>
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<tbody>
<tr>
<td></td>
<td>Affinity (nM)</td>
<td>Bmax (pmol/g protein)</td>
</tr>
<tr>
<td>D3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.34 ± 0.24*</td>
<td>3920 ± 370a</td>
</tr>
<tr>
<td>High</td>
<td>0.57 ± 0.12</td>
<td>1530 ± 270</td>
</tr>
<tr>
<td>Low</td>
<td>4.4 ± 1.3</td>
<td>3140 ± 320</td>
</tr>
<tr>
<td>PTX treated</td>
<td>2.74 ± 0.23</td>
<td>1230 ± 100</td>
</tr>
<tr>
<td>D2A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>4.20 ± 0.70</td>
<td>180 ± 20</td>
</tr>
<tr>
<td>PTX treated</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The radioligand binding studies were performed and the dissociation constants (Kd) and receptor densities (Bmax) were calculated as described in "Methods." The results are means ± S.E.M of three to four experiments. The buffer contained 50 mM Tris-HCl, 1 mM EDTA, 5 mM KCl, 4 mM MgCl2 and 120 mM NaCl for the [3H]raclopride binding or 120 mM NMDG for the [3H]quinpirole binding. High and low represent the Kd and Bmax values of high and low affinity agonist binding site, respectively. PTX; pertussis toxin. NS; no specific binding.

* Values analyzed by a one-site model. A significantly better fit was achieved with a two-site model.

Fig. 4. Effect of pertussis toxin on [3H]quinpirole binding to cloned human dopamine D3 receptors. The saturation studies were performed as described in "Methods." Representative saturation binding curves for [3H]quinpirole to untreated (A) and pertussis toxin-treated (C) D3 receptor membranes. The saturation curve A is fitted to a two-site model. B and D are the corresponding Scatchard plots of the specific [3H]quinpirole binding. The Kd and Bmax values determined from the Scatchard analysis were in good agreement with the values determined with the LIGAND program. The Scatchard plot B is clearly biphasic and was analyzed by nonlinear regression.

The Kd value of 2.74 nM corresponds to its affinity for the low affinity binding site for untreated D3 receptors (4.40 nM, P > 0.05) (table 3). The Bmax value of 1230 pmol/g protein for the pertussis toxin-treated D3 receptor membranes was not significantly different (P > 0.05) from the Bmax value of 1150 pmol/g determined with [3H]raclopride (table 3).

Competition of [3H]raclopride binding by dopamine and haloperidol. To investigate the effect of G protein coupling on the affinities of the agonist dopamine and the inverse agonist haloperidol, competition studies with [3H]raclopride were performed. The experiments were done in the presence and in the absence of sodium with both untreated and pertussis toxin-treated D3 and D2A receptor membranes. The results are summarized in table 4A (D3) and 4B (D2A).

As expected, the analysis of dopamine competition curves for [3H]raclopride binding revealed high and low affinity

The radioligand binding studies were performed and the dissociation constants (Kd) and receptor densities (Bmax) were calculated as described in "Methods." The results are means ± S.E.M of three to four experiments. The buffer contained 50 mM Tris-HCl, 1 mM EDTA, 5 mM KCl, 4 mM MgCl2 and 120 mM NaCl for the [3H]raclopride binding or 120 mM NMDG for the [3H]quinpirole binding. High and low represent the Kd and Bmax values of high and low affinity agonist binding site, respectively. PTX; pertussis toxin. NS; no specific binding.

* Values analyzed by a one-site model. A significantly better fit was achieved with a two-site model.

Fig. 4. Effect of pertussis toxin on [3H]quinpirole binding to cloned human dopamine D3 receptors. The saturation studies were performed as described in "Methods." Representative saturation binding curves for [3H]quinpirole to untreated (A) and pertussis toxin-treated (C) D3 receptor membranes. The saturation curve A is fitted to a two-site model. B and D are the corresponding Scatchard plots of the specific [3H]quinpirole binding. The Kd and Bmax values determined from the Scatchard analysis were in good agreement with the values determined with the LIGAND program. The Scatchard plot B is clearly biphasic and was analyzed by nonlinear regression.
binding sites at both D$_3$ and D$_{2A}$ receptors. As mentioned above, there is a larger separation between the high (1.96 nM) and the low (379 nM) affinity binding sites at D$_{2A}$ receptors (table 4B), than at D$_3$ receptors (0.50 and 15.3 nM, respectively, table 4A). The proportion of D$_{2A}$ receptors in the high affinity conformation was similar for both dopamine (29%, table 4B) and [H]quinpirole (20%, table 3). The same was true for the D$_3$ receptor (53 and 45%, table 4A and 3, respectively).

The addition of sodium did not alter the affinity of dopamine for the high affinity binding sites at D$_3$ receptors. The proportion of the high affinity binding sites was, however, reduced from 53% to 24% leading to a 3-fold higher proportion of the high affinity binding sites was, however, present in the presence of sodium for reliable $K_{high}$ and $K_{low}$ values.

The results are means ± S.E. of two to four experiments. -Na and +Na; the buffer contained 120 mM NMDG and 120 mM NaCl, respectively. $K_{high}$ and $K_{low}$ represent the affinity values of high and low affinity binding sites, respectively, and are given when the data were best described with a two-site analysis. $R_{high}$ indicates the percentage of receptors in the high affinity state. PTX: pertussis toxin.

| TABLE 4 | The effect of pertussis toxin on dopamine and haloperidol competition of [H]raclopride binding to D$_3$ (A) and D$_{2A}$ (B) receptors |
|---------|-------------------------------------------------|------------------|-----------------|------------------|------------------|------------------|
|         | Untreated (+Na)                                | PTX Treated (+Na) |                  |                  |
| D$_3$ receptors | Affinity (nM) | Affinity (nM) |                  |                  |
| Dopamine | 4.92 ± 1.96 | 16.2 ± 2.7 | 4.02 ± 0.59 | 24.4 ± 2.2 |
| $K_{high}$ | 0.50 ± 0.23 | 0.45 ± 0.16 |                  |                  |
| $K_{low}$  | 15.3 ± 2.9 | 28.7 ± 1.2 |                  |                  |
| $R_{high}$ | 53 ± 5%  | 24 ± 5%  | 0.54 ± 0.20 | 2.21 ± 0.26 |
| $R_{low}$  | 0.74 ± 0.12 | 1.77 ± 0.07 |                  |                  |
| Haloperidol | 0.12 ± 0.02 | 0.44 ± 0.01 | 0.09 ± 0.04 | 0.36 ± 0.06 |

The affinity of dopamine to D$_2A$ receptors was also reduced by sodium ions. Although the dopamine binding curves were better fitted to a two-site model it was not possible to obtain reliable $K_{high}$ and $K_{low}$ values due to the low proportion of receptors in the high affinity conformation in the presence of sodium. Hence, only the affinity values from a one-site analysis (4.92 nM compared to 16.2 nM). The affinity of dopamine to D$_{2A}$ receptors was also reduced by sodium ions. However, the addition of sodium did not alter the affinity of dopamine for the high affinity binding sites at D$_3$ receptors. The proportion of the high affinity binding sites was, however, reduced from 53% to 24% leading to a 3-fold higher affinity for D$_{2A}$ receptors than for D$_3$ receptors. The addition of sodium decreased the affinity of haloperidol 2- to 4-fold for both untreated and pertussis toxin treated D$_3$ and D$_{2A}$ receptors (table 4, A and B). Pertussis toxin treatment did not, however, affect the affinity of haloperidol for D$_3$ or D$_{2A}$ receptors.

**Discussion**

The equilibrium for ligand-receptor-G protein interactions is described by the “ternary complex model” (Kent et al., 1980; De Lean et al., 1980). In this model, an agonist stabilizes the receptor-G protein coupled state (RG) by binding with a higher affinity to this conformation, as compared to the free receptor (R). RG is the active form which may lead to a biological response. An inverse agonist is believed to stabilize R by binding with a higher affinity to this receptor form, thereby inhibiting the basal activity, i.e., the spontaneous receptor-G protein interaction (Costa et al., 1992). Ligands that bind with the same affinity to both receptor conformations and thus do not affect the basal activity are classified as (silent) antagonists. More recently, an extension of the ternary complex model, the so-called “allosteric ternary complex model,” was proposed (Samama et al., 1993; Leffkowitz et al., 1995). This version introduces a spontaneous, G protein-independent, isomerization step of R to R*. R* is an active receptor conformation that may couple to the G protein. The extended model is based on results from experiments with mutated receptors that possess constitutive activity (i.e., agonist-independent activity). In addition, high expression levels of receptors or G proteins may lead to constitutive activity (Burstein et al., 1997; Tiberi and Caron, 1994; Samama et al., 1993; Leffkowitz et al., 1993).

The receptor-mediated activation of G proteins is the first step in the signaling pathway. Agonist binding to the receptor stimulates the GDP/GTP exchange and can therefore be studied by measuring the incorporation of the non-hydrolyzable GTP analogue [35S]GTPγS (Gardner and Strange, 1995; Wieland and Jakobs, 1994; Lazareno et al., 1993). Thus, agonists promote an exchange of GDP for [35S]GTPγS whereas inverse agonists inhibit the GDP→[35S]GTPγS exchange. In this study, the receptor-G protein interactions of cloned human D$_3$ and D$_{2A}$ receptors have been investigated using [35S]GTPγS and receptor binding studies.

**Agonist and inverse agonist activity at the D$_3$ receptor.** At D$_3$ receptors dopamine and quinpirole produced maximal stimulation of the [35S]GTPγS binding whereas (+)-7-OH-DPAT and (-)-3-PPP behaved as partial agonists. At D$_{2A}$ receptors only dopamine was a full agonist. The reported intrinsic activities for (+)-7-OH-DPAT and (-)-3-PPP have varied, from full to partial agonists, depending on the second messenger response measured (Chio et al., 1994; Sautel et al., 1995). In addition, the potency order of the agonists seems to...
vary depending on the functional response measured. However, in common for the various D3 receptor-mediated functional responses ([35S]GTPγS binding, cyclic AMP and mitogenesis studies) has been that (+)-7-OH-DPAT is the most potent agonist (present study, Chio et al., 1994; Sautel et al., 1995).

In our study, five dopamine receptor antagonists (haloperidol, raclopride, clozapine, (+)-UH-232 and (+)-AJ-76) from four different chemical classes were tested in the [35S]GTPγS binding assay. At the D3 receptor all compounds inhibited basal [35S]GTPγS binding. Clozapine had a lower intrinsic activity than the other inverse agonists (70% of haloperidol-induced inhibition). Recently several dopamine antagonists were reported to behave as inverse agonists by inhibiting basal [3H]thymidine incorporation in NG 108-15 cells expressing the D3 receptor (Griffon et al., 1996). Haloperidol and raclopride behaved as inverse agonists whereas (+)-AJ-76 and clozapine were inactive. Furthermore, (+)-UH-232 stimulated the mitogenesis and was classified as a weak partial agonist (Griffon et al., 1995, 1996). Thus, (+)-UH-232 has been shown to behave as an inverse agonist in one system and a partial agonist in another. This type of behavior has been explained by high and low basal activities, respectively, in the two systems (Kenakin, 1995).

Interestingly, neither the IC50 values of various inverse agonists tested nor their rank order of potency did correlate with the Kd values determined with receptor binding studies in the same cell line (present study and Griffon et al., 1996). Haloperidol, raclopride and (+)-UH-232 bind to D3 receptors with about 2 to 3 nM affinity whereas (+)-AJ-76 and clozapine have at least 10-fold lower affinity (Malmberg et al., 1993). In the [35S]GTPγS binding experiments haloperidol, (+)-AJ-76, (+)-UH-232 and clozapine were about equipotent and raclopride was about 2- to 3-fold less potent. The different assay conditions in receptor binding and [35S]GTPγS binding experiments (e.g., buffer composition and presence of guanine nucleotides) may explain some of the discrepancies.

Among the dopamine receptor antagonists studied, only haloperidol has been reported to behave as an inverse agonist at the D2 receptor (Nilsson and Eriksson, 1993; Malmberg et al., 1996). However, no significant inhibitory effect of the compounds could be detected when [35S]GTPγS binding was measured, although there was a trend for a slight inhibition of the basal [35S]GTPγS binding by haloperidol. It remains to be shown whether these compounds would behave as inverse agonists at the D3 receptor if the receptor density was as high as for the D3 receptor.

Pertussis toxin treatment abolishes the effect of agonists and inverse agonists. Pertussis toxin binds covalently to Gαi, Gαo proteins and thereby disrupts the receptor-G protein coupling and inactivates the G protein. In this study, the pertussis toxin treatment of the cells abolished the responses induced by dopamine and haloperidol on D3 and D2A receptor-mediated [35S]GTPγS binding. This indicates that the effects of agonists as well as inverse agonists are Gαi,Gαo protein-dependent.

Effect of pertussis toxin treatment on the receptor binding characteristics of various ligands. The extended ternary complex model proposes two forms of free receptors, R and R*, which may thus both exist in pertussis toxin treated cells. It has, however, not been possible to differentiate between these forms in receptor binding experiments (Samama et al., 1993; Gether et al., 1997). To further examine the hypothesis that agonists possess higher affinity for the G protein coupled receptor and inverse agonists possess higher affinity for the free receptor, we studied the receptor binding characteristics of [3H]quinpirole, dopamine, [3H]raclopride and haloperidol using both untreated and pertussis toxin treated (G protein uncoupled receptors) cell membranes.

The antagonist [3H]raclopride bound to a single class of binding sites with high affinity (about 1 nM) at both D3 and D2A receptors (Malmberg et al., 1993). Pertussis toxin treatment reduced the density of [3H]raclopride binding sites (Bmax) to both D3 and D2A receptors. The reason for this reduction is not known, but it could be explained by a decreased receptor expression due to the toxin treatment. It should be noted that the Bmax value of [3H]quinpirole also was reduced to the same extent as the Bmax value of [3H]raclopride at the D3 receptor. The affinity of [3H]raclopride to the remaining sites was not changed. Thus, although raclopride behaved as an inverse agonist inhibiting the basal [35S]GTPγS binding via the D3 receptor its binding characteristics were similar to those of silent antagonists.

[3H]Quinpirole labeled a high affinity binding site at both D3 and D2A receptors. In contrast to D2A receptors, [3H]quinpirole bound a biphasic binding to D3 receptors (Malmberg and Mohell, 1995). The Kd values of the high and low affinity binding sites were 0.57 and 4.4 nM, respectively. No specific [3H]quinpirole binding was detected in pertussis toxin treated D2A receptor membranes. In pertussis toxin treated D3 receptor membranes, [3H]quinpirole labeled a single low affinity binding site, which corresponds to the low affinity site in untreated D3 receptor membranes. Thus, pertussis toxin treatment abolished the high affinity agonist binding at both D2A and D3 receptors. Because the dopamine-induced D3 and D2A receptor-mediated [35S]GTPγS binding was lost in cell membranes treated with pertussis toxin it can be suggested that the presence of the high affinity agonist binding is necessary for receptor function.

Sodium has been shown to bind to an allosteric site on the receptor protein (Horton et al., 1990; Neve et al., 1991), thereby counteracting the stabilization and formation of the high affinity agonist complex (agonist-receptor-G protein). The sodium-induced shift toward the low affinity agonist conformation has been shown for both D2 receptors (Watanabe et al., 1985; Urwyler, 1989; Malmberg and Mohell, 1995; Hamblin and Creese, 1982; Grigoriadis and Seeman, 1985) and D3 receptors (Malmberg and Mohell, 1995). The effects of sodium and pertussis toxin treatment on the affinities of dopamine and haloperidol were investigated in competition experiments using [3H]raclopride.

Dopamine displayed biphasic binding both in the presence and absence of sodium to untreated D3 and D2A receptor membranes. However, it was not possible to reliably determine the high and low affinity values for dopamine in the presence of sodium at D2A receptors, probably due to the low proportion of high affinity agonist binding sites under these conditions. Addition of sodium did not affect the affinity of dopamine for the high affinity binding site at D3 receptors, although a lower proportion of receptors displayed high affinity for dopamine in the presence of sodium (24% as compared to 53% in the absence of sodium). Pertussis toxin treatment completely abolished the high affinity agonist binding site for both receptor subtypes. Furthermore, addition of sodium decreased the affinity of do-
pamine 3- to 6-fold to the uncoupled, pertussis toxin-treated, D3 and D2A receptors. The results in table 4A indicate that sodium only reduces the affinity of dopamine to the uncoupled D3 receptor. The Kd_high values are not affected whereas the Kd_low values (which should correspond to the affinity for the uncoupled receptor) and the K values for pertussis toxin treated membranes are increased by sodium.

Haploperidol bound with the same affinity to untreated and pertussis toxin-treated D3 and D2A receptors identified with [3H]quinpirole. Addition of sodium, however, decreased the affinity of haploperidol 2- to 4-fold. Haploperidol was expected to bind with higher affinity in the presence of sodium (promoting higher proportions of free receptors) as well as to pertussis toxin-treated receptor membranes (uncoupled receptors). Thus, the binding characteristics of haploperidol did not support the hypothesis that an inverse agonist binds with higher affinity to the uncoupled receptor.

It has been shown that the constitutive activity increases with increased receptor density and that agonists bind with higher affinity to constitutively active receptors (Tiberi and Caron, 1994; Lefkowitz et al., 1993). We found that dopamine was more potent when membranes with high D3 receptor expression (Bmax = 10700 pmol/g protein) were used whereas the IC50 value of haploperidol was not changed. Preliminary experiments showed, however, that the affinity of [3H]quinpirole for the high and the low affinity binding sites was the same as in membranes with the lower D3 receptor density (Bmax = 3410 pmol/g protein). (The percentage of receptors in high affinity state for [3H]quinpirole was lower with high receptor density.) To adequately investigate how different Bmax values affect the affinities and potencies, several expression levels need to be tested.

In conclusion, we found that all dopamine D2 receptor antagonists tested behaved as inverse agonists by inhibiting the basal [35S]GTPgS binding at the D3 receptor. The high affinity agonist binding site as well as the agonist and inverse agonist effects were abolished by pertussis toxin treatment, which in- indicates involvement of Gi/G0 proteins. The inverse agonists tested behaved as inverse agonists by inhibiting the basal [35S]GTPgS binding at the D3 receptor.

References


Send reprint requests to: Dr. Åsa Malmberg, Department of Molecular Pharmacology, Preclincal R & D, AstrA AB, S-151 85 Södertälje, Sweden.