Dopamine D2 receptors internalize in their low-affinity state

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The present study was done in order to determine whether dopamine internalizes D2 receptors in CHO cells and whether the high-affinity or the low-affinity state of the dopamine D2 receptor is associated with dopamine-induced internalization of dopamine D2 receptors. Using [3H]sulpiride to label D2 receptors in CHO cells, it was found that dopamine lowered the binding of [3H]sulpiride by 20%. Although the high-affinity states of D2 were converted to low-affinity states by guanine nucleotide, the latter had no effect in blocking the dopamine-induced reduction in [3H]sulpiride binding, indicating that the dopamine-induced internalization of D2 receptors occurred with D2 in the low-affinity state. *NeuroReport* 13:1017–1020 © 2002 Lippincott Williams & Wilkins.

**Key words:** D2 receptor; Dopamine; High-affinity state of D2; Internalization; Schizophrenia

INTRODUCTION

Many types of G protein-linked receptors become desensitized upon continuous exposure to agonists. A variety of molecular events occurs during the course of desensitization [1,2], including receptor phosphorylation [3], receptor conversion from the high-affinity to the low-affinity state [4], receptor internalization or endocytosis [5], and down-regulation of receptor site density [6]. The internalization of receptors appears necessary for the re-sensitization of receptors [5,7]. Although dopamine receptors become desensitized upon exposure to dopamine agonists [8,9], there is little information on which of these various molecular events are associated with desensitization to dopamine. In particular, although there is information on desensitization of dopamine D1 receptors [9–11], there are only a few studies on dopamine D2 receptors [12–14]. These latter studies looked at the sequestration of the D2 receptors through the G-protein-coupled receptor kinases (GRK)-mediated pathways by co-expression of G-protein-coupled receptor kinases and dopamine D2 receptors, then examining the subsequent [3H]sulpiride binding.

It is of considerable clinical interest to determine the basic mechanism of dopamine D2 receptor down-regulation, because Laruelle et al. and Breier et al. [15,16] have found that the dopamine-releasing action of amphetamine causes a greater apparent fall in D2 receptors in schizophrenia patients than in control individuals. The imaging data from these studies suggest that there is a higher release of endogenous dopamine in schizophrenia patients, a finding compatible with the dopamine over-activity hypothesis of schizophrenia [17]. With an increase in dopamine release pre-synaptically, this would lead to a heightened displacement or decrease in radioligand binding. If such a fall in receptor density is attributable to internalization, then it is important to know the factors that contribute to the process of internalization. For example, although it is known that β2-adrenergic receptors are internalized in their low-affinity state, no such information is available for dopamine D2 receptors [5]. Because it is known that the high-affinity state of the dopamine D2 receptor is the functional state of the D2 receptor in controlling the release of prolactin [18], one might expect that the functional state of the dopamine D2 receptors in the brain and tissue culture cells would also function in their high-affinity state. Therefore, one would expect that dopamine D2 receptors would be internalized in their high-affinity state upon exposure to dopamine. The present findings, however, reveal that the dopamine D2 receptors are internalized in their low-affinity state.

METHODS AND MATERIALS

**Cell culture:** CHO cells stably transfected with the D2Long receptor were grown on large Falcon tissue culture dishes (100 × 20 mm) to ~90% confluence in AlphaMEM + penicillin/streptomycin (Tissue Culture Media Preparation Service, University of Toronto, Toronto, ON) and 10% fetal bovine serum; Gibco-BRL products, Life Technologies, Burlington, ON, containing 1 mg/ml Gentamicin (GIBCO-BRL). Cells were maintained at 37°C in a CO₂ incubator. For each experiment, unless otherwise mentioned, 24-well plates were seeded with the CHO cells
stably transfected with the D2Long receptor and allowed to grow for an additional 3 days at 37°C.

**Radioligand binding to D2 receptors in intact tissue culture cells:** On the day of the experiment, cells adhering to the bottom of the 24-well plates were washed three times with 0.5 ml cold phosphate-buffered saline (PBS), after which 0.5 ml buffer (50 mM Tris–HCl, pH 7.4 at 20°C, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂·6H₂O, 1 mM EDTA and 120 mM NaCl) or dopamine (supplemented with 5.7 mM L-ascorbic acid) was added. The dopamine concentrations tested were 100 nM, 200 nM, 500 nM and 1 μM. Cells were exposed to dopamine at both room temperature and at 37°C and for varying time periods ranging from 5 min to 60 min. After exposure to dopamine, the buffer containing dopamine was removed and the wells were washed three times with 0.5 ml cold PBS to remove the dopamine. Preliminary tests were carried out using [³H]dopamine to ensure that all the dopamine was removed from the wells. Non-specific binding for dopamine D2 receptors was defined by the presence of 100 nM haloperidol or 100 nM domperidone.

In order to determine the optimal concentration of the radioligand [³H]sulpiride (70.3 Ci/mmol; NEN Life Science Products, Boston, MA) to label dopamine D2 receptors in the CHO cells, preliminary experiments were done using [³H]sulpiride concentrations ranging from 1.4 nM to 10 nM (final concentrations). A final concentration of 5 nM [³H]sulpiride (Kᵢ 5 nM) was used to measure the binding of [³H]sulpiride to the D2-transfected CHO cells.

The cells were incubated with the radioligand for 4 h at 4°C, after which trypsin–EDTA ( Gibco-BRL) was added to the cells. The cells were scraped off the wells and placed into minivials (Packard Instruments, Chicago, IL). Scintillant (4 ml; CytoScint, ICN, CA) was added per minivial and the vials were then monitored for tritium in a Beckman scintillation spectrometer at 55% efficiency.

**Guanine nucleotide effect in intact tissue culture cells:** The same experiments as described above were carried out in the presence of 200 μM final concentration of guanylylimidodiphosphate (Gpp[NH]p) included with dopamine during its exposure with the intact tissue culture cells.

**Effect of sucrose and concanavalin A on intact tissue culture cells:** The same experiments as described above were carried out in the presence of 0.5 mg/ml concanavalin A, included with dopamine during its exposure with the intact tissue culture cells. The same experiments were also repeated with 0.6 M final concentration of sucrose.

**D2High state in disrupted CHO cells:** Competition experiments were carried out between dopamine and [³H]raclopride in the following manner. Incubation tubes (12 × 75 mm glass) received 0.5 ml buffer (with increasing concentrations of dopamine or Gpp[NH]p, final concentration 200 μM; or with a final concentration 10 μM S-sulpiride), 0.5 ml [³H]raclopride (76.8 Ci/mmol, final concentration 2 nM) and 0.5 ml of a suspension of disrupted CHO cells. The cells were not washed, centrifuged or homogenized, though they were disrupted since they were scraped off the plate. The tubes containing a total volume of 1.5 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 s (7.5 ml buffer). The filters were pushed out and placed in scintillation minivials. The minivials received 4 ml each of scintillant and were monitored 6 h later for tritium in a Packard 4660 scintillation spectrometer at 55% efficiency. Non-specific binding for dopamine D2 receptors was defined as that which occurred in the presence of 10 μM S-sulpiride.

**D2High state in intact CHO cells:** CHO cells with the D2Long receptor were seeded on 24-well plates. Competition experiments were carried out between dopamine and [³H]raclopride on the intact cells in the 24-well plates. Cells adhering to the bottom of the wells were washed three times with 0.5 ml cold PBS, and the following were added to each well: 0.5 ml buffer containing increasing concentrations of dopamine (or a final concentration of 10 μM S-sulpiride), 0.5 ml [³H]raclopride (76.8 Ci/mmol, final concentration 2 nM) and 1 ml buffer (or Gpp[NH]p, final concentration 200 μM). The wells, containing a total volume of 2 ml, were incubated for 2 h at room temperature (20°C), after which they were washed three times with 0.5 ml cold PBS, trypsinized with trypsin–EDTA (Gibco-BRL) and scraped off the wells and placed into the minivials. Four ml of scintillant was added per minivial and these were then monitored for tritium in a Beckman scintillation spectrometer at 55% efficiency.

**RESULTS**

Optimizing conditions to study the in vitro action of dopamine on radioligand binding to D2 receptors in intact tissue culture cells: In order to study the dopamine-induced internalization, conditions were optimized. To establish the conditions under which dopamine exposure would have a consistent effect in the range 20–30%, we tested the variables of dopamine concentration, temperature, and length of exposure time to dopamine. After testing each variable, we found that exposure to 1 μM dopamine for 1 h at room temperature produced the maximal decrease in [³H]sulpiride binding. The optimum concentration of [³H]sulpiride was 5 nM, which in fact was the Kᵢ of this ligand.

Effects of concanavalin A in the presence of dopamine on [³H]sulpiride binding to D2 receptors in intact tissue culture cells: Table 1 shows that 1 μM dopamine decreased [³H]sulpiride binding significantly by 21 ± 2.7% and that this decrease in binding was blocked by 0.5 mg/ml concanavalin A.

Establishing the presence of the D2High state in disrupted CHO cells: To establish that the high-affinity state of the D2 receptor was present in disrupted CHO cells, competition experiments were carried out between dopamine and [³H]raclopride. As shown in Fig. 1a, the high-affinity state of the D2 receptor was present in disrupted cells and this state
Table 1. Dopamine decreased $[^{3}H]$sulpiride binding (5 nM) to D2-transfected cells, an effect blocked by concanavalin A. Values are mean ± s.e.m.

<table>
<thead>
<tr>
<th>Number of experiments</th>
<th>D2-specific binding (d.p.m./mg protein)</th>
<th>Decrease in specific binding caused by 1 μM dopamine (d.p.m./mg protein)</th>
<th>% of control</th>
<th>% decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63</td>
<td>153 500 ± 2150</td>
<td>0</td>
<td>100 ± 1.4</td>
</tr>
<tr>
<td>1 μM dopamine</td>
<td>32</td>
<td>123 270 ± 3300**</td>
<td>32 230 ± 3300**</td>
<td>79 ± 2.2</td>
</tr>
<tr>
<td>1 μM dopamine + 200 μM Gpp[NH]p</td>
<td>44</td>
<td>119 730 ± 2000**</td>
<td>33 770 ± 2000**</td>
<td>70 ± 1.3</td>
</tr>
<tr>
<td>1 μM dopamine + 0.5 mg/ml Con A</td>
<td>28</td>
<td>153 000 ± 4300</td>
<td>~0</td>
<td>100 ± 2.8</td>
</tr>
<tr>
<td>1 μM dopamine + 0.6 M sucrose</td>
<td>4</td>
<td>155 000 ± 6900</td>
<td>~0</td>
<td>100 ± 4.5</td>
</tr>
</tbody>
</table>

*a Non-specific binding in the presence of 100 nM haloperidol = 1200 ± 50 d.p.m./mg protein (n = 63).

*b Statistically significantly different from control, p < 0.005 (t-test).

![Graph](image)

**Fig. 1.** Demonstrating the existence of high-affinity states of dopamine D2 receptors in CHO cells, and their conversion to low-affinity states. (a) Disrupted cells: although dopamine recognized high- and low-affinity states, the high-affinity sites were converted to low-affinity sites in the presence of 200 μM guanilylimidodiphosphate (Gpp[NH]p). Representative experiment. (b) Intact cells: dopamine recognized high- and low-affinity sites for D2 receptors, with 10 nM dopamine inhibiting 50% of the $[^{3}H]$raclopride binding to the high-affinity sites. In the presence of 200 μM Gpp[NH]p, the proportion of high-affinity sites was reduced by > 50%. Representative experiment.

was converted into the low-affinity state in the presence of guanine nucleotide.

Establishing the presence of the D2High state in intact CHO cells: In intact cells, dopamine recognized both the high-affinity sites (~40%), and the low-affinity sites (~60%; 2.7%).

Fig. 1b. The concentration of dopamine for 50% inhibition of $[^{3}H]$raclopride at the high-affinity site was 10 nM. Guanine nucleotide only partially inhibited the high-affinity state receptors (40–15%) [18], as is clearly seen by the competition curve spanning > 2 log units.

Effects of guanine nucleotide and sucrose, respectively, in the presence of dopamine on $[^{3}H]$sulpiride binding to D2 receptors in intact tissue culture cells: Dopamine decreased the dopamine D2 receptor binding of $[^{3}H]$sulpiride by 21 ± 2.7% (n = 32) when compared with controls (Table 1). Surprisingly, in the presence of dopamine and guanine nucleotide, the decrease in $[^{3}H]$sulpiride binding was not reversed, but remained decreased by 22 ± 1.8% compared with controls. Sucrose completely blocked the dopamine-induced decrease in $[^{3}H]$sulpiride binding (Table 1).

**DISCUSSION**

The main observation of this study is that guanine nucleotide was unable to inhibit the dopamine-induced internalization of dopamine D2 receptors labeled by $[^{3}H]$sulpiride, suggesting that the D2 receptors are internalized in their low-affinity state.

One disadvantage of the study was that dopamine was only able to internalize 21% of the D2Long receptors, as revealed by the reduction of $[^{3}H]$sulpiride binding. Nevertheless, this reduction was completely inhibited by either 0.6 M sucrose or 0.5 mg/ml concanavalin A, indicating that dopamine genuinely caused internalization of D2 receptors. Although sucrose inhibited the dopamine-induced reduction in $[^{3}H]$sulpiride binding, this effect is not especially selective because 0.6 M sucrose is hypertonic and would be expected to inhibit a wide variety of cellular processes. The experiment with sucrose, however, served as an excellent non-specific control to determine whether dopamine induced a non-specific reduction in $[^{3}H]$sulpiride binding. Thus, the fact that dopamine did not reduce the binding of $[^{3}H]$sulpiride in the presence of sucrose indicated the absence of nonspecific internalization in the presence of dopamine.

Concanavalin A, however, is selective in disaggregating microfibrils responsible for receptor endocytosis and preventing receptor dephosphorylation, supporting the interpretation that dopamine induces internalization of dopamine D2 receptors.

Although the magnitude of the dopamine-induced internalization was only 21–22%, it is possible that this may be
attributed to the low level (~40%) of the proportion of D2 receptors in the high-affinity state, as indicated by the \[^{3}H\]raclopride/dopamine competition experiments. At the same time, it may be argued that guanine nucleotide did not fully eliminate the high-affinity states in the intact cells, and that this incomplete action of guanine nucleotide may explain why the guanine nucleotide was not effective in inhibiting dopamine-induced internalization.

As noted above, the main finding of this study was that Gpp[NH]p was unable to inhibit the dopamine-induced internalization of \[^{3}H\]sulpiride. This negative finding did not occur because the Gpp[NH]p was not effective or not capable of entering the cell. In fact, the \[^{3}H\]raclopride/dopamine competition experiments clearly showed that 30–40% of the D2 receptors were in the high-affinity state (Fig. 1a,b) and, in addition, that 200 μM Gpp[NH]p significantly reduced the high-affinity states in both the disrupted CHO cells and the intact CHO cells. Nevertheless, although the presence of 200 μM Gpp[NH]p converted the high-affinity states to low-affinity states, the presence of the guanine nucleotide had no effect in blocking the dopamine-induced reduction (internalization) in \[^{3}H\]sulpiride binding, indicating that the dopamine-induced internalization of D2 receptors was mediated by the low-affinity state of the D2 receptor. Collectively, these data indicate, therefore, that the dopamine-induced internalization of D2 receptors was mediated in the low-affinity state of the D2 receptor.

**CONCLUSION**

Dopamine-induced internalization of dopamine D2 receptors into CHO cells was not affected by the absence of D2 receptors in their high-affinity state, and that the internalization occurred with the D2 receptors in their low-affinity state for dopamine.

**REFERENCES**


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