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EVIDENCE FOR ESSENTIAL THIOL GROUPS AND DISULFIDE BONDS IN AGONIST AND ANTAGONIST BINDING TO THE DOPAMINE RECEPTOR

Erick T. Suen, Ennio Stefanini, and
Yvonne C. Clement-Cormier*

Department of Pharmacology
University of Texas Medical School
Houston, Texas 77025

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SUMMARY Dithiothreitol (DTT), a disulfide reducing agent, diminished the specific binding of [^{3}H] dopamine to partially purified calf striatal membranes (P_2) but did not have an effect on [^{3}H] spiroperidol binding. The thiol reagents, p-chloromercuribenzoate (PCMB), N-ethylmaleimide (NEM) and iodoacetamide (IA), were also tested for inhibitory effects on agonist and antagonist binding to the dopamine receptor. PCMB inhibited both [^{3}H] dopamine and [^{3}H] spiroperidol binding by changing the affinity (K_d) and the number of binding sites (B_{max}) for both of these ligands. This effect of PCMB was reversed by the addition of DTT. NEM inhibited binding to the dopamine agonist site but not to the antagonist site,while IA was ineffective on either site. These results indicate that a DTT-reducible disulfide bond may be an essential component for agonist binding to the dopamine receptor. Furthermore, the experiments with PCMB, NEM and IA suggest that the exposure of thiol groups in the dopamine receptor may play an important role in agonist and antagonist binding.

INTRODUCTION

The dopamine receptor, which has been shown to play an important role in central nervous system function (1), has been studied by several laboratories (2-7). These investigations have made considerable progress toward a better understanding of the biochemical characteristics of the dopamine receptor, including its recent solubilization and partial purification (6-7). However, one concept that has not been clarified from these studies is the biochemical nature of dopamine agonist and antagonist binding sites. Some investigators propose that these receptor sites are

*Direct correspondence to: Dr. Yvonne C. Clement-Cormier, Department of Pharmacology, University of Texas Medical School, Houston, Texas 77025.

Abbreviations: PCMB; p-chloromercuribenzoate, DTT; dithiothreitol, NEM; N-ethylmaleimide, IA; iodoacetamide, DA; dopamine, Spiro; spiroperidol.

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separate entities with distinct cellular locations (8) whereas others suggest that the receptor is a unitary complex, composed of agonist and antagonist sub-unit binding sites (9).

Recent neurotransmitter receptor studies have clarified some aspects of the chemical nature of agonist and antagonist receptor sites. For example, the agonist conformation of the β-adrenergic receptor in turkey erythrocytes possesses an essential disulfide group which can be reduced by dithiothreitol (DTT) (10). Similarly, the agonist conformation of the muscarinic acetylcholine receptor can be inactivated by alkylating agents such as N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB). Moreover, studies on the muscarinic cholinergic receptor demonstrate that reductive alkylation agents can also discriminate between subclasses of agonist receptors by converting the low affinity form of the receptor to a high affinity form (11). Overall, these studies imply that thiol groups and the disulfide bond have an important role in neurotransmitter receptor binding and that reductive alkylation may be a useful tool for characterizing agonist and antagonist binding sites.

This paper reports on the chemical properties of agonist and antagonist binding sites of the dopamine receptor following reductive alkylation of the receptor with NEM, PCMB and iodoacetamide (IA). The reversibility of this reaction using dithiothreitol (DTT) is also discussed.

MATERIAL & METHODS

[^{3}H] Spiroperidol (23-27 Ci/mmol) was purchased from New England Nuclear (Boston, Massachusetts). [^{3}H] Dopamine (41-45 Ci/mmol) was obtained from Amersham/Searle (Arlington Heights, Illinois). (+)- and (-)- Butaclamol were gifts from Ayerst Laboratories (Toronto, Ontario, Canada), and pargyline, p-chloromercuribenzoate (PCMB), N-ethylmaleimide (NEM), iodoacetamide (IA) were purchased from Sigma Chemical Co. (St. Louis, Missouri). DTT was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). All other reagents and solvents were of analytical grade or better.

Frozen calf brains were obtained from Pel Freeze (Rogers, Arkansas). The caudate nuclei were dissected and frozen at -80°. The tissue was homogenized in 10 vol. of 0.32 M sucrose-15 mM potassium phosphate buffer (pH 7.4) using a polytron (Brinkman) at setting 7 for 20 sec. The homogenate was centrifuged at 900 x g for 10 min. The pellet, P_1, was washed once and both supernatants were combined and then centrifuged again at 11,500 x g for 20 min. The pellet,

P_2, was collected and washed with half of the original volume of homogenizing buffer and recentrifuged for 20 min. The resulting pellet was stored at -80° until use.

The P_2 fraction was thawed, washed once with 20 mM of sodium phosphate buffer (pH 7.4) and homogenized to yield a suspension of 0.4-0.6 mg/ml protein. The standard assay mixture for binding studies contained 20 mM sodium phosphate (pH 7.4) with 0.002% ascorbic acid. Pargyline (10^{-6}M) was added only to the standard assay mixture for [^{3}H] dopamine binding studies. Incubation was in the presence and absence of 10^{-6}M(+)-butaclamol for [^{3}H] spiroperidol binding or 10^{-5}M(+)-butaclamol for [^{3}H] dopamine binding. Samples were assayed at 37° for 10 min in triplicate. To terminate the reaction, the assay samples were then filtered rapidly under vacuum over Whatman GF/B filters and rinsed three times with 5 ml ice-cold sodium phosphate buffer. Finally, the filters were placed in counting vials with scintillation fluor and analyzed by liquid scintillation spectrometry. The final protein concentration for each assay was 240-300 µg for [^{3}H] spiroperidol and 800-1000 µg for [^{3}H] dopamine. In the standard assay system, specific binding was defined as the differences between total binding and non-specific binding under those conditions in which non-specific binding remained linear. Under ideal conditions, the specific binding represents 60-70% of total binding. Protein concentration was determined essentially as described by Lowry et al (14).

RESULTS

The effect of the pretreatment of calf striatal membranes with dithiothreitol (DTT), a disulfide bond reducing agent, on the high affinity stereospecific dopamine agonist and antagonist binding site is shown in Table 1. DTT, at concentrations from 0.5-2.0 mM, diminished the specific binding of the agonist ligand, [^{3}H] dopamine. A concentration of 2 mM DTT

TABLE 1. Effect of DTT on Agonist and Antagonist Binding

DTT (mM)	[^{3}H] DOPAMINE		[^{3}H] SPIROPERIDOL	
	CPM	%	CPM	%
0	986	100	1411	100
0.5	378	38	1448	103
1.0	345	35	-	-
2.0	269	27	1313	93

Table 1. The concentration for [^{3}H] dopamine was 4 x 10^{-10}M and [^{3}H] spiroperidol 9 x 10^{-10}M. Membranes were pretreated with DTT for 20 min at 4°. The reaction was started by the addition of the radioligand as described in Material and Methods. The values presented are the averages of triplicates from three separate experiments.

TABLE 2. Recovery of DTT Inhibition by H_2O_2

ADDITION	[^{3}H] DOPAMINE (cpm)	%
None	947	100%
DTT	419	44%
H_2O_2 (20 mM)	949	100%
DTT + H_2O_2 (5 mM)	892	92%
DTT + H_2O_2 (10 mM)	900	95%

Table 2. The concentrations of DTT and [^{3}H] dopamine were 10^{-3}M and 4.8 x 10^{-10}M respectively. Membranes were pretreated with DTT for 20 min at 4° prior to the addition of H_2O_2. Following the addition of H_2O_2, the membranes were pre-incubated for 20 min at 4°C. The reaction was started by the addition of [^{3}H] dopamine as described in Materials and Methods.

reduced the specific binding to 27% of that measured in untreated membranes. In a separate set of experiments, we determined that the reduction in specific binding due to DTT pretreatment did not alter the pharmacological response of the remaining sites for dopamine agonists and antagonists. In these studies, the calculated inhibition constant (Ki) for apomorphine displacement of agonist binding was identical in untreated and treated membranes but the level of specific binding was diminished by DTT pretreatment. The specific binding for the antagonist ligand [^{3}H] spiroperidol was not significantly inhibited in the presence of DTT at concentrations up to 2 mM. The DTT inactivation of the agonist binding site was reversed by hydrogen peroxide (Table 2). To examine the involvement of thiol groups in dopamine receptor binding, we used a potent -SH group alkylating agent, p-chloromercuribenzoate (PCMB), which attacks essential thiol groups (15). Figure 1 shows that PCMB, at concentrations as low as 2 x 10^{-5}M, inhibited both agonist and antagonist binding in calf striatal membranes. There was slightly more inhibition for agonist binding with low concentrations of PCMB, but at a higher concentration, 5 x 10^{-5}M, PCMB produced a 75-80% reduction in both agonist and antagonist binding. Scatchard analysis (Fig. 2) of the [^{3}H] spiroperidol

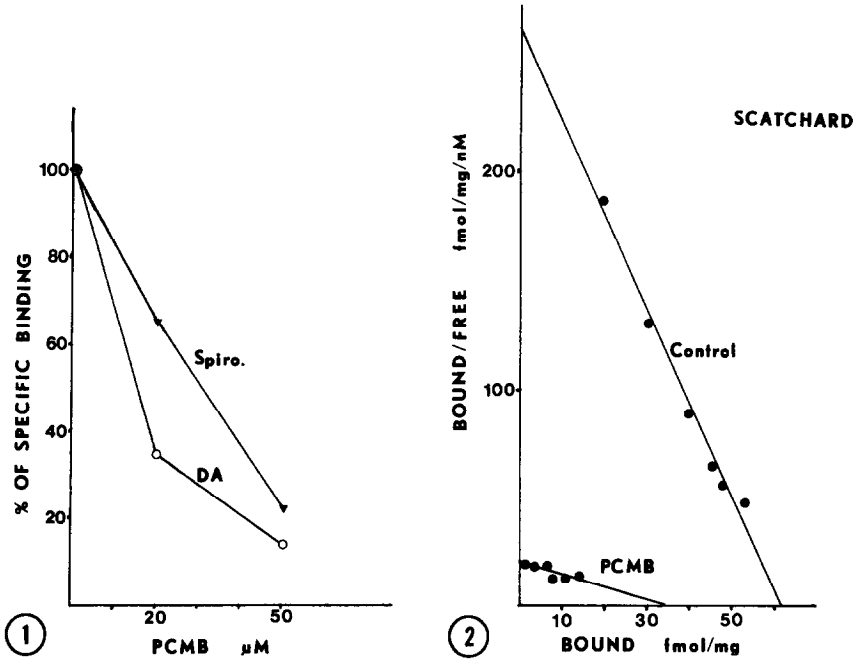

Fig. 1. Inactivation of spiroperidol and dopamine binding by PCMB. The concentration for spiroperidol was 7 x 10^{-10}M (▼) and for dopamine (0) was 6 x 10^{-10}M. Each point represents the average from two separate experiments, each done in triplicate which differ by less than 10%.

Fig. 2. Scatchard plot of [^{3}H] spiroperidol saturation curves with and without PCMB. The concentration for PCMB was 5 x 10^{-5}M. Each point represents the mean of two experiments done in triplicate.

saturation curve revealed that this inhibition was due to both a decrease in receptor affinity and a decrease in the number of binding sites. The K_d value increased from 0.27 nM in untreated membranes to 1.6 nM in the presence of 5 x 10^{-5}M of PCMB and the B_{max} decreased from 65 fmol/mg protein to 32 fmol/mg. PCMB (5 x 10^{-5}M) also caused about a 70% decrease in the B_{max} value for [^{3}H] dopamine binding to calf striatal membranes (data not shown). To verify that the PCMB was reacting with thiol groups, we tested the reversibility of this reaction by pretreating membranes with DTT prior to the addition of PCMB. The results, as shown in Table 3, demonstrate that the PCMB inhibition of [^{3}H] spiroperidol binding was completely reversed by adding the reducing agent DTT. Partial reversal of binding activity was observed for the agonist ligand [^{3}H] dopamine (Table 2). The lack of

TABLE 3. Recovery of PCMB Inhibition by DTT

ADDITION	PERCENT OF BINDING	
	[^{3}H] DOPAMINE[a]	[^{3}H] SPIROPERIDOL[b]
None	100	100
PCMB 50 μM	25	44
PCMB 100 μM	5	21
PCMB 50 μM +0.5mM DTT	39	81
PCMB 100 μM +0.5mM DTT	38	99

Table 3. a. The concentration of [^{3}H] dopamine was 1.2 x 10^{-9}M, (100% = 740 cpm).
b. The concentration of [^{3}H] spiroperidol was 1.3 x 10^{-9}M, (100% = 894 cpm).
DTT was added to the incubation mixture for 20 min in ice before the addition of PCMB.

complete recovery for agonist binding by DTT may be due to the inhibitory effect of DTT on agonist binding as shown in Table 1.

Another thiol agent, N-ethylamaleimide (NEM), was also tested for its ability to alkylate the dopamine receptor. Figure 3 shows that unlike PCMB,

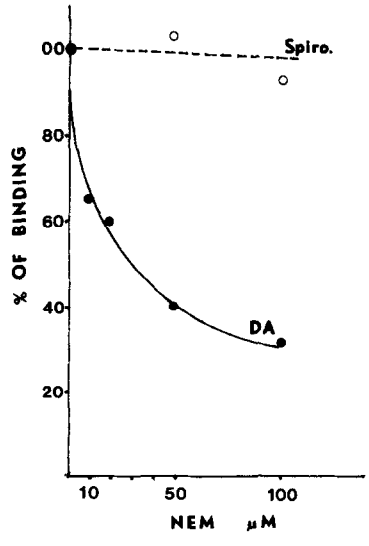

Fig. 3. Inactivation of spiroperidol and dopamine binding by NEM. The concentration for spiroperidol (○) was 9 x 10^{-10}M and for dopamine (●) was 4 x 10^{-10}M. The data represent the average of triplicates from a representative experiment which was repeated three times with less than a 10% difference.

NEM only inhibited the agonist binding site. A 60% reduction in [^{3}H] dopamine binding was observed in the presence of 10^{-4}M NEM. There was no affect on [^{3}H] spiroperidol binding in the presence of NEM (Fig. 3). Pre-treatment of the membranes with the weak thiol agent, iodoacetamide (IA), however, showed that neither the agonist nor the antagonist binding site was affected (data not shown).

DISCUSSION

There is considerable evidence suggesting that the radiolabeled agonists and antagonists, which are used to study the dopamine receptor, bind to different sites (2,4,8,9). Recently, several investigators have attempted to biochemically distinguish these sites through the chemical modifications of the receptor protein. Kayaalp and Neff (15) have shown that the reducing agents, ascorbic acid and sodium bisulfite, selectively inhibit dopamine agonist but have no effect on antagonist binding. Ascorbate also inhibits dopamine-sensitive adenylate cyclase in this preparation (18). Recently, other investigations, using [^{3}H] spiroperidol, have shown that divalent cations and guanine nucleotides regulate agonist binding in caudate membranes without affecting antagonist binding (16,17). In this report, we used thiol-reducing and alkylating agents to study agonist and antagonist binding to the dopamine receptor. We determined that the alkylating agent NEM selectively inactivated dopamine agonist binding sites but had no effect on antagonist binding. Inactivation of both agonist and antagonist binding sites also occurred with the potent alkylating agent, PCMB. These data suggest a differential orientation of thiol groups for dopamine agonist and antagonist binding sites in the membrane. The results are also consistent with the hypothesis that the position of the agonist binding site is more to the outside of the membrane (i.e., an extrinsic protein) than is the antagonist binding site (i.e., an intrinsic protein).

The results presented also show that two categories of dopamine agonist binding sites exist in the membrane. Only 60% of the dopamine agonist bind-

ing sites were sensitive to pretreatment with the alkylating agent NEM (see Fig. 3). These data support recent biochemical and pharmacological studies that identify at least two types of dopamine agonist receptors in the membrane. One of these agonist receptor sites is coupled to the enzyme adenylate cyclase (18). Recent studies have shown that both the β-adrenergic-sensitive and the dopamine-sensitive adenylate cyclase can be modified by reducing agents (19,20). Because it has been suggested that conformational changes produced in the β-adrenergic receptor by reducing agents may decrease the coupling of the receptor to adenylate cyclase (20), studies are in progress to determine whether similar conformational changes in the dopamine agonist receptor alter its ability to couple to adenylate cyclase.

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