Importance of thiol groups in ligand binding to D₂ dopamine receptors from brain and anterior pituitary gland

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The effects of the thiol group reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) on D₂ dopamine receptors have been examined in three brain regions (caudate nucleus, putamen and olfactory tubercle), and in the anterior and neurointermediate lobes of the pituitary gland. Whereas the receptors in brain were insensitive to DTNB, a dose-dependent inhibition of [³H]spiperone binding to D₂ receptors in both lobes of the pituitary gland was observed. The effects in the pituitary could be reversed by subsequent treatment with dithiothreitol and could be prevented by prior occupancy of the receptor binding site with a ligand. The effect of DTNB was to decrease the number of ligand-binding sites without altering the affinity of those remaining. The results show that modification of a thiol group of the D₂ dopamine receptor in the two lobes of the pituitary gland tested here significantly affects ligand binding. There is therefore a difference between the D₂ dopamine receptor populations in brain and pituitary in their sensitivity to modification by DTNB, and this may imply the existence of different receptor isoforms in the two tissues.

INTRODUCTION

Many neurotransmitters and hormones act via receptors linked to guanine nucleotide regulatory proteins (G-proteins) (Lefkowitz & Caron, 1988; Strange, 1991a; Tota *et al.*, 1991). From gene cloning studies it is now clear that G-protein-linked receptors for small molecules such as acetylcholine, dopamine and noradrenaline form a homologous family based on common structural motifs. The basic motif comprises seven transmembrane spanning α -helices linked by extracellular and intracellular loops. The seven α -helices are bundled together to form the ligand-binding domain, and an understanding of the structure of this domain will be critical for drug design.

Considerable information has emerged on the amino acid residues that contribute to ligand binding through the application of site-specific mutagenesis to the receptor genes (Fraser *et al.*, 1989; Tota *et al.*, 1991). In particular, for the cationic amine ligands this approach has identified an aspartic acid residue in the third membrane-spanning α -helix that may form the counter-anion for the cationic amine. A complementary approach is chemical modification or protein labelling with reagents specific for particular amino acid side chains.

Using chemical modification and protein labelling, evidence has been obtained for the importance of a carboxyl group in ligand binding to D₁ and D₂ dopamine receptors (Williamson & Strange, 1990; Hollis & Strange, 1991) and muscarinic acetylcholine receptors (Kurtenbach *et al.*, 1990), and this may correspond to the aspartic acid residue mentioned earlier. In addition, modification of thiol groups affects ligand binding to D₁ dopamine receptors (Sidhu *et al.*, 1986; Dewar & Reader, 1990; Hollis & Strange, 1991), but for D₂ dopamine receptors there is some disagreement. Whereas studies with the thiol reagent *N*-ethylmaleimide have demonstrated an effect of thiol group modification on antagonist binding to D₂ dopamine receptors from both brain and pituitary gland (Kilpatrick *et al.*, 1982; Freedman *et al.*, 1982; Scheuhammer & Chierian, 1985; Holden-Dye *et al.*, 1985), when the alternative thiol reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used, modification of thiol groups was without effect on ligand binding to brain D₂

dopamine receptors (Williamson & Strange, 1990). In this paper we have examined the effects of DTNB on D₂ dopamine receptors more carefully.

MATERIALS AND METHODS

Except where otherwise stated, all chemicals and other materials were obtained as outlined in Leonard *et al.* (1987).

Preparation of mixed mitochondrial/microsomal membrane preparations

Mixed mitochondrial/microsomal membrane preparations were prepared from the different bovine tissues essentially as described by Leonard *et al.* (1987). Briefly, bovine brain regions and pituitary lobes were dissected and homogenized in 0.32 M-sucrose solution containing 20 mM-Hepes, pH 7.4 (9 ml/g wet wt. of tissue) with 10 strokes of a Teflon/glass homogenizer at 4 °C. The homogenate was centrifuged (1500 *g*, 10 min) and the supernatant was kept at 4 °C while the pellet was re-homogenized in 4.5 vol. of homogenization solution. After centrifugation the supernatants were combined and re-centrifuged (126000 *g*, 60 min). The supernatant was discarded and the pellet was resuspended (2 ml/g of tissue) in ice-cold sodium phosphate buffer (20 mM, pH 7.5). Protein was determined by the method of Lowry *et al.* (1951) using BSA as a standard. The tissue homogenates were stored at –80 °C.

Modification with DTNB

The procedure was based on that of Means & Feeney (1971). Mixed mitochondrial/microsomal membranes (0.75 mg of protein) were incubated with DTNB [diluted from a stock of freshly prepared DTNB (10 mM)] in a total volume of 1 ml of 20 mM-sodium phosphate buffer, pH 7.5, for 2 h at 22 °C. The membranes were then washed twice by centrifugation (12000 *g*, 10 min, 4 °C) and resuspension in 1 ml of phosphate buffer. The membranes were finally resuspended in 0.75 ml of phosphate buffer and assayed for [³H]spiperone binding. Parallel control experiments were performed with membranes that had not been treated with DTNB but otherwise were treated identically.

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); IC₅₀, concn. causing 50 % of maximal inhibition; EC₅₀, half-maximal effective dose.

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In order to reverse the effect of DTNB, 0.75 ml of membrane suspension, modified as described above, was incubated with 0.75 ml of 10 mM-dithiothreitol in phosphate buffer and the mixture was then incubated at 22 °C for a given time. The membranes were collected by centrifugation and washed twice as described above before resuspension in 0.75 ml of phosphate buffer, and were assayed for [³H]spiperone binding. Control experiments were performed on membranes that had not been treated with DTNB but which had otherwise been treated identically.

Protection of the ligand-binding site was carried out by incubating membranes (0.75 mg of protein) with either haloperidol or domperidone at a given concentration in phosphate buffer for 1 h at 22 °C. Modification with DTNB was then performed as above, but including four washes with 1 ml of phosphate buffer supplemented with BSA (1 mg/ml).

Ligand-binding assays with [³H]spiperone

Mixed mitochondrial/microsomal membranes (approx. 100 µg of protein) were incubated with [³H]spiperone (26.0 Ci/mmol, 0.01–2.5 nM; Amersham International) in a final volume of 1 ml of phosphate buffer for 60 min at 22 °C. Mianserin (0.3 µM) was included to block the binding of [³H]spiperone to 5HT₂ 5-hydroxytryptamine receptors (Withy *et al.*, 1981), and specific [³H]spiperone binding was defined as that binding inhibited by 3 µM-(+)-butaclamol. Assays were terminated by rapid filtration over Whatman GF/B filter paper strips in a Dynatech Automash 2000 Cell Harvester, and the filters were washed with 15 ml of ice-cold buffer (137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.4) before determination of radioactivity.

RESULTS

In initial experiments the effect of a single concentration of DTNB (2.5 mM) was tested on [³H]spiperone binding to a range of bovine tissues. Whereas there was no effect in the brain regions tested (caudate nucleus, putamen and olfactory tubercle), there was a significant decrease in [³H]spiperone binding to membranes from the anterior and neurointermediate lobes of the pituitary gland (Table 1). The effects of DTNB on [³H]spiperone binding to the pituitary anterior and neurointermediate lobes were dose-dependent (Fig. 1). IC₅₀ values (concns. causing 50% of maximal inhibition) of 3.2 ± 1.8 mM and 2.5 ± 0.7 mM (means ± S.D.; three experiments) were obtained for DTNB in the anterior and neurointermediate lobes respectively. In the same experiment,

the use of a range of DTNB concentrations confirmed the lack of an effect on [³H]spiperone binding to the caudate nucleus.

We examined whether the effects of DTNB were reversible by applying dithiothreitol (5 mM) to anterior pituitary membranes pretreated with DTNB. This showed that the DTNB modification could be reversed by treatment with dithiothreitol. The effects of dithiothreitol were time-dependent, requiring up to 3 h for full reversal (Table 2). There was a small effect of dithiothreitol alone on [³H]spiperone binding to D₂ dopamine receptors.

Saturation analysis of [³H]spiperone binding to anterior pituitary membranes treated with DTNB was performed (Fig. 2).

Fig. 1. Inhibition by DTNB of [³H]spiperone binding to D₂ dopamine receptors in the brain and the pituitary gland

Mixed mitochondrial/microsomal membranes from brain caudate nucleus (△) and from the anterior (▲) and neurointermediate (○) lobes of the pituitary gland were treated with different concentrations of DTNB for 2 h at 22 °C. [³H]Spiperone binding to the membranes was determined as described in the Materials and methods section, and expressed as a proportion of that present in untreated membranes processed in parallel experiments. The data are expressed as means ± S.D. from three experiments, with points determined in triplicate.

Table 1. Effect of DTNB on [³H]spiperone binding in different tissues

Membranes from various tissues were incubated with 2.5 mM-DTNB for 2 h at 22 °C and assayed for [³H]spiperone binding as described in the Materials and methods section. Data are expressed as means ± S.D. from three experiments and are given as the [³H]spiperone binding remaining in DTNB-treated membranes as a percentage of that present in untreated membranes in parallel experiments.

Tissue	[³ H]Spiperone binding (% of control)
Brain	
Caudate nucleus	96.3 ± 16.4
Putamen	91.8 ± 3.6
Olfactory tubercle	85.5 ± 6.5
Pituitary gland	
Anterior lobe	44.9 ± 10.7
Neurointermediate lobe	53.0 ± 3.7

Table 2. Reversal of DTNB inhibition of [³H]spiperone binding by dithiothreitol

Membranes of the anterior lobe of the pituitary gland were treated with DTNB (2.5 mM) as described in the Materials and methods section, followed by dithiothreitol (DTT, 5 mM) for various times. [³H]Spiperone binding was determined and expressed as a percentage of that in untreated membranes. Data are expressed as means ± S.D. (three experiments).

Treatment	[³ H]Spiperone binding (% of control)
None	100
DTNB (2.5 mM)	41.4 ± 4.4
DTT (5 mM, 1 h)	92.0 ± 9.6
DTNB (2.5 mM) + DTT (5 mM, 1 h)	44.0 ± 10.0
DTT (5 mM, 2 h)	102.0 ± 2.3
DTNB (2.5 mM) + DTT (5 mM, 2 h)	65.8 ± 1.2
DTT (5 mM, 3 h)	77.9 ± 6.4
DTNB (2.5 mM) + DTT (5 mM, 3 h)	80.5 ± 3.9

Fig. 2. Saturation analysis of [³H]spiperone binding to control and DTNB-treated membranes from the anterior lobe of the pituitary gland

Saturation analysis of [³H]spiperone binding was performed as described in the Materials and methods section on untreated membranes (○) and membranes treated with 1 mM-DTNB (●). The data are from a representative experiment replicated three times, and the curve is the best fit to a one-binding-site model. Binding parameters: control membranes, $B_{\max}$, 154.0 ± 3.8 fmol/mg, K_d 0.146 ± 0.028 nM; DTNB-treated membranes, $B_{\max}$, 95.6 ± 15.9 fmol/mg, K_d 0.090 ± 0.045 nM (means $\pm$ s.d., three experiments).

Table 3. Protection against DTNB inhibition of [³H]spiperone binding

Membranes of the anterior lobe of the pituitary gland were preincubated with haloperidol or domperidone at the stated concentration before the effects of DTNB (2.5 mM) were determined as described in the Materials and methods section. Data are given as means $\pm$ s.d. (three experiments) and are expressed as the percentage protection afforded by the antagonists against the inhibition of [³H]spiperone binding by DTNB.

Antagonist	Protection (%)
Domperidone (10 nM)	39.6 ± 8.8
Domperidone (100 nM)	80.5 ± 13.9
Haloperidol (10 nM)	46.8 ± 14.9
Haloperidol (100 nM)	95.4 ± 6.5

This showed that the effects of DTNB were to decrease the number of [³H]spiperone-binding sites without affecting the affinity of those sites remaining. In order to examine whether the effects of DTNB were at the ligand-binding site of the receptor, experiments were performed where the receptor was preincubated with D₂ dopamine receptor antagonists (haloperidol, domperidone). These antagonists protected the receptors against modification by DTNB in a dose-dependent manner (Table 3). The concentrations of the ligand that gave protection were comparable with their K_i values for binding to D₂ dopamine receptors. Values for the concentrations of the drugs that gave 50% protection can be estimated from the data in Table 3 as 20 nM (domperidone) and 10 nM (haloperidol); the K_i values are 7 nM (domperidone) and 20 nM (haloperidol) (Leonard *et al.*, 1987). One interpretation of these data is that the modification by DTNB that is responsible for inhibition of [³H]spiperone binding is occurring at the ligand-binding site of the receptor.

DISCUSSION

In this paper we describe a specific effect of the thiol reagent DTNB on [³H]spiperone binding to D₂ dopamine receptors in

the anterior and neurointermediate lobes of the pituitary gland. Under identical conditions there is no effect on the brain D₂ dopamine receptor.

The assays in this report were performed using the radioligand [³H]spiperone for labelling D₂ dopamine receptors. The interpretation of these experiments has been complicated by the recent cloning of D_{2(short)}, D_{2(long)}, D₃ and D₄ dopamine receptors, each of which will bind [³H]spiperone with high affinity (Strange, 1990, 1991b; Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991). D₃ and D₄ dopamine receptors are present in lower numbers than D₂ receptors in the brain regions chosen, based on the distribution of mRNA. In the pituitary gland, the D₃ receptor is absent and the distribution of the D₄ receptor has not been reported.

D_{2(short)} and D_{2(long)} dopamine receptors are present in all of the tissues studied here but to different extents (Gandelman *et al.*, 1991); however, these two isoforms do not differ appreciably pharmacologically and have identical amino acid sequences in the putative ligand-binding regions. Therefore it will be assumed here that the assays are detecting a receptor with a D₂-dopamine-receptor-like pharmacological profile but which could be either D_{2(short)} or D_{2(long)}. In addition, [³H]spiperone has also been reported to label 5HT₂, 5-hydroxytryptamine- and spirodecane-binding sites, but in other analyses it has been shown that spirodecane-binding sites are not included under the conditions used here for defining specific [³H]spiperone binding (Leonard *et al.*, 1987). 5HT₂, 5-hydroxytryptamine receptors are absent in the pituitary gland (Simmonds *et al.*, 1986), and in the brain [³H]spiperone binding to these receptors was suppressed by the inclusion of mianserin. Therefore in the present paper the binding of [³H]spiperone is exclusively to D₂ dopamine receptors.

We chose to use DTNB as the thiol reagent in these experiments for certain reasons. It is more specific than other thiol reagents; for example, *N*-ethylmaleimide reacts under certain conditions with free amino groups or histidine residues. Also, the reaction of DTNB is reversible upon addition of a thiol reagent, whereas that of *N*-ethylmaleimide is not (Means & Feeney, 1971).

In the present experiments the specificity of DTNB is apparent. There was no effect of DTNB on [³H]spiperone binding to D₂ dopamine receptors from three brain regions (caudate nucleus, putamen and olfactory tubercle), whereas under identical conditions there was 76% inhibition of [³H]SCH23390 binding to D₁ dopamine receptors in caudate nucleus (Hollis & Strange, 1991). It seems clear, therefore, that antagonist binding to D₂ dopamine receptors in brain is not affected by modification of thiol groups, at least in the three regions we have tested.

In contrast, in the anterior and neurointermediate lobes of the pituitary gland there was a clear dose-dependent effect of DTNB on [³H]spiperone binding to D₂ dopamine receptors. The potency of DTNB was, however, less than that for its effects on D₁ dopamine receptors in the brain [EC₅₀ values (half-maximal effective doses) of 0.18 mM (Dewar & Reader, 1990), 0.45 mM (Sidhu *et al.*, 1986) and 0.07 mM (C. M. Hollis & P. G. Strange, unpublished work)], which presumably reflects the properties of the different receptors.

The effects of DTNB on the D₂ dopamine receptor in the anterior lobe of the pituitary gland can be reversed by the addition of a thiol reagent (dithiothreitol), so that the effect cannot be due to an irreversible denaturation of the receptor. The DTNB effect is to decrease the number of D₂ dopamine receptor binding sites and is not due to an alteration in receptor affinity, and can be prevented by prior occupancy of the receptors with specific antagonists. These observations suggest that modification of a thiol group at the receptor binding site affects ligand binding. As we have not demonstrated this directly, however, it is important to consider alternative interpretations of the results. One alternative possibility is that modification by DTNB is not

within the ligand-binding site, but that it alters the conformation of the receptor, preventing subsequent ligand binding. In this case it is necessary to explain the prevention of modifications by receptor antagonists. This could be accommodated by the receptor existing in two conformations, one that binds ligands but where the modifiable thiol group is not accessible, and one that does not bind ligands but where the modifiable thiol group is accessible.

In other experiments using *N*-ethylmaleimide, specific effects on [³H]spiperone binding to D₂ dopamine receptors in the anterior pituitary gland have been reported (Kilpatrick *et al.*, 1982), in agreement with the present study. Furthermore, two studies in which D₂ dopamine receptors were labelled by [³H]sulpiride reported an effect on receptors in both pig (Holden-Dye *et al.*, 1985) and rat (Freedman *et al.*, 1982) brain. This discrepancy cannot be explained, but certainly in the present study, using the more specific thiol reagent DTNB, a clear difference between receptors from brain and pituitary gland was seen. The possibility, however, of a species variation in D₂ dopamine receptors resulting in the observed discrepancy cannot be discounted.

The results of the present study suggest, therefore, that there is a difference between D₂ dopamine receptors in the brain and the pituitary gland, and that a thiol group is present on the pituitary receptor whose modification by DTNB affects ligand binding. This thiol group is either absent or inaccessible to DTNB in the brain receptor. This in turn implies that the population of bovine D₂ dopamine receptors assayed by [³H]spiperone binding in the pituitary gland in these experiments differs from that in brain. This could reflect the presence of different receptor isoforms in the two sites. According to the arguments presented earlier, the isoforms likely to be detected in the present experiments are D_{2(short)} and D_{2(long)}, although the distributions of receptor isoforms have been determined mainly for the rat, and the present experiments employed bovine tissues in which the relative distributions of D₂, D₃ and D₄ receptors may be different. Recent molecular biological evidence suggests that marked distribution differences in the mRNAs for the two D₂ dopamine receptor isoforms [D_{2(short)} and D_{2(long)}] exist between rat and human species in some tissues (O'Malley *et al.*, 1990; Gandelman *et al.*, 1991).

The D_{2(short)} and D_{2(long)} isoforms differ only by the presence in the longer form of a 29-amino-acid insertion in a region of the receptor not thought to be important for ligand binding. Indeed, they do not differ in amino acid sequence in the putative ligand-binding region and do not differ pharmacologically to an appreciable extent, so it is not easy to see how different distributions of D_{2(short)} and D_{2(long)} in pituitary and brain could explain the present results. It should be noted, however, that in the longer isoform the insertion does contain cysteine residues, and if this were important in explaining the present results it implies either that the insertion plays a role in forming the ligand-binding site or that modification of one of these cysteine residues affects ligand binding indirectly.

A final question concerns possible mechanisms that might lead to inhibition of ligand binding if the modifiable thiol group is at the ligand-binding site. One possibility is that modification occludes part of the binding site but that the thiol group itself does not directly participate in ligand binding. Alternatively, the thiol group may actually participate in ligand binding and its modification could interfere with this. There are a number of cysteine residues within the putative transmembrane spanning region of D₂ dopamine receptors, so there is ample scope for these ideas. Whatever the explanation, the results reported here suggested differences in the D₂ dopamine receptor populations in the brain and the pituitary gland.

We thank the M.R.C. for financial support and Sue Davies for preparing the manuscript.

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