Biochemical Pharmacology, Vol. 44, No. 2, pp. 325–334, 1992.
Printed in Great Britain.

0006-2952/92 $5.00+0.00
© 1992. Pergamon Press Ltd

STUDIES ON THE STRUCTURE OF THE LIGAND-BINDING SITE OF THE BRAIN D_1 DOPAMINE RECEPTOR

CLARE M. HOLLIS and PHILIP G. STRANGE*
Biological Laboratory, The University, Canterbury, Kent CT2 7NJ, U.K.

(*Received* 27 *February* 1992; *accepted* 10 *April* 1992)

Abstract—A series of group-specific modifying reagents were tested for their effects on [^{3}H]SCH23390 binding to brain D_1 dopamine receptors in order to identify amino acid residues at the ligand binding site of the D_1 dopamine receptor that are critical for ligand binding. The dependence of ligand binding on the pH of the incubation medium was also examined. The histidine-selective reagent, diethylpyrocarbonate did affect ligand binding but this is probably not due to an effect at the ligand binding site. Experiments with *N*-acetylimidazole and ethylacetimidate indicated that modification of tyrosine and amino residues did not exert major influences at the ligand binding site. The use of the thiol dithiothreitol indicated that breakage of a disulphide bond altered ligand binding, probably by affecting the receptor conformation, and the use of the sulphydryl reagent 5,5′-dithio-bis-nitrobenzoic acid showed that modification of a sulphydryl group on the receptor inhibited ligand binding. The carboxyl reagent *N,N′*-dicyclohexyl carbodiimide (DCCD) potently inhibited ligand binding and the effect could be prevented by occupancy of the receptor site by an agonist or antagonist so that there is an important carboxyl group at the receptor binding site. The total number of D_1 receptors was reduced after the modification by DCCD and 70% of the residual receptors showed a reduced affinity for binding [^{3}H]SCH23390, the remainder having the same affinity as untreated receptors.

[^{3}H]SCH23390 binding is also reduced by a decrease of pH and this effect seems to depend on the protonation of a group of pK_a 6.9. Saturation analysis of [^{3}H]SCH23390 binding performed at pH 7.5 shows a single class of high affinity sites whereas at pH 6.0, two classes of sites with higher and lower affinities are seen. These studies suggested a model whereby [^{3}H]SCH23390 binding is to two receptor isoforms with different pH dependencies for [^{3}H]SCH23390 binding.

From the application of molecular genetic techniques, it is now clear that there exists a large family of structurally related receptors for neurotransmitters and hormones that signal via G-proteins (guanine nucleotide regulatory proteins) [1, 2]. Each receptor shows a common motif of seven presumed membrane spanning α-helices and between receptors there are significant homologies at the amino acid level. The ligand binding site for receptors for small molecule ligands appears to be formed from the transmembrane α-helical regions which bundle to form a cavity in the membrane. The ligands then bind in this cavity and interactions with certain amino acid side chains are imporant in realizing the binding energy.

The amino acids that contact ligands at the ligand binding site can be inferred in several ways. Amino acids that are found only in receptors for related ligands may play important roles. For example, an aspartic acid residue in the third putative transmembrane region is found only in receptors for cationic amines [3] and may form the counter ion for the positively charged ligand. Site specific mutagenesis of the cloned gene can confirm such speculation and this has been used for β-adrenergic [4] and muscarinic acetylcholine receptors [5]. An alternative, complementary approach is to use chemical modification and protein labelling studies. These approaches have been used to obtain direct information on the importance of the same aspartic acid residue for ligand binding to muscarinic acetylcholine receptors [6] and have inferred its importance for ligand binding to D_2 dopamine receptors [7].

In this report we have studied the ligand binding site of another G-protein-linked receptor, the D_1 dopamine receptor, in detail using chemical modification and pH-dependence studies with [^{3}H]-SCH23390 binding as our assay. D_1 dopamine receptors have been classically defined as the receptors for dopamine linked to stimulation of adenylyl cyclase [8]. D_1 dopamine receptors are now known to be important for a variety of behavioural effects mediated by dopamine [9]. Three D_1 dopamine-like receptors have been cloned and sequenced (D_{1a}: [10–13]; D_{1b}: [14]; D_5 [15]). At present it seems that the cloned D_{1a} receptor is the principal D_1-like receptor present in the striatum. In this report we have used striatal tissue (caudate nucleus) so that the results obtained should reflect the structure of the ligand binding site of the cloned D_{1a} dopamine receptor.

MATERIALS AND METHODS

Preparation of mixed mitochondrial/microsomal membrane preparation of bovine caudate nucleus. Caudate nuclei were dissected from the brains of freshly slaughtered cattle and either used directly or frozen at −80°. The caudate nuclei were homogenized at 4° using a teflon/glass homogenizer (15 strokes, 850 rpm) in 9 vol. of buffer [sodium phosphate, 20 mM, sucrose 0.3 M, trypsin inhibitor 0.001% (w/v), phenylmethanesulphonylfluoride, 0.1 mM,

* Corresponding author. Tel. (0227) 764000 ext. 3697; FAX (0227) 763912.

pH 7.5]. The homogenate was centrifuged (10 min, 1500 *g*, 4°), the supernatant retained and the pellet rehomogenized in 4.5 vol. of buffer as above. This homogenate was centrifuged (10 min, 1500 *g*, 4°), the pellet discarded and the two supernatants combined and centrifuged (45,000 *g*, 60 min, 4°). The resulting pellet was resuspended by homogenization (8 strokes, 850 rpm) in 2 mL/g wet weight of the tissue in buffer (sodium phosphate, 20 mM, pH 7.5) containing a mixture of protease inhibitors [0.05% (w/v) leupeptin, chymostatin, aprotinin, pepstatin A, antipain] and stored at −80°.

pH dependence of [^{3}H]*SCH*23390 *binding.* The procedure used was based on that of Ref. 7. Aliquots of bovine caudate nucleus membrane (1 mg of protein) were diluted into 1 mL of citrate buffer [citric acid (28.5 mM), diethylbarbituric acid (28.5 mM), potassium dihydrogen orthophosphate (28.5 mM), boric acid (28.5 mM), magnesium sulphate (0.8 mM), NaCl (110 mM)] at the chosen pH (between 3.0 and 10.0). The membranes were incubated for 1 hr at 22° before [^{3}H]SCH23390 binding was assayed. In order to determine whether the effect of pH on [^{3}H]-SCH23390 binding was reversible, membranes incubated at a given pH were centrifuged (12,000 *g*, 8 min, 4°) and resuspended in 1 mL of citrate buffer, pH 7.5, prior to assay of [^{3}H]SCH23390 binding.

Chemical modification with 5,5′-dithio-bis-nitrobenzoic acid (DTNB).* The procedure used was modified from the method described [16]. Caudate nucleus membrane protein (0.75 mg) was incubated with DTNB (0.001–2.5 mM) in 1 mL of 20 mM phosphate buffer pH 7.5, for 2 hr at 22°. The membranes were washed by centrifugation (12,000 *g*, 8 min, 4°) and resuspended in 1 mL of 20 mM phosphate buffer, pH 7.5, followed by centrifugation (as above) and resuspension in 0.75 mL of 20 mM phosphate buffer, pH 7.5. [^{3}H]SCH23390 binding was then assayed.

In experiments where the modification by DTNB was reversed with dithiothreitol (DTT), membranes were treated with DTNB and washed twice as described above, resuspended in 1 mL of 2 mM DTT and incubated for 1 hr at 22°. The membranes were then washed twice as described previously before [^{3}H]SCH23390 binding was assayed.

Chemical modification with DTT. Caudate nucleus membranes (0.75 mg of protein) were incubated with DTT (0.1–50 mM) in 1 mL of 20 mM phosphate buffer, pH 7.5, for 1 hr at 22°. The membranes were then washed as described above for DTNB-treated membranes.

Chemical modification with N,N′-dicyclohexyl carbodiimide (DCCD). The procedure used was based on the method of Ref. 7. Caudate nucleus membrane protein (0.75 mg) was centrifuged (12,000 *g*, 8 min, 4°) and resuspended in 20 mM phosphate buffer, pH 6.0 (1 mL final volume) containing DCCD (0.1–100 μM). Incubation with DCCD was carried out for 3 hr at 15°. The membranes were then washed as described for modification with DTNB except that the first resuspension was at pH 6.0, and then [^{3}H]SCH23390 binding was assayed. DCCD (10 mM) was stored in ethanol at −20° and was diluted 1:100 in 20 mM phosphate buffer, pH 6.0, immediately prior to use.

For saturation binding studies, 9 mg of membrane protein was modified with 20 μM DCCD and the membranes were washed with 12 mL of 20 mM phosphate buffer, pH 6.0 with the final resuspension in 9 mL of 20 mM phosphate buffer, pH 7.5. In order to protect sulphydryl residues from DCCD modification, membranes (1 mL) were pretreated with 1 mM DTNB and washed to remove excess reagent (as described above) before treatment with DCCD (0.1–100 μM) as described above. The membranes were then washed twice and incubated in 1 mL of 2 mM DTT in 20 mM phosphate buffer, pH 7.5, for 1 hr at 22° to restore free sulphydryl groups. The membranes were washed twice and resuspended in 0.75 mL of 20 mM phosphate buffer, pH 7.5 before [^{3}H]SCH23390 binding was assayed.

For binding site protection experiments, caudate nucleus membranes (0.75 mg of protein) were pre-incubated in duplicate in 1 mL of 20 mM phosphate buffer, pH 6.0, containing haloperidol (10^{-8}–10^{-5} M) or (+)-SKF38393 (10^{-9}–10^{-4} M) for 1 hr at 22°. Half of the membranes were then treated with 20 μM DCCD (by addition of 250 μL of 100 μM DCCD in 20 mM phosphate buffer, pH 6.0) for 3 hr at 15° and the remainder, to which 250 μL of 20 mM phosphate buffer, pH 6.0, was added, served as controls. Five washing steps were used in order to remove the protecting ligand and these consisted of two washes with 1 mL of 20 mM phosphate buffer, pH 6.0, containing 1 mg bovine serum albumin (BSA)/mL, two washes with 1 mL of 20 mM phosphate buffer, pH 7.5, containing 1 mg BSA/mL and resuspension after the final centrifugation in 0.75 mL of 20 mM phosphate buffer, pH 7.5.

Chemical modification with N-acetylimidazole. This procedure was based on the method described in Ref. 7. Caudate nucleus membranes (0.75 mg of protein) were incubated with *N*-acetylimidazole (0.1–100 mM) in 1 mL of 20 mM phosphate buffer, pH 7.5, for 2 hr at 22°. The membranes were then washed three times, as described for modification with DTNB.

Chemical modification with ethylacetimidate. This method was adapted from the procedure described in Ref. 17. Caudate nucleus membranes (0.75 mg of protein) were centrifuged (12,000 *g*, 8 min, 4°) and resuspended in 1 mL of 0.2 M triethanolamine hydrochloride, pH 8.5, containing ethyl acetimidate (0.01–0.4 M). Membranes were incubated with ethylacetimidate for 2 hr at 22° followed by three washes essentially as described for modification with DTNB with the first resuspension in 0.2 M triethanolamine hydrochloride, pH 8.5, and subsequent resuspensions in 20 mM phosphate buffer, pH 7.5.

For binding site protection experiments, duplicate aliquots of caudate nucleus membranes (0.75 mg of protein) were centrifuged (as described above) and resuspended in 1 mL of 0.2 M triethanolamine

* Abbreviations: DCCD, *N,N′*-dicyclohexyl carbodiimide; DEP, diethylpyrocarbonate; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; DIDS, 4,4′diisothiocyanostilbene-2,2′-disulphonate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

hydrochloride, pH 8.5, containing haloperidol (10^{-8}–10^{-5} M) followed by incubation for 1 hr at 22°. Half of the membranes were then treated with 0.3 M ethylacetimidate for 2 hr at 22° and the other half served as controls. The membranes were subsequently washed as described for binding site protection experiments with DCCD above except that triethanolamine hydrochloride pH 8.5, containing 1 mg BSA/mL was used for the first two washes.

Chemical modification with diethylpyrocarbonate (DEP). The procedure used was adapted from the method described in Ref. 8. Modification with DEP was carried out in 20 mM phosphate buffer both at pH 6.0 and at pH 7.5. For modification at pH 6.0, membranes were centrifuged (12,000 *g*, 8 min, 4°) and resuspended in 20 mM phosphate buffer, pH 6.0, prior to treatment with DEP. Duplicate aliquots of caudate nucleus membranes (0.75 mg of protein) were incubated with DEP (5–100 mM) in 0.75 mL of 20 mM phosphate buffer for 20 min at 4°. Membranes were then centrifuged (as described previously) and half were resuspended in 0.75 mL of 0.1 M hydroxylamine in 20 mM phosphate buffer, pH 7.0, in order to reverse the modification with DEP, whilst the remainder, as controls, were resuspended in 20 mM phosphate buffer, pH 7.0. The membranes were incubated for a further 20 min at 4° followed by centrifugation (as described above) and resuspension in 0.75 mL of 20 mM phosphate buffer, pH 7.5, prior to assay of [^{3}H]SCH23390 binding.

[^{3}H]*SCH*23390 *binding assay for D_1 dopamine receptors.* Membrane suspension (100 μL; approximately 0.1 mg of protein, assuming no loss during washing) was incubated with [^{3}H]SCH23390 (0.5 nM for competition binding studies; 0.05–5 nM for saturation analysis) in 20 mM phosphate buffer, pH 6.0 or 7.5 (1 mL final volume) for 1 hr at 22°. Non-specific binding was defined as that binding inhibited by 10 μM (+)-butaclamol. The assay was terminated by filtration through Whatman 934AH glass fibre filter sheets mounted on a Dynatech Automash 2000 cell harvester, the filters then being washed with 15 mL of ice-cold phosphate buffer (12.3 mM Na_2HPO_4, 87.7 mM NaH_2PO_4), pH 6.0 or ice-cold buffer (20 mM phosphate, 150 mM NaCl) pH 7.5 according to the pH of the assay buffer. Radioactivity trapped on the filters was determined by liquid scintillation counting.

Assay procedure modified for [^{3}H]*SCH*23390 *binding to membranes derived from pH dependence experiments.* Membrane suspension (100 μL; approximately 0.1 mg of protein) was incubated with 0.5 nM [^{3}H]SCH23390 (65–75 Ci/mmol, Amersham) in citrate buffer (1 mL final volume) at the desired pH for 1 hr at 22°. Non-specific binding was defined using 10 μM (+)-butaclamol. The assay was terminated by the addition of 5 mL of citrate buffer at 4° and immediate filtration through Whatman GF/B glass fibre filter discs mounted on a Millipore filtration manifold. Each filter was immediately given two 5 mL washes of buffer and radioactivity on the filter was determined.

Data analysis. Saturation and competition binding data were analysed by non-linear least-squares curve fitting using the computer programs EBDA and LIGAND [19, 20]. For the effects of pH on ligand binding the hydrogen ion concentration was treated as if it were a competing ligand. For binding site protection experiments, the percentage protection of the ligand binding site for each ligand concentration was calculated and treated as if it were percentage occupancy of receptor.

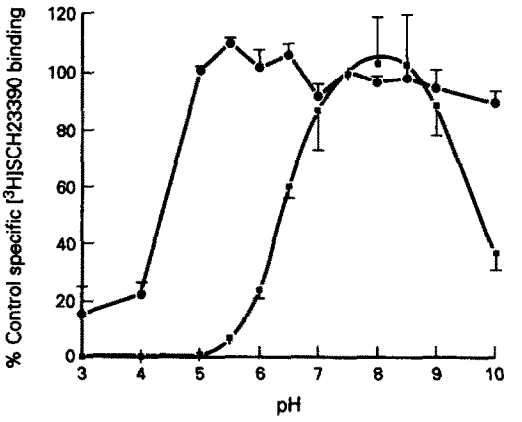

Fig. 1. pH dependence of [^{3}H]SCH23390 binding. Bovine caudate nucleus membranes were incubated at the chosen pH for 1 hr at 22°. Binding of [^{3}H]SCH23390 (0.5 nM) was assayed either at the chosen pH (■) or following adjustment of the pH to 7.5 (●). Values shown represent the means ± range of two experiments and are expressed as a percentage of specific [^{3}H]SCH23390 binding to membranes incubated at pH 7.5.

RESULTS

pH dependence of [^{3}H]*SCH*23390 *binding*

In preliminary experiments the pharmacological profile of [^{3}H]SCH23390 binding to bovine caudate nucleus membranes was established using a series of specific competing ligands. This showed that the specific binding of [^{3}H]SCH23390 as in the present report is exclusively to D_1 dopamine receptors with no component of binding to $5HT_2$ serotonin receptors (data not shown).

Specific [^{3}H]SCH23390 binding to D_1 dopamine receptors was then determined following pre-incubation of caudate nucleus membranes at pH values ranging from 3 to 10 for 1 hr at 22° (Fig. 1). The optimal pH for [^{3}H]SCH23390 binding was in the range of pH 8.0–8.5 and binding decreased markedly as the pH was decreased from 8 to 5, with a 50% reduction in binding at pH 6.4 ± 0.1 (mean ± range, N = 2). The shape of the pH dependence curve in the range pH 5–8 is consistent with interaction of [^{3}H]SCH23390 with a single ionizable group. An increase in pH from 8.5 to 10 resulted in a loss of approximately 60% of control-specific [^{3}H]SCH23390 binding which was probably due to deprotonation of the ligand. The effects of pH on [^{3}H]SCH23390 binding were reversed by adjustment of the pH to 7.5 for membranes which had been incubated in the pH range 5–10. However,

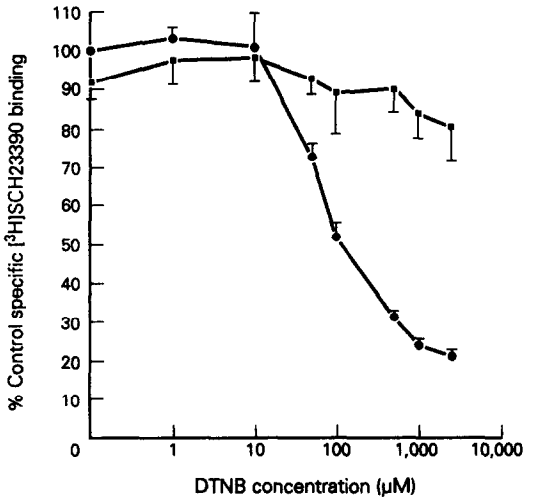

Fig. 2. Effect of DTNB on [^{3}H]SCH23390 binding. Specific [^{3}H]SCH23390 binding to caudate nucleus membranes was determined after treatment with increasing concentrations of DTNB alone (●) or DTNB followed by 2 mM DTT (■) as described in Materials and Methods. Values shown are the mean ± SD of three experiments and are expressed as a percentage of the control specific [^{3}H]SCH23390 binding to membranes treated in the absence of DTNB and DTT.

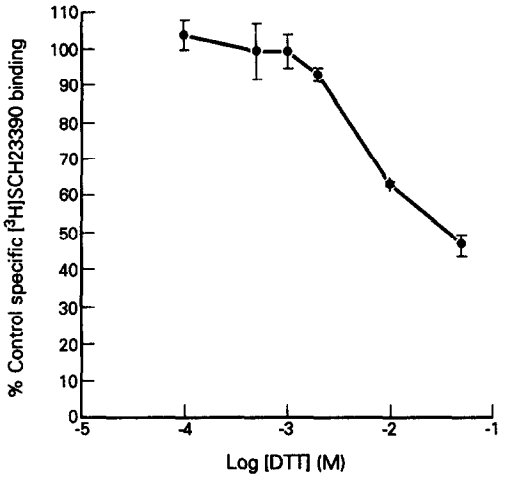

Fig. 3. Effect of DTT on [^{3}H]SCH23390 binding. Specific [^{3}H]SCH23390 binding to caudate nucleus membranes was determined after treatment with increasing concentrations of DTT for 1 hr at 22°. Values shown represent the means ± SD of three experiments and are expressed as a percentage of the specific binding to membranes treated in the absence of DTT.

an irreversible reduction in [^{3}H]SCH23390 binding (to 15–23% of the control) was observed for membranes incubated at pH 3 and 4, which may in part be due to the aggregation of membrane protein which was noted under these conditions.

Effect of DTNB treatment on [^{3}H]*SCH*23390 *binding*

The effect of modification of sulphydryl residues on [^{3}H]SCH23390 binding to the D_1 dopamine receptor was examined by treatment with the sulphydryl-specific reagent DTNB. A decrease in specific [^{3}H]SCH23390 binding was observed with increasing DTNB concentration in a dose-dependent manner (Fig. 2). At a concentration of 2.5 mM DTNB, a plateau was reached with a maximum loss of approximately 80% of [^{3}H]SCH23390 binding and the concentration of DTNB required to achieve 50% of this maximum reduction in [^{3}H]SCH23390 binding (EC_{50}) was 69 ± 15 μM (mean ± SD, N = 3). Eighty to one hundred percent of control [^{3}H]SCH23390 binding was recovered after the addition of 2 mM DTT to DTNB-treated membranes in order to release thionitrobenzoate ions from the protein and thus restore free sulphydryl residues.

Effect of DTT on [^{3}H]*SCH*23390 *binding*

[^{3}H]SCH23390 binding to caudate nucleus membranes treated with DTT was examined and a dose-dependent decrease in binding for DTT concentrations above 1 mM was observed (Fig. 3). The highest concentration of DTT tested (50 mM) resulted in a loss of approximately 50% of control specific [^{3}H]SCH23390 binding and the concentration of DTT used to reverse DTNB modification (2 mM) caused a 7% reduction in binding.

Effect of DCCD on [^{3}H]*SCH*23390 *binding*

The effect of DCCD treatment on [^{3}H]SCH23390 binding to caudate nucleus membranes was examined using conditions of DCCD treatment (3 hr, 15°, pH 6.0) such that predominantly carboxyl and sulphydryl residues would be modified [21, 22]. A dose-dependent decrease in specific [^{3}H]SCH23390 binding to membranes was observed as the DCCD concentration was increased (Fig. 4), with a 50% reduction in binding (EC_{50}) at 7.5 ± 2.5 μM (mean ± SD, N = 3). Protection of sulphydryl residues with DTNB, prior to DCCD treatment and subsequent removal of the protecting groups with DTT, resulted in a dose-dependent decrease in [^{3}H]SCH23390 binding with increasing DCCD concentration similar to that observed for DCCD alone (EC_{50} = 4.4 ± 1.8 μM; mean ± SD, N = 3). DTT had no effect on the loss of binding associated with DCCD treatment of the membranes (EC_{50} = 6.3 ± 3.2 μM; mean ± SD, N = 3).

Saturation binding analysis of [^{3}H]SCH23390 binding to control and DCCD-treated membranes (20 μM DCCD) (Fig. 5) revealed a marked reduction in the B_{max} of [^{3}H]SCH23390 binding to DCCD-treated membranes compared with the control. In addition, two affinities of [^{3}H]SCH23390 binding to DCCD-treated membranes (K_d = 0.08 ± 0.05 and 2.72 ± 1.42 nM; mean ± SD, N = 3) were observed compared with a single site of binding to control membranes (K_d = 0.10 ± 0.01 nM; mean ± SD, N = 4) (Table 1). As DCCD treatments of caudate nucleus membranes were carried out at pH 6.0, saturation binding of [^{3}H]SCH23390 to membranes at this pH was examined (Fig. 6) and this study revealed two classes of binding site with K_d values (0.12 ± 0.08 and 1.16 ± 0.19 nM; mean ± SD, N = 3) similar to those observed for DCCD-treated

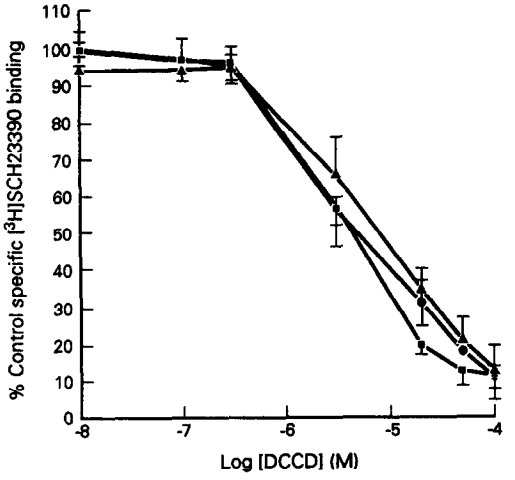

Fig. 4. Effect of DCCD on [^{3}H]SCH23390 binding. Specific [^{3}H]SCH23390 binding to caudate nucleus membranes was determined after treatment with increasing concentrations of DCCD (▲), pretreatment with DTNB followed by treatment with DCCD and removal of sulphydryl group protection with DTT (■) or treatment with DCCD and DTT (●). Values shown are the means ± SD of three experiments and are expressed as a percentage of the control-specific [^{3}H]SCH23390 binding to membranes treated in the absence of DCCD.

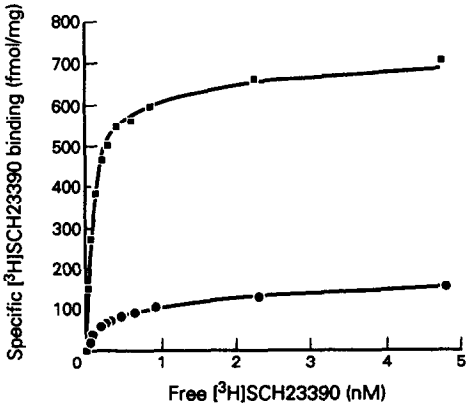

Fig. 5. Saturation analysis of [^{3}H]SCH23390 binding to control and DCCD-treated membranes. Specific [^{3}H]-SCH23390 binding to control membranes (■) and membranes treated with 20 μM DCCD (●) was determined in the presence of increasing concentrations of free radioligand. Non-specific binding was defined using 10 μM (+)-butaclamol. The figure shows data from a single representative experiment, with determinations in triplicate. The experiment was replicated as described in Table 1 which also gives the derived ligand binding parameters.

membranes but with a much greater binding capacity for the lower affinity sites (Table 1).

Binding site protection experiments with DCCD

The dopamine receptor antagonist haloperidol

Table 1. Parameters of [^{3}H]SCH23390 binding to control and DCCD-treated membranes, and membranes assayed at pH 6.0

Condition	N	K_d (nM)	B_{max} (fmol/mg)
Control (pH 7.5)	4	0.10 ± 0.01	867 ± 136
DCCD-treated (pH 7.5)	3	0.08 ± 0.05 2.72 ± 1.42	96 ± 30 226 ± 91
pH 6	3	0.12 ± 0.08 1.16 ± 0.19	106 ± 130 603 ± 90

Dissociation constants (K_d) and maximal binding capacity (B_{max}) from saturation binding of [^{3}H]SCH23390 to control membranes, DCCD-treated membranes and membranes at pH 6.0 are shown.

Values are mean ± SD for the number of experiments (N) indicated in the table.

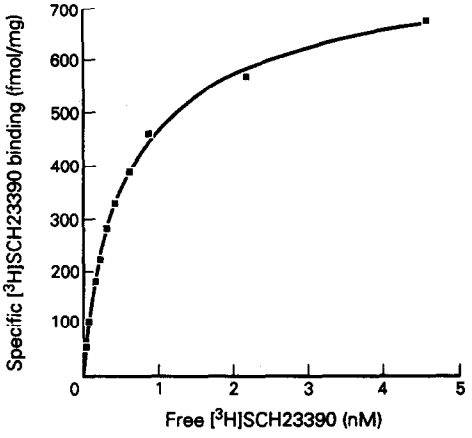

Fig. 6. Saturation analysis of [^{3}H]SCH23390 binding to caudate nucleus membranes at pH 6.0. Caudate nucleus membranes were incubated with increasing concentrations of [^{3}H]SCH23390 (0.05–5 nM) at pH 6.0, for 1 hr at 22°. Non-specific binding was defined using 10 μM (+)-butaclamol. The figure shows data from a single representative experiment, with determinations in triplicate. The experiment was replicated as in Table 1 which also gives the derived ligand binding parameters.

and D_1 dopamine receptor agonist (+)-SKF38393 were used as protecting ligands in experiments to determine whether the reduction in [^{3}H]SCH23390 binding associated with DCCD treatment was due to a modification at the ligand binding site of the receptor. The choice of haloperidol may at first sight seem unusual as it is not a typical D_1 receptor ligand. When, however, more potent D_1 dopamine receptor selective ligands were used in protection experiments e.g. (+)-butaclamol, SCH23390, *cis*-piflutixol, it proved impossible to wash the ligands away after treatment of the membranes. Haloperidol, with its moderate affinity for the D_1 dopamine receptor, could be washed away satisfactorily as could (+)-SKF38393. Preincubation of membranes with haloperidol and (+)-SKF38393 (Fig. 7) for 1 hr at

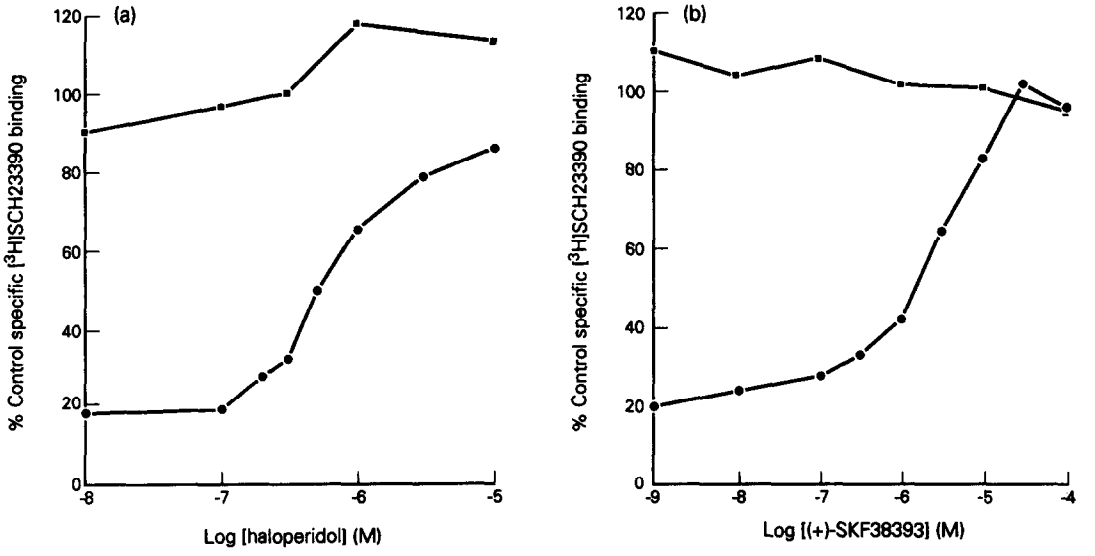

Fig. 7. Inhibition of the effect of DCCD with protecting ligands. The percentage of the maximum DCCD effect observed in membranes preincubated with increasing concentrations of haloperidol (a) and (+)-SKF38393 (b) is shown, after treatment with 20 μM DCCD (●) or in control membranes (■). Data were analysed by LIGAND and conformed to a one-site model ($P > 0.05$) for both ligands. The figure shows data from a single representative experiment, with determinations in triplicate. The experiment has been replicated with similar results at least three times.

22° afforded protection against the effects of 20 μM DCCD. Dose-related protection was observed with increasing concentrations of both ligands. In experiments with protecting ligands but omitting the DCCD treatment, 100% of control-specific binding was observed, indicating that the ligands were removed from the membranes by the washing procedure used. Analysis of the ligand protection data showed that in affording protection the ligands appeared to interact with single classes of binding sites and this analysis gave EC_{50} values of 0.59 ± 0.19 μM (mean ± SD, N = 4) for haloperidol and 3.79 ± 1.14 μM (mean ± SD, N = 3) for (+)-SKF38393. The corresponding IC_{50} values for these ligands in competition experiments versus [^{3}H]-SCH23390 (0.5 nM) binding to caudate nucleus membranes at pH 6.0 were: haloperidol 88.9 ± 31.3 nM, (+)-SKF38393 416 ± 34 nM (mean ± SD, N = 3).

Effect of N-acetylimidazole on [^{3}H]SCH23390 binding

Modification with *N*-acetylimidazole was carried out under conditions (pH 7.5, 2 hr, 22°) such that predominantly tyrosyl residues would be acetylated [23] and the reagent would be most stable [16]. However, caudate nucleus membranes treated with *N*-acetylimidazole, at concentrations of up to 100 mM, showed no reduction in [^{3}H]SCH23390 binding (data not shown).

Effect of ethylacetimidate on [^{3}H]SCH23390 binding

The effect of modification of amino groups on [^{3}H]SCH23390 binding to the D_1 dopamine receptor was examined by treatment with ethyl acetimidate, a reagent specific for α- and ε-amino groups [24]. A decrease in specific [^{3}H]SCH23390 binding was observed with increasing reagent concentration with an EC_{50} of 0.18 ± 0.02 M (mean ± SD, N = 3) (Fig. 8). In binding site protection experiments, however,

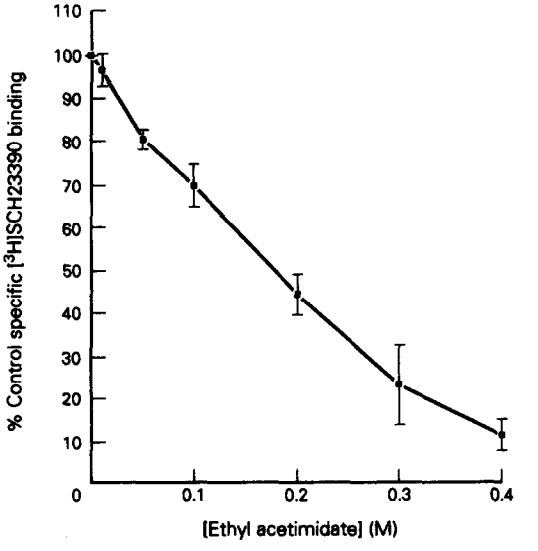

Fig. 8. Effect of ethylacetimidate on [^{3}H]SCH23390 binding. Specific [^{3}H]SCH23390 binding to caudate nucleus membranes was determined after treatment with increasing concentrations of ethylacetimidate for 2 hr at 22°. Values shown are the means ± SD of three experiments and are expressed as a percentage of the control-specific [^{3}H]-SCH23390 binding to membranes treated in the absence of ethylacetimidate.

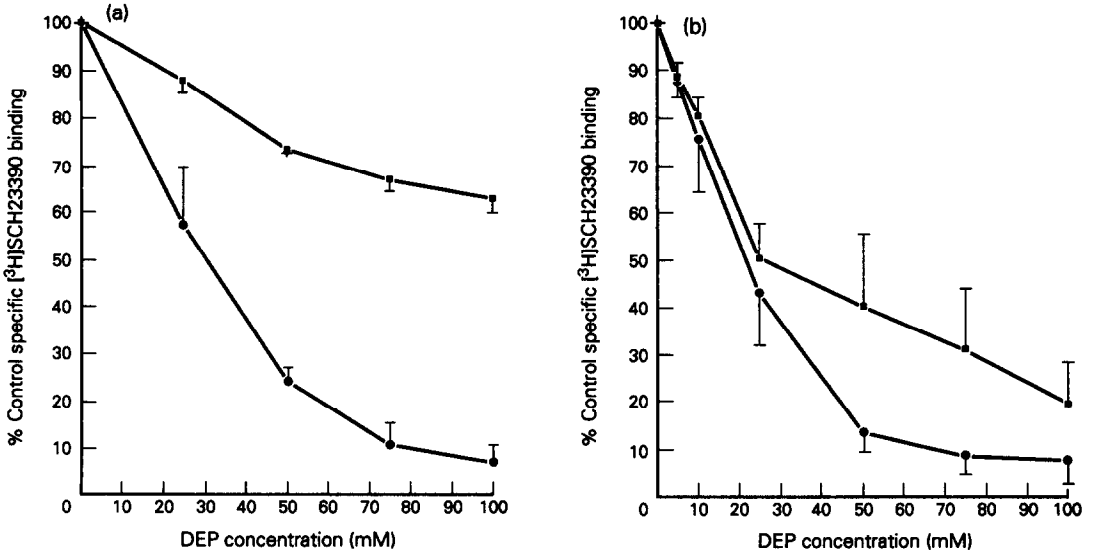

Fig. 9. Effect of DEP on [^{3}H]SCH23390 binding. [^{3}H]SCH23390 binding to caudate nucleus membranes treated with increasing concentrations of DEP at pH 6.0 (a) and pH 7.5 (b) for 20 min at 4° is shown. [^{3}H]SCH23390 binding was determined after incubation of the membranes in the presence (■) or absence (●) of 0.1 M hydroxylamine. Values are the means ± SD of three experiments and are expressed as a percentage of the control specific [^{3}H]SCH23390 binding to membranes treated in the absence of DEP.

haloperidol (up to 10 μM) failed to protect against the reduction in [^{3}H]SCH23390 binding caused by treatment with 0.3 M ethylacetimidate.

Effect of DEP on [^{3}H]*SCH*23390 *binding*

Modification with DEP was carried out using conditions (pH 6.0 or 7.5, 20 min, 4°) which would be expected to result in a selective attack on histidyl and primary amino groups [16]. A dose-dependent reduction in specific [^{3}H]SCH23390 binding to membranes treated with increasing concentrations of DEP was observed at both pH 6.0 and 7.5 (Fig. 9). The reagent was slightly more potent at pH 7.5 (EC_{50} = 22 ± 5 mM; mean ± SD, N = 3) than at pH 6.0 (EC_{50} = 30 ± 5 mM; mean ± SD, N = 3). The reduction in [^{3}H]SCH23390 binding caused by DEP treatment at pH 6.0 was partly reversed (60–70%) by subsequent incubation of the membranes with 0.1 M hydroxylamine. However, the modification at pH 7.5 was reversed to a much smaller extent (0–30%) by hydroxylamine.

DISCUSSION

In this report we provide, using chemical modification and pH dependence studies on the D_1 dopamine receptor, evidence for the importance of sulphydryl residues and acidic ionizable groups at the ligand binding site of this receptor. These residues may be important in ligand binding to this receptor. D_1 dopamine receptors were assayed in this report using specific [^{3}H]SCH23390 binding which in bovine caudate nucleus is exclusively to D_1 dopamine receptors.

The specific tyrosine modifying agent, *N*-acetylimidazole failed to affect ligand binding to D_1 dopamine receptors. Since tyrosyl residues in carboxypeptidase A were modified by *N*-acetylimidazole under similar conditions [23], this suggests that tyrosine residues are not important for [^{3}H]SCH23390 binding. An analogous study on D_2 dopamine receptors also indicated a lack of importance of tyrosyl residues in ligand binding to that receptor [7]. There are, however, conserved tyrosine residues within the presumed ligand binding regions of receptors for cationic amines and Hulme *et al.* [25] have proposed that at least for the muscarinic acetylcholine receptor, tyrosine residues may be involved in a conformational switch after ligand binding. Srivastava and Mishra [26] have reported effects of tyrosine-modifying agents on ligand binding to both D_1 and D_2 dopamine receptors. In their study, however, teranitromethane and 4-nitrobenzene sulphonylfluoride were used and tetranitromethane has additional effects on residues other than tyrosine [17]. Therefore, although the present data suggest that tyrosine residues are unimportant for ligand binding to D_1 dopamine receptors it would be prudent to await the results of mutagenesis studies in order to clarify the situation. It could be, for example, that tyrosine residues in the D_1 receptor are inaccessible to *N*-acetylimidazole under the conditions used.

Amidination of amino groups with ethylacetamidate resulted in a reduction of [^{3}H]SCH23390 binding to D_1 dopamine receptors. The effect was, however, not prevented by occupancy of the receptor by a protecting ligand. It, therefore, is not due to modification of a group at the ligand binding site and probably reflects effects on the conformation of

the receptor protein following modification of accessible external amino groups. Similar data have been reported using the lysine modifying reagent DIDS [27].

The sulphydryl reagent DTNB markedly reduced [^{3}H]SCH23390 binding in the present study and the effects of DTNB could be reversed by subsequent treatment with DTT indicating a specific effect at a free sulphydryl residue. DTT itself also reduced [^{3}H]SCH23390 binding albeit at rather high concentrations so that the integrity of a disulphide bond may be important for ligand binding. These data are in agreement with those of Dewar and Reader [28] and Sidhu *et al.* [29] which also provided evidence for the importance of free sulphydryl groups and disulphide bonds in D_1 dopamine receptors. There is considerable evidence that a disulphide bond between the putative first and second extracellular loops is important in forming the ligand binding conformation of G-protein linked receptors [3, 30]. Perhaps it is the breaking of this bond that accounts for the effects of DTT. In Refs 28 and 29 it was shown that the effects of DTNB on D_1 dopamine receptors could be prevented by occupancy of the ligand binding site by an agonist and partially by antagonists suggesting that the effects of DTNB are to modify a sulphydryl residue at the ligand binding site. Cysteine residues are found in the putative third and sixth membrane spanning domains of the D_1 dopamine receptor.

The results of the experiments suggest that there is a cysteine at the ligand binding site whose alkylation affects ligand binding. The differential protection by agonists versus antagonists suggests that the cysteine involved may be closer to the agonist binding region and this may be subtly different from the antagonist binding region. The role of such a cysteine residue is unclear but it could be involved in hydrogen bonding or, if the pK_a of the cysteine were sufficiently reduced by the microenvironment, in forming an ionic interaction with the cationic ligand.

Treatment of caudate nucleus membranes with DEP at pH 6.0 and 7.5 resulted in a dose-dependent reduction in [^{3}H]SCH23390 binding. At acidic pH, DEP reacts predominantly with amino and histidyl groups of proteins although reactions with sulphydryl, tyrosyl and serine residues have been observed [18]. It is possible to distinguish between modification of amino and histidyl residues as hydroxylamine treatment can only reverse the modification of histidine groups. The results obtained indicate that 60–70% of the reduction in binding observed after DEP treatment at pH 6 is reversed by hydroxylamine and so this loss of binding represents histidine modification. The irreversible reduction in binding at pH 6 could be due to modification of amino groups, assuming that the reduction is due to a conformational change, as suggested from the experiments using ethylacetimidate treatment, with modification of amino groups outside the ligand binding site leading to reductions in ligand binding (see above). An alternative possibility is that a number of dicarbethoxyhistidyl residues were formed during DEP treatment and that addition of hydroxylamine resulted in ring cleavage thus causing an irreversible modification. At pH 7.5, where DEP treatment resulted in a largely irreversible loss of [^{3}H]SCH23390 binding, reaction with other groups such as sulphydryl residues must be considered in addition to the possibilities discussed above. The lower level of reversibility at pH 7.5 compared to pH 6 reflects a pH-dependent shift in the selectivity of the DEP reaction either from the formation of carbethoxyhistidine residues to formation of dicarbethoxyhistidine residues or to modification of amino and other residues. Binding site protection experiments to determine whether the reactive histidine residue at pH 6 was at the ligand binding site of the receptor were not performed as previous work on the D_2 dopamine receptor [7] showed that the modification with DEP at pH 6 was spontaneously reversed during the washing procedure to remove protecting ligands. It does appear unlikely, however, that histidine residues are involved in ligand binding to the D_1 dopamine receptor from the circumstantial evidence that no histidine residues are present in the putative transmembrane regions of the protein where the ligand binding site is thought to be situated.

The results of experiments with DCCD, however, did provide evidence for the involvement of carboxyl residues in ligand binding to the D_1 dopamine receptor. These results support earlier preliminary indications of a role for carboxyl groups in ligand binding to D_1 dopamine receptors using the reagent EEDQ [31]. DCCD treatment of caudate nucleus membranes in the present study resulted in a marked reduction in [^{3}H]SCH23390 binding which can be attributed to modification of carboxyl residues as, although DCCD can also react with sulphydryl groups, protection of cysteine residues by DTNB treatment had no effect on the subsequent loss of [^{3}H]SCH23390 binding. The ability of the antagonist ligand, haloperidol, and the agonist (+)-SKF38393 to protect against modification by DCCD would indicate that the loss of [^{3}H]SCH23390 binding was due to modification of a carboxyl residue at the ligand binding site of the receptor. However, the EC_{50} values for protection with these ligands were 6–10-fold higher than the IC_{50} values of the ligands measured in ligand binding assays at pH 6. Similar discrepancies were observed between EC_{50} values for protection and K_i values of ligands in competition experiments in DCCD modification experiments on the D_2 dopamine receptor [7] using a range of antagonist ligands for binding site protection. The suggested explanation of the discrepancies in that study was that if protecting ligands were less hydrophobic than the DCCD then they would wash out of the tissue more quickly and higher ligand concentrations would be required to protect the receptor against DCCD treatment.

The results of saturation binding experiments also indicated that DCCD modification affected carboxyl residues at the ligand binding site as a marked decrease in the B_{max} of [^{3}H]SCH23390 binding was observed. However, when partial modification was achieved approximately 70% of the binding sites remaining after treatment with DCCD also exhibited a lower affinity for [^{3}H]SCH23390 (K_d = 2.72 nM) compared with the control (K_d = 0.1 nM). A possible

explanation for this decrease in binding affinity will be discussed later in the light of the results of experiments performed to determine the effect of pH on [^{3}H]SCH23390 binding to the receptor.

The pH dependence profile of [^{3}H]SCH23390 binding to D_1 dopamine receptors showed that binding was reversibly inhibited by a decrease in pH. The effect of pH in the range 5–8 could be analysed in terms of the protonation of a single ionizing group so that hydrogen ions were behaving as if they were competitive inhibitors of ligand binding. Making this assumption, the pK_a of the ionizing group can be estimated as 6.9 after correcting for occupancy of the receptors by radioligand. The simplest rationalization of these data is that the ionizing group is the same carboxyl modified by DCCD and that this corresponds to the aspartic acid residue (Asp 103) in the third transmembrane spanning region which has been shown for other cationic amine receptors to be essential for ligand binding [4, 6]. A pK_a of 6.9 is rather high for an aspartic acid residue and the pK_a of the corresponding residue has been inferred as 5.4 at the muscarinic acetylcholine receptor [32] and 5.5 at the D_2 dopamine receptor [7]; after correction for binding site occupancy. The microenvironment of the receptor site could be different for the D_1 dopamine receptor and mutagenesis studies will be needed to confirm these speculations. There is also considerable evidence for the participation of groups of higher pK_a, which may also be aspartic acid residues, in the binding of selective ligands to muscarinic and D_2 dopamine receptors [7, 32, 33].

Further indirect evidence for the importance of ionizing groups at the receptor site being involved in ligand binding came from saturation analysis of [^{3}H]SCH23390 binding to D_1 dopamine receptors that had been treated with DCCD. Whereas saturation analysis of [^{3}H]SCH23390 binding to control membranes (at pH 7.5) indicated a single class of high affinity binding sites (K_d = 0.1 nM), when DCCD treated membranes were tested (at pH 7.5), although overall there were fewer binding sites, the residual binding sites exhibited two affinities for [^{3}H]SCH23390 (K_d = 0.08 nM, 2.72 nM). As the DCCD modification was performed at pH 6 saturation analysis was then performed at pH 6 and two affinity states were seen whose affinities (K_d = 0.12 nM, 1.16 nM) agreed quite closely with those seen in DCCD-treated membranes.

These observations can be rationalized in the following model. [^{3}H]SCH23390 binding to D_1 dopamine receptors depends on interaction with several groups including an electrostatic interaction with a carboxyl group (Asp 103 in the D_1 sequence). In bovine caudate nucleus there are two forms of D_1 receptors in which the pK_a of this carboxyl group differs. In the minor form (≈15%) the pK_a is about 5, in the other predominant form (≈85%) the pK_a is about 7. Either form binds [^{3}H]SCH23390 with high affinity (K_d about 0.1 nM) when the carboxyl group is fully ionized. Using the equation for the pH dependence of the ligand dissociation constant derived in Ref. 7 it can be shown that at pH 7.5 both forms of receptor will display high affinity (K_d about 0.1 nM) for [^{3}H]SCH23390 whereas at pH 6 there will be two affinities (K_d = 0.1 nM, 1.1 nM approx.). This provides an explanation of the effects of pH on the K_d for [^{3}H]SCH23390 binding. In the experiment where the pH dependence of the binding of a single concentration of [^{3}H]SCH23390 was examined there was no evidence of more than one ionizing group affecting ligand binding. This may be because in this experiment the pH dependence of the predominant form (pK_a 7) will dominate the apparent pH dependence.

As for the effects of DCCD, this will modify carboxyl groups on the receptor and where this is the carboxyl group of Asp 103 this will eliminate ligand binding. When there is partial modification by DCCD but some of the residual receptors have an altered K_d it may be that there is modification of another more reactive carboxyl group. In the receptors with this carboxyl group modified, ligand binding is not inhibited but the pK_a of the ligand-binding carboxyl group (Asp 103) is altered in one form of the receptor, the predominant form (from a pK_a of 7 to a pK_a of about 8.8 which would represent a carboxyl group in a highly perturbed environment). This form would then have a K_d of about 2 nM at pH 7.5. Partial modification of the minor receptor form does not affect its pK_a and so the K_d at pH 7.5 is still about 0.1 nM. The two forms of the receptors in this scheme would differ at the amino acid level and so would correspond to distinct isoforms. The relation of these to the presently cloned species is unclear.

In conclusion, therefore, this study has shown the power of the chemical modification approach in identifying residues at the active site of the D_1 dopamine receptor. The information will be important in producing models of the receptor binding site, in guiding mutagenesis experiments and ultimately in drug design.

Acknowledgements—We thank Schering AG and the SERC for financial support, Dr E. Schillinger and Dr J. Turner for encouragement and interest and Miss Sue Davies for preparing the manuscript.

REFERENCES

1. Lefkowitz RJ and Caron MG, Adrenergic receptors. Models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J Biol Chem* **263:** 4993–4996, 1988.
2. Strange PG, Receptors for neurotransmitters and related substances. *Curr Opinion Biotechnol* **2:** 269–277, 1991.
3. Dixon RAF, Sigal IS and Strader CD, Structure–function analysis of the β-adrenergic receptor. *Cold Spring Harbor Symposia* **53:** 487–497, 1988.
4. Tota MR, Candelore MR, Dixon RAF and Strader CD, Biophysical and genetic analysis of the ligand-binding site of the β-adrenoceptor. *Trends Pharmacol Sci* **12:** 4–6, 1991.
5. Fraser CM, Wang C, Robinson DA, Gocayne D and Venter JC, Site directed mutagenesis of m_1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* **36:** 840–847, 1989.
6. Kurtenbach E, Curtis CAM, Pedder EK, Aitken A, Harris ACM and Hulme EC, Muscarinic acetylcholine receptors. Peptide sequencing identifies residues

involved in antagonist binding and disulphide bond formation. *J Biol Chem* **265:** 13702–13708, 1990.
7. Williamson RA and Strange PG, Evidence for the importance of a carboxyl group in the binding of ligands to the D_2 dopamine receptor. *J Neurochem* **55:** 1357–1365, 1990.
8. Kebabian JW and Calne DB, Multiple receptors for dopamine. *Nature* **277:** 93–96, 1978.
9. Waddington JL and O'Boyle KM, The D1 dopamine receptor and the search for its functional role: from neurochemistry to behaviour. *Rev Neurosci* **1:** 157–184, 1987.
10. Dearry A, Gingrich JA, Falardeau P, Fremeau RT, Bates MD and Caron MG, Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature* **347:** 72–76, 1990.
11. Monsma FJ, Mahan LC, McVittie LD, Gerfen CR and Sibley DR, Molecular cloning and expression of a D_1 dopamine receptor linked to adenylyl cyclase activation. *Proc Natl Acad Sci USA* **87:** 6723–6727, 1990.
12. Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL, Gelernter JE, Rozmahel R, Yang Y, Israel Y, Seeman P and O'Dowd BF, Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5. *Nature* **347:** 80–83, 1990.
13. Zhou QY, Grandy DK, Thambi L, Kushner JA, Van Tol HHM, Cone R, Pribnow D, Salon J, Bunzow JR and Civelli O, Cloning and expression of human and rat D_1 dopamine receptors. *Nature* **347:** 76–80, 1990.
14. Tiberi M, Jarvie KR, Silvia C, Falardeau P, Gingrich JA, Godinot N, Bertrand L, Yang Feng TL, Fremeau RT and Caron MG, Cloning, molecular characterisation and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: differential expression patterns in rat brain compared with the D1A receptor. *Proc Natl Acad Sci USA* **88:** 7491–7495, 1991.
15. Sunahara RK, Guan HC, O'Dowd, BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol, HHM and Niznik HB, Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature* **350:** 614–619, 1991.
16. Means GE and Feeney RE, *Chemical Modification of Proteins*. Holden Day, San Francisco, 1971.
17. Glazer AN, *Chemical Modification of Proteins* North Holland, Amsterdam, 1975.
18. Wilson-Miles E, Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol* **47:** 431–432, 1977.
19. Munson PJ and Rodbard D, A versatile computerised approach for the characterisation of ligand binding systems. *Anal Biochem* **107:** 220–239, 1980.
20. McPherson GA, Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *J Pharmacol Method* **14:** 213–228, 1985.
21. Carraway KL and Koshland DE, Carbodiimide modification of proteins. *Methods Enzymol* **25:** 616–623, 1972.
22. Nalecz MJ, Casey RP and Azzi A, Use of *N,N'*-dicyclohexyl carbodiimide to study membrane bound enzymes. *Methods Enzymol* **125:** 86–108, 1986.
23. Simpson RT, Riordan JF and Vallee BL, Functional tyrosyl residues in the active centre of bovine pancreatic carboxypeptidase A. *Biochemistry* **2:** 616–622, 1963.
24. Hunter MJ and Ludwig ML, Amidination. *Methods Enzymol* **25:** 585–596, 1972.
25. Hulme EC, Birdsall NJM and Buckley NJ, Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* **30:** 633–673, 1990.
26. Srivastava LK and Mishra RK, Chemical modification reveals involvement of tyrosine in ligand binding to dopamine D_1 and D_2 receptors. *Biochem Int* **21:** 705–714, 1990.
27. Bzowej NH, Niznik HB and Seeman P, Dopamine D_1 receptors with enhanced agonist affinity and reduced antagonist affinity revealed by chemical modification. *Biochem Biophys Res Commun* **152:** 933–939, 1988.
28. Dewar KM and Reader TA, Specific [^{3}H]SCH23390 binding to D_1 dopamine receptors in cerebral cortex and neostriatum: role of disulphide and sulphydryl groups. *J Neurochem* **52:** 472–482, 1989.
29. Sidhu A, Kassis S, Kebabian J and Fishman PH, Sulphydryl groups in the ligand binding site of the D-1 dopamine receptor: specific protection by agonist and antagonist. *Biochemistry* **25:** 6695–6701, 1986.
30. Dohlman HG, Caron MG and Lefkowitz RJ, A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* **26:** 2657–2664, 1987.
31. Hess EJ and Creese I, Biochemical characterisation of dopamine receptors. In: *Dopamine Receptors* (Eds. Creese I and Fraser CM), pp. 1–27. A.R. Liss, New York, 1987.
32. Birdsall NJM, Chan S, Eveleigh P, Hulme EC and Miller KW, The modes of binding of ligands to cardiac muscarinic receptors. *Trends Pharmacol Sci* **10**(Suppl): 31–34, 1989.
33. Presland JP and Strange PG, pH dependence of sulpiride binding to D_2 dopamine receptors from bovine brain. *Biochem Pharmacol* **41:** R9–R12, 1991.