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Purification of brain D_2 dopamine receptor

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D_2 dopamine receptors have been extracted from bovine brain using the detergent cholate and purified ~20 000-fold by affinity chromatography on haloperidol-sepharose and wheat germ agglutinin-agarose columns. The purified preparation contains D_2 dopamine receptors as judged by the pharmacological specificity of [^{3}H]spiperone binding to the purified material. The sp. act. of [^{3}H]spiperone binding in the purified preparation is 2.5 nmol/mg protein. The purified preparation shows a major diffuse band at M_r 95 000 upon SDS-polyacrylamide gel electrophoresis and there is evidence for microheterogeneity either at the protein or glycosylation level. Photoaffinity labelling of D_2 dopamine receptors also shows a species of M_r 95 000. The D_2 dopamine receptor therefore is a glycoprotein of M_r 95 000.

Key words: brain/dopamine/molecular characterization/purification/receptor

Introduction

From a variety of experimental approaches including protein isolation, mechanistic studies and gene cloning, it has become apparent that cell surface receptors linked to guanine nucleotide binding proteins (G proteins) form a super-family of proteins based on homologous gene sequences (Dohlman *et al.*, 1987; Strange, 1988). Receptors for the neurotransmitter dopamine (D_1, D_2) are linked to G-proteins but have not been fully characterized yet. The D_2 dopamine receptor is of particular interest owing to its association with certain clinical conditions, e.g. schizophrenia, Parkinson's disease or their drug treatments (Iversen, 1976; Seeman, 1980) and several groups have solubilized this receptor and achieved partial purification (for review, see Strange, 1987). Here we describe our studies on the purification and molecular characterization of this important receptor and site of drug action.

Results

Solubilization of D_2 dopamine receptors

The effect of different concentrations of sodium cholate on the solubilization of D_2 dopamine receptors from a mixed mitochondrial-microsomal preparation of bovine caudate nucleus in the presence of NaCl (1M) is shown in Figure 1. The yield of solubilized receptors increased as the cholate was increased to 0.3% at which point the maximum yield of receptors was obtained (this preparation was used for all subsequent work). Further increase in cholate led to lower yields of solubilized receptors. In contrast the yield of solubilized protein increased continuously across the range of cholate concentrations.

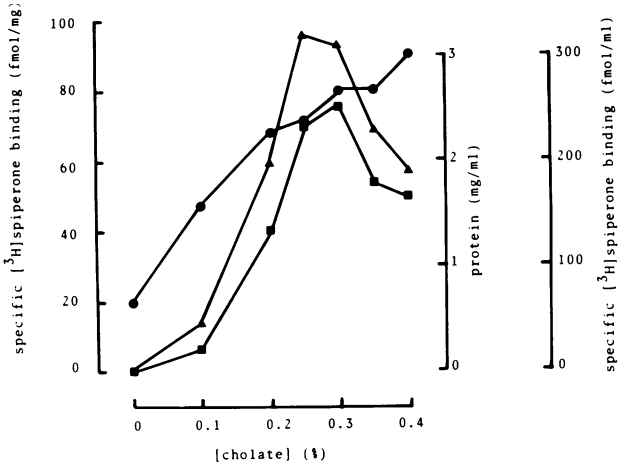

Fig. 1. Solubilization of D_2 dopamine receptors from bovine caudate nucleus. Solubilization of D_2 dopamine receptors was carried out as described in the Materials and methods section but using different final concentrations of sodium cholate. Protein (●) and specific [^{3}H]spiperone binding solubilized (■) were determined at each cholate concentration and sp. act. data (▲) are also given. The data shown are the means of three experiments.

Table I. [^{3}H]Spiperone binding to crude solubilized and purified D_2 dopamine receptors

Competing substance	K_i (nM)	
	Crude solubilized	Purified
Apomorphine	285 ± 38	–
(+)-Butaclamol	11 ± 1	15 ± 1
(−)-Butaclamol	3203 ± 97	>1000
Clebopride	122 ± 6	–
Domperidone	26 ± 2	11 ± 1
Haloperidol	33 ± 3	–
Metoclopramide	898 ± 236	–
Mianserin	2103 ± 306	>1000
Prazosin	>10,000	>1000
Spiperone	5 ± 2	–
Sulpiride	1886 ± 386	1130 ± 690

Specific [^{3}H]spiperone binding was determined in the crude solubilized preparation and in the preparation obtained after affinity and lectin chromatography in the presence of different concentrations of competing ligands. Competition data were analysed using non-linear least squares computer curve fitting as in Withy *et al.* (1981) and a one-binding site model was the best fit to the data in all cases. K_i values (inhibition constants) were determined from competition data as in Withy *et al.* (1981). Data are mean ± SEM for three or more experiments on the crude solubilized material and mean ± range (two experiments) for the purified material except for domperidone which is mean ± SEM (3).

Pharmacological characterization of the preparation obtained in 0.3% cholate/1 M NaCl using [^{3}H]spiperone binding and a series of competing drugs indicated that specific binding was exclusively to D_2 dopamine receptors (Table I). Thus D_2 dopamine receptor antagonists competed with high affinity and in a homogeneous manner (pseudo Hill coefficients of unity) whereas $5HT_2$ serotonin and α_1-adrenergic antagonists competed with low affinity. Solubilized D_2 dopamine receptors obtained using 0.3% cholate/1 M NaCl were unstable [$t_{1/2}$, 4°C, 40.2 ± 3.8 h, mean ± SEM (3)] and the stability was further reduced when receptors were incubated with sepharose, conditions that more closely reflect those in affinity chromatography ($t_{1/2}$ 20.4 ± 0.1 h). These half-lives could be increased ~2.5-fold by dilution of the preparation immediately after solubilization (to 0.225% cholate/0.75 M NaCl) and the diluted solubilized preparation was used for all subsequent procedures owing to its greater stability.

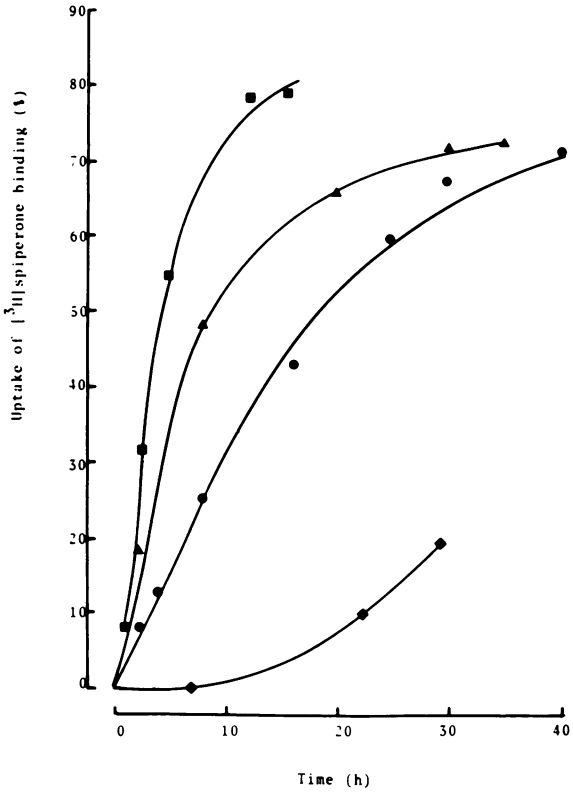

Fig. 2. Uptake (adsorption) of solubilized D_2 dopamine receptor on to affinity matrices. Soluble receptor preparation was incubated with affinity matrices [type I spiperone (■), type I haloperidol (●), type II haloperidol (▲)] or AH-sepharose (◆) as described in the Materials and methods section. At different times the matrix was allowed to settle and samples of supernatant were taken. [^{3}H]Spiperone binding was determined in the supernatants and corrected for that recoverable in a buffer wash of the matrix at each time point. The data shown are the mean of three experiments, variation < 10%.

Affinity chromatography of D_2 dopamine receptor

Affinity chromatography columns were synthesized based on carboxymethoxyl oxime derivatives of the D_2 dopamine receptor-selective ligands haloperidol or spiperone (type I coupling, Juszczak and Strange, 1985) or the hemisuccinate derivative of haloperidol (type II coupling, Juszczak and Strange, 1987) linked to amino hexyl (AH) sepharose. The amount of haloperidol coupled with AH sepharose was about three times greater using type II coupling than using type I coupling (see Materials and methods). The affinities of the coupled ligands for D_2 dopamine receptors were estimated using equivalent ligands coupled to bovine serum albumin in competition binding assays with [^{3}H]spiperone. Affinities of coupled ligands were reduced 55- to 60-fold by type I coupling and 30-fold by type II coupling relative to the free ligands (data not shown).

The affinity matrices each bound soluble D_2 dopamine receptor (Figure 2) and uptake (adsorption) was more rapid on the type I spiperone and type II haloperidol columns compared with the type I haloperidol. Minimal uptake was seen with the AH-sepharose matrix. Preincubation of solubilized receptor with the dopamine antagonists (+)-butaclamol, domperidone, haloperidol (10 μM) or metoclopramide (1 mM) suppressed receptor uptake whereas mianserin ($5HT_2$ serotonin antagonist) prazosin (α_1 adrenergic antagonist) and (−)-butaclamol (inactive stereoisomer) did not (Williamson *et al.*, 1987 and

Table II. Purification of D_2 dopamine receptor from bovine brain

	Specific [^{3}H]spiperone binding (fmol)	Protein (mg)	Yield of [^{3}H]spiperone binding (expressed as % of that bound to affinity column)	Sp. act. of [^{3}H]spiperone binding (pmol/mg)	Fold purification
Soluble receptor preparation	11 537 ± 1909	95.4 ± 16.1	–	0.124 ± 0.008	1.0
Unbound fraction from affinity column	5588 ± 1586	94.7 ± 16.1	–	–	–
Material bound	5949 ± 590	–	100	–	–
Metoclopramide eluate of affinity column	1758 ± 530	0.0345 ± 0.007	25.2 ± 4.8	51.0	411
N-acetylglucosamine eluate of WGA-agarose column	736 ± 159	0.0003 ± 0.00003	13.0 ± 2.7	2453	19782

The data shown for [^{3}H]spiperone binding are from 14 experiments except for metoclopramide eluates which are from 4 of these experiments where this fraction was analysed. Owing to the limited amounts of protein present, protein values were obtained in three separate experiments for the metoclopramide eluates and WGA-eluates and sp. act. values calculated accordingly. Data are mean ± SEM. The data for protein in WGA eluates are from quantitative silver staining on SDS−polyacrylamide gels. A very similar value was obtained using quantitative staining with Coomassie Blue after separation in a mini gel system [0.33 ± 0.06 μg (3)].

unpublished results). At equilibrium, 50–70% of the applied receptors and < 1% of the applied protein were bound to the affinity matrix. The type II haloperidol column has been used for further work as it gives more rapid uptake of receptor and the slightly lower affinity of haloperidol for the receptor (relative to spiperone) may assist in elution.

Receptor bound to the type II haloperidol column was eluted over two 24 h periods using the D_2 dopamine receptor-selective antagonist metoclopramide. Dialysis of eluates for 72 h against five changes of 100 volumes of buffer, followed by determination of [^{3}H]spiperone binding showed that 25% of the receptors bound to the affinity column could be recovered with metoclopramide elution, the majority (70%) being eluted in the first 24 h. Sp. act. data indicated a purification of ~400-fold (Table II). Sephadex G-25 gel filtration could also be used to remove metoclopramide giving similar recoveries and degrees of purification (data not shown).

The preparation obtained by affinity chromatography was further fractionated by application to wheat germ agglutinin (WGA)–agarose columns. The receptors were allowed to bind to the WGA–agarose which was washed extensively and eluted with 10 mM N-acetylglucosamine. [^{3}H]Spiperone binding was detected in the eluate and competition binding experiments (Table I, Figure 3) showed that specific [^{3}H]spiperone binding was to D_2 dopamine receptors with a pharmacological profile very similar to that observed in the crude solubilized preparation. Thus high affinity homogeneous competition curves were observed for D_2 dopamine antagonists whereas $5HT_2$ serotonin and α_1-adrenergic antagonists competed only weakly. In addition there was good agreement between affinities for competing ligands in the purified preparation and in the crude solubilized preparations.

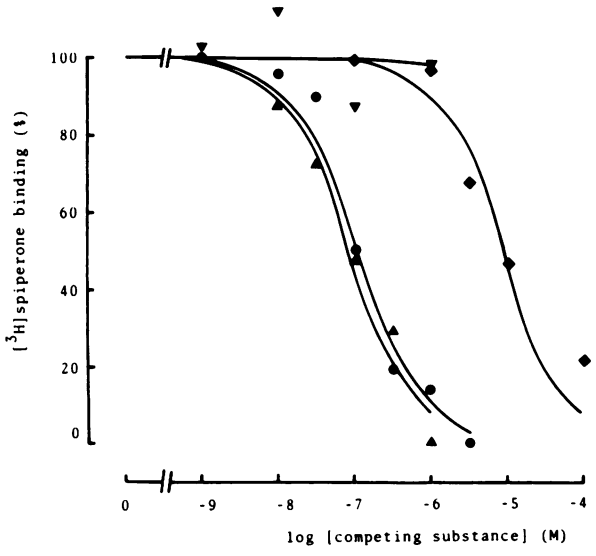

Fig. 3. Pharmacological characterization of [^{3}H]spiperone binding to purified D_2 dopamine receptors. Specific [^{3}H]spiperone (3 nM approx.) binding to purified preparations obtained after affinity and lectin chromatography was determined in the presence of increasing concentrations of (+)-butaclamol (●), (−)-butaclamol (▼), domperidone (▲), sulpiride (◆). Data for mianserin and prazosin are not shown but are essentially identical to those obtained for (−)-butaclamol. The data shown are from single experiments replicated as in Table I. The curves shown are the best fit curves to a one-binding site model.

The yield of [^{3}H]spiperone binding (Table II) was 13% after the two-step procedure and the sp. act. of [^{3}H]spiperone binding indicated a purification of ~20 000.

Molecular characterization of purified and photoaffinity labelled receptor by electrophoresis

SDS–polyacrylamide gel electrophoretic analyses of the preparation obtained after the two-step procedure are shown in Figure 4. A simple pattern is seen with a major diffuse band that often appears as a closely spaced doublet at M_r 95 000. Occasionally very faint bands in the M_r 50 000–65 000 region can also be seen. The M_r 95 000 species is suppressed if receptor is preincubated with metoclopramide before application to the affinity column or if the soluble receptor preparation is incubated at 22°C for 24 h to eliminate [^{3}H]spiperone binding (Figure 4).

The D_2 dopamine receptor in bovine caudate nucleus membranes was also labelled with the photoaffinity probe [^{3}H]azidomethylspiperone as described by Niznik *et al.* (1986). Specific labelling was defined as that prevented by (+)-butaclamol. SDS–PAGE of the labelled membranes consistently indicated specific labelling of a species of M_r 95 000 (Figure 5). Occasionally a M_r 32 000 species was also labelled specifically and this may be a proteolytic fragment of the larger band. The efficiency of labelling of the M_r 95 000 band was variable (0.6–10.1%). Non-specific labelling of a number of species including a M_r 58 000 band was also seen.

Discussion

This paper describes the first report of the purification of the brain D_2 dopamine receptor. The results described should be of great use in the further understanding of this important receptor and site of drug action.

The success of the procedure depends on a number of factors. Firstly efficient solubilization of D_2 dopamine

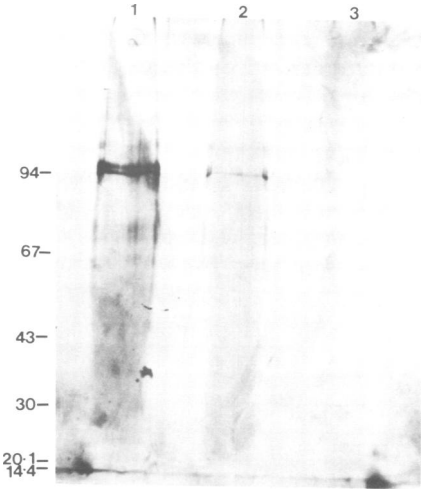

Fig. 4. SDS–PAGE of D_2 dopamine receptor. Samples of purified receptor obtained after affinity and lectin chromatography were analysed and gels were silver stained as described. **Lane 1** is for the standard procedure, **lane 2** is for receptors that have been pretreated with metoclopramide (1 mM) before purification, **lane 3** is for receptor that has been left at 22°C for 24 h to eliminate [^{3}H]spiperone binding before purification. This experiment has been replicated, as shown, twice with similar results. M_r values for standard proteins are also shown.

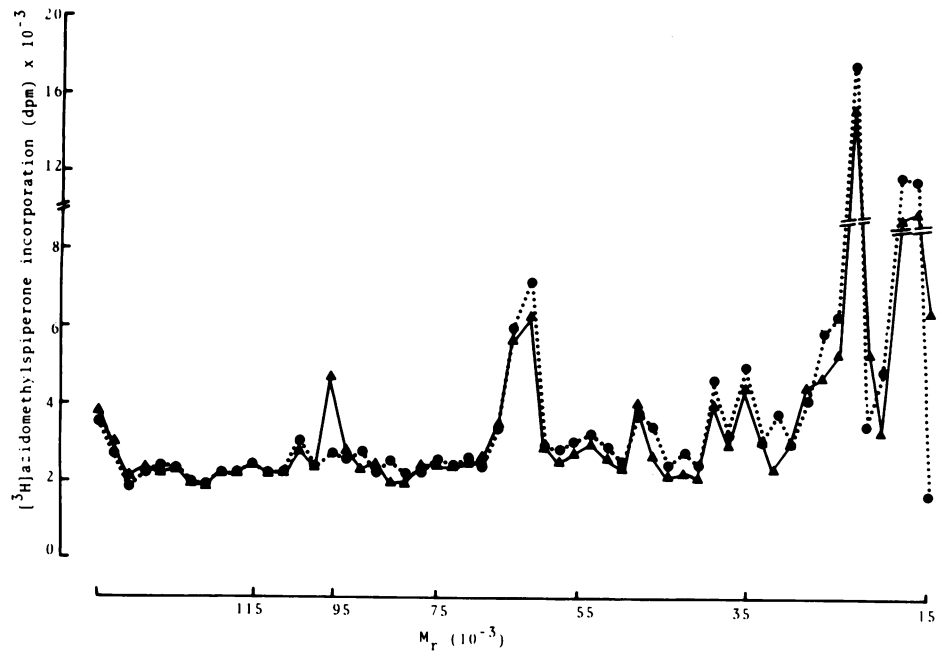

Fig. 5. SDS–PAGE of D_2 dopamine receptor photoaffinity labelled with [^{3}H]azidomethylspiperone. D_2 dopamine receptors were photoaffinity labelled as described and analysed electrophoretically. The data are from a single experiment performed in the absence (▲) and presence (●) of 1 μM (+)-butaclamol. The experiment has been replicated six times with similar results.

receptors was achieved with 0.3% cholate/1 M NaCl and the preparation stabilized by partial dilution immediately after solubilization. In our previous work with D_2 dopamine receptors solubilized with 0.2% cholate/1 M NaCl, [^{3}H]spiperone binding was to D_2 dopamine and $5HT_2$ serotonin receptors (Hall *et al.*, 1983) but here only D_2 dopamine receptors were detected by [^{3}H]spiperone binding. It seems that the soluble $5HT_2$ serotonin receptor is selectively unstable to raising the cholate concentration.

Secondly an efficient affinity chromatography procedure was required and we have provided this based on the D_2 dopamine receptor antagonist haloperidol linked as a hemisuccinate to AH-sepharose. This binds soluble D_2 dopamine receptor in a biospecific manner. Thirdly elution of bound receptor was achieved using metoclopramide. We chose this substance as it has a high selectivity for D_2 dopamine receptors but only a moderate affinity (K_i 0.9 μM) and so should be readily removed from eluted preparations. Fourthly further purification was achieved by fractionation on WGA–agarose using the method we have described previously (Abbott and Strange, 1985). The yield of the two-step procedure is moderate (13%) but the preparation when analysed by gel electrophoresis shows a single diffuse band which often appears as a doublet (M_r 95 000).

This diffuse band is likely to represent the D_2 dopamine receptor for the following reasons. The band (M_r 95 000) is largely suppressed if receptors are preincubated with metoclopramide before purification or inactivated by a room temperature incubation. A small amount of the M_r 95 000 species appears in the purified preparation even if receptors are preincubated with metoclopramide. This may reflect the very high apparent affinity of the affinity column for receptors owing to the high local concentration of haloperidol. Photoaffinity labelling of the receptor with [^{3}H]azidomethylspiperone shows specific labelling of an M_r 95 000 species and similar data have been obtained by other groups using this and other photoaffinity labels (Amlaiky and Caron, 1985; Lew *et al.*,1985; Redouane *et al.*, 1985; Niznik *et al.*, 1986). The purified preparation contains D_2 dopamine receptors as defined by [^{3}H]spiperone binding and the pharmacological profile is preserved upon purification. The sp. act. of [^{3}H]spiperone binding (2.5 nmol/mg protein) in the purified material is less than the theoretical value for an M_r 95 000 protein (10.5 nmol/mg). This is likely to be due to the instability of the receptor during purification and inaccuracies in determining the low protein concentrations. Similar discrepancies have been reported for other purified receptors, e.g. GABA/benzodiazepine receptor (Sigel *et al.*, 1983), glycine receptors (Pfeiffer *et al.*, 1982). D_2 dopamine receptors do seem to be rather labile to purification and we have shown in separate experiments that D_2 dopamine receptors partially purified (~10-fold) by WGA–agarose chromatography show a decreased stability relative to crude solubilized receptors ($t_{1/2}$ 11 h).

The receptor runs as a diffuse band which often separates into a doublet upon SDS–PAGE. The doublet bands seem to represent D_2 dopamine receptor as both are suppressed under conditions that prevent uptake of active receptor on to the affinity column. By analogy with other receptors linked to G-proteins, the receptor is likely to be a single subunit. Therefore these bands could represent variants at the protein level (either different gene products or proteolytic variants) or glycosylation variants. We favour the latter explanation at present as we have shown that there are at least two different glycosylation variants of the receptor based on differential binding to WGA–agarose (Leonard *et al.*, 1988).

Affinity chromatography procedures for fractionation of D_2 dopamine receptors have been described by ourselves and other groups previously (Antonian *et al.*, 1986; Ramwani and Mishra, 1986; Senogles *et al.*, 1986; Soskic and Petrovic, 1986; Worrall *et al.*, 1986) but this is the first report of a complete fractionation and molecular characterization of the purified receptor. The results reported here should be of great use in the further characterization

of this receptor particularly using immunological and gene cloning techniques.

Materials and methods

Reagents

[^{3}H]dinitrofluorobenzene and [^{3}H]azidomethylspiperone were from Dupont. Metoclopramide was a generous gift from Beecham Pharmaceuticals. Other materials were obtained from suppliers detailed in Juszczak and Strange (1985, 1987) and Leonard *et al.* (1987).

Solubilization of D_2 dopamine receptors

A mixed mitochondrial−microsomal membrane preparation of bovine caudate nucleus was prepared as in Withy *et al.* (1981) except that tissue was homogenized in a buffer that contained additionally 0.1 mM phenylmethylsulphonylfluoride (PMSF), EDTA (10 mM) and EGTA (1 mM). Membranes were resuspended in 20 mM Hepes, pH 7.4, 10 mM EDTA, 1 mM EGTA (buffer II) containing pepstatin A, leupeptin hemisulphate, aprotinin, chymostatin and antipain dihydrochloride (5 μg/ml).

For solubilization, mitochondrial−microsomal membranes were diluted in buffer II to 8 mg protein/ml and mixed with an equal volume of buffer II containing sodium cholate (generally 0.6% w/v), NaCl (2 M), PMSF (0.2 mM), pepstatin A, aprotinin, antipain dihydrochloride, chymostatin and leupeptin hemisulphate (all at 10 μg/ml) for 1 h, 4°C. Solubilized receptors were obtained as the supernatant after centrifugation (200 000 *g*, 1 h, 4°C) and assayed by [^{3}H]spiperone binding as in Hall *et al.* (1983).

Preparation of affinity matrices

Carboxymethoxyloxime derivatives of spiperone and haloperidol were synthesized as described in Juszczak and Strange (1985). The oxime derivatives (40 mg, dissolved in 2 ml ethanediol) were coupled to AH-sepharose (10 ml, Pharmacia) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (400 mg) at pH 5.0 for 15 h at 4°C. The coupled matrix (type I) was washed sequentially with 50% ethanol (2 L), 2 M NaCl (3 L), and water (500 ml). Haloperidol hemisuccinate was synthesized, activated using isobutylchloroformate and triethylamine and coupled to AH-sepharose at pH 9.0 as described in Juszczak and Strange (1987). The coupled matrix (type II) was washed as above but with an additional wash of 50% dioxane (200 ml) before the ethanol wash. The amounts of drugs immobilized were determined from the absorption spectra of the matrices suspended in ethanediol and the known extinction coefficients of the drugs (type I haloperidol 229 ± 29 nmol/ml gel; type I spiperone 282 ± 55 nmol/ml gel; type II haloperidol 709 ± 114 nmol/ml gel, mean ± SEM, 3−5 experiments). The structures of the derivatives are shown in Juszczak and Strange (1985, 1987).

Affinity chromatography of solubilized D_2 dopamine receptor

Soluble receptor preparation diluted to 0.225% cholate, 0.75 M NaCl (45 ml) and supplemented with sodium acetate (2 mM) was incubated at 4°C for 16−20 h with 10 ml of affinity matrix (generally type II haloperidol sepharose) in a column made from a 50 ml syringe equipped with a tap. The matrix was then washed with 500 ml of buffer III over 2−3 h. Buffer III is buffer II containing additionally sodium acetate (2 mM), soyabean phosphatidylcholine (0.045%), sodium cholate (0.225%), NaCl (0.75 M).

After washing, the columns were eluted over two 24 h periods with buffer III containing metoclopramide (1 mM). Data in Table II are the sum of these two eluates. Portions of each eluate were supplemented to 0.3% cholate, 1 M NaCl and incubated with WGA−agarose (90 min, 4°C). After washing (buffer III, 30 ml) the WGA−agarose was eluted with buffer III (10 ml) containing N-acetylglucosamine (10 mM) (90 min, 4°C). [^{3}H]Spiperone binding was determined in the different fractions as in Hall *et al.* (1983). In some experiments aliquots of metoclopramide eluates were dialysed against buffer III (5 changes of 100 volumes over 72 h).

Protein concentrations were determined in soluble preparation and unbound fraction by a modification of the method of Lowry *et al.* (1951) as described in Wheatley and Strange (1983). For metoclopramide eluates the method of Schultz *et al.* (1978) with [^{3}H]dinitrofluorobenzene was used and for WGA-eluates protein levels were estimated by quantitative silver staining of eluates run on SDS−polyacrylamide gels. Bovine serum albumin was used as standard throughout.

SDS−PAGE of D_2 dopamine receptor after purification and after photoaffinity labelling

Eluates were prepared for electrophoresis by chloroform−methanol extraction (Wessel and Flugge, 1984) and run on a discontinuous gel system (5% stacking gel, 8% resolving gel) (Laemmli, 1970). Gels were silver stained as in Morrissey (1981). For photoaffinity labelling, bovine caudate nucleus membranes (0.4 mg/ml) were incubated with [^{3}H]azidomethylspiperone (~2.5 nM) in the presence of mianserin (0.3 μM) and pepstatin A, aprotinin, antipain, chymostatin, leupeptin (all at 5 μg/ml) for 60 min at 22°C in buffer II. The incubation mixture was then cooled to 4°C and exposed to light from a water cooled mercury lamp (400 W) filtered through 6 mm of pyrex glass for 10 min. Samples were separated by SDS−PAGE as above and the gel cut into 2 mm slices and radioactivity determined after incubation with 10 ml of 3% protosol in econofluor (Packard) (24 h, 45°C). Parallel labelling experiments were performed with and without 1μM (+)-butaclamol.

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