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ANTISERA RAISED AGAINST THE DRUG HALOPERIDOL

REGINA JUSZCZAK and PHILIP G. STRANGE*
Department of Biochemistry, The Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

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Abstract—Antisera against haloperidol coupled to albumin have been raised in two rabbits. Both antisera bind haloperidol with high affinity but differ in their selectivity for binding other substances. A sub-population of anti-haloperidol antibodies has been partially purified from one antiserum by affinity chromatography. This sub-population resembles the D_2 dopamine receptor in its ability to bind some D_2 receptor selective antagonists.

Receptors for neurotransmitters or hormones are designed to recognize the neurotransmitter or hormone specifically and elicit a response in the appropriate cell. The recognition function is also shared by antibodies directed against the neurotransmitter or hormone, and it is of interest to compare the specificities of receptors and corresponding anti-ligand antibodies. In the present paper we have raised antisera against the D_2 dopamine receptor-selective drug haloperidol, in order to probe the structural requirements needed for recognition of this substance.

EXPERIMENTAL SECTION

Materials

[^{3}H]Haloperidol (14.7 Ci/mmole) was obtained from Amersham International p.l.c. (Amersham, U.K.), human serum albumin, carboxymethoxylamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were obtained from Sigma Chemical Company (Poole, U.K.) Haloperidol, domperidone and spiperone were generous gifts from Janssen Pharmaceutica (Beerse, Belgium). All other chemicals were obtained from commercial sources and were of the highest purity available.

Methods

Coupling of haloperidol to albumin. Haloperidol (40 mg) and carboxymethoxylamine (100 mg) were dissolved in a mixture of 2 ml methanol and 2 ml sodium acetate solution (1 M in water) and left at room temperature in the dark for three days. Thin-layer chromatography (silica gel in methanol/ethyl acetate, 1:1 v/v) indicated complete conversion of haloperidol to the carboxymethoxyl oxime. This was extracted using ethyl acetate, after the addition of 1 ml water to the reaction mixture and acidification with hydrochloric acid (1 M). The ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen.

Half the haloperidol carboxymethoxyloxime was dissolved in 0.5 ml ethanol, mixed with water (0.8 ml) and the pH adjusted to 5.5. Human serum albumin (6 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (4.5 mg) were added, adjusting the pH to 5.5 after each addition. This mixture was left at room temperature for 36 hr, adjusting the pH to 5.5 after 18 hr. The mixture was then placed in a dialysis sac together with two rinses (1 ml) of buffer [sodium phosphate (10 mM), sodium chloride (150 mM), pH 7.4] and dialysed against 1 l. changes of this buffer for 72 hr, changing the buffer five times, until the white precipitate had disappeared.

The haloperidol–albumin conjugate was stored in aliquots at −70° and the number of haloperidol molecules coupled per albumin was determined from the absorption at 247 nm relative to control samples.

Immunisation of rabbits. Female rabbits (Californian, New Zealand White Cross) were injected intramuscularly at four-weekly intervals with haloperidol–albumin emulsified in Freunds Complete Adjuvant. The two initial injections contained 0.5 mg protein whereas subsequent injections had only 0.25 mg protein per injection. Approximately one week after each injection blood samples were taken from the ear vein, allowed to clot overnight at room temperature and the serum fraction prepared by centrifugation.

[^{3}H]*haloperidol binding assay.* Rabbit serum at the stated dilution was incubated with [^{3}H]haloperidol (approx. 1 nM except for saturation analyses where a range between 0.25 and 15 nM was used) in a final volume of 525 μl of buffer [sodium phosphate (10 mM), sodium chloride (154 mM), sodium azide (15 mM) and gelatine (0.1%) pH 7.4] with displacing substances where appropriate. After 15 hr at 4° or 1 hr at 25°, when equilibrium was attained, free [^{3}H]haloperidol was removed by the addition of 100 μl of a charcoal–albumin mixture as described in [1], the mixture centrifuged (12,000 *g*, 2.5 min) and bound [^{3}H]haloperidol in a 300 μl aliquot of the supernatant determined by liquid scintillation spectrometry after addition of Fisofluor I.

Affinity chromatography of anti-haloperidol antibodies. A spiperone-amino-hexyl-sepharose affinity column (2 ml packed gel, 110 nmoles spiperone bound/ml gel, column diameter approx. 1.5 cm,

* To whom correspondence should be addressed.

packed gel height approx. 1.5 cm) [P. G. Strange, unpublished results] was initially washed with 20 volumes of buffer [sodium phosphate (10 mM), sodium chloride (150 mM), pH 7.4], the flow was stopped, 1 ml of buffer and 1–2 ml of antiserum-1 were applied, and the column was sealed and turned end-over-end for 4 hr at 4° in order to mix the contents thoroughly. The flow was restarted collecting the run-through fraction and then the column was washed with buffer, collecting 2.5 ml fractions until the absorption at 280 nM was less than 0.02. The column was then eluted with acetic acid (0.1 M, pH 3) and fractions of 2.5 ml collected and immediately neutralised with disodium hydrogen phosphate (1 M). [^{3}H]Haloperidol binding and protein were determined in each fraction.

RESULTS

Immunisation of rabbits against haloperidol

In order to raise antisera to haloperidol the drug was coupled to albumin. Firstly the drug was derivatised via its carbonyl group using carboxymethoxylamine (Fig. 1). The derivative was then coupled to albumin using the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The efficiency of coupling varied between experiments, coupling ratios of between 1:1 and 16:1 [haloperidol:albumin (mole:mole)] being

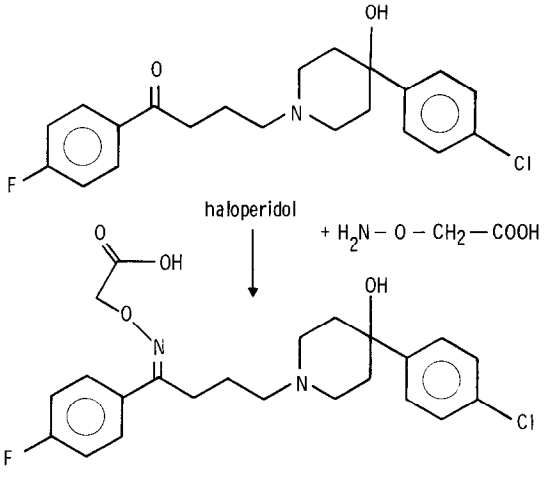

Fig. 1. Activation of haloperidol.

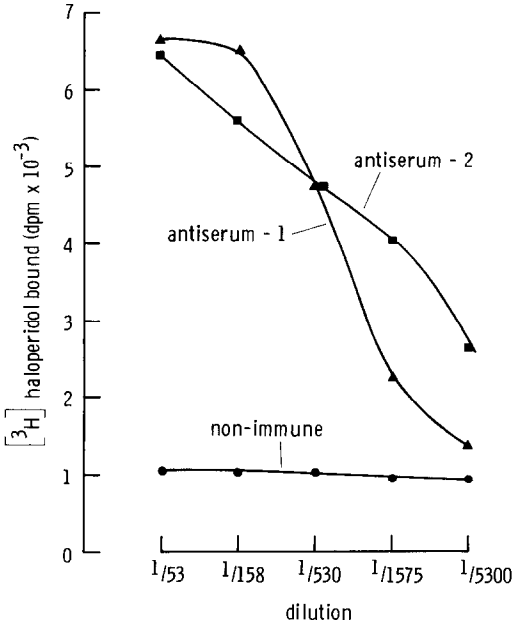

Fig. 2. [^{3}H]Haloperidol binding to antisera. [^{3}H]Haloperidol (approx. 1 nM) binding was determined as described to antiserum-1 or 2 or non-immune serum at the specified dilutions. The data are from a single experiment with the points being determined in duplicate. The apparent [^{3}H]haloperidol binding in non-immune serum represents the assay blank which has been subtracted from all subsequent data. [^{3}H]Haloperidol binding refers to the 300 µl aliquot taken from each assay tube (see Methods). It can be calculated that at the 1/53 dilution both antisera bind about 85% of the added [^{3}H]haloperidol.

Table 1. Binding of substances to rabbit antisera, partially-purified antibodies and D_2 dopamine receptors

Substance	IC_{50} (nM) for inhibition of [^{3}H]haloperidol binding			K_i (nM) at D_2 dopamine receptor
	Antiserum-1	Antiserum-2	Partially-purified antibody from antiserum-1	
(+)-Butaclamol	>100,000	16,000	—	1.1 (a)
(−)-Butaclamol	>100,000	>100,000	—	1960 (a)
Domperidone	71,000	>100,000	61	3.9 (c)
Dopamine	>100,000	>100,000	—	1250 (a)
Haloperidol	2.8	4.2	—	4.5 (b)
Mianserin	39,000	>100,000	—	2050 (a)
Spiperone	1400	120,000	63	0.14 (a)

IC_{50} (concentration of substance that inhibits half the binding of [^{3}H]haloperidol) values were determined for the substances shown for antiserum-1 or antiserum-2 or the partially-purified antibodies from antiserum-1. The data are the means of duplicate determinations. K_i values are for binding to D_2 dopamine receptors of bovine caudate nucleus from (a) ref. [11], (b) M. Leonard and P. G. Strange, unpublished, or of bovine anterior pituitary from (c) S. H. Simmonds and P. G. Strange, unpublished.

achieved in different batches. The haloperidol–albumin conjugate was used to immunise two rabbits and anti-haloperidol antibodies were assayed by binding of [^{3}H]haloperidol.

[^{3}H]Haloperidol binding activity, indicating the presence of anti-haloperidol antibodies, could be detected in sera from the two rabbits after one booster injection. Dilution curves for the two antisera (Fig. 2) were of different shapes. The curve shape is dependent on the number of binding sites in the antiserum and their affinity and as is shown below, these are different for the two antisera. Both antisera bound [^{3}H]haloperidol above the binding shown by non-immune controls at all dilutions used down to as low as 1/5000.

Characterisation of [^{3}H]haloperidol binding to antisera

The specificity of [^{3}H]haloperidol binding to the two antisera was determined by displacement of bound [^{3}H]haloperidol by a series of substances (Fig. 3). IC_{50} values have been determined for the displacing substances (Table 1) and these showed that both antisera bound haloperidol with high affinity. Antiserum-1 also bound spiperone and to a lesser extent domperidone whereas antiserum-2 was fairly specific for haloperidol. In the case of antiserum-1, these must be considered as average values from a mixed population of antibodies, since displacement curves for this serum were quite flat indicating the presence of mixtures of antibodies binding [^{3}H]haloperidol with different specificities. The different antibodies would nevertheless have similar affinities for haloperidol as displacement by haloperidol was characterised by steep displacement curves.

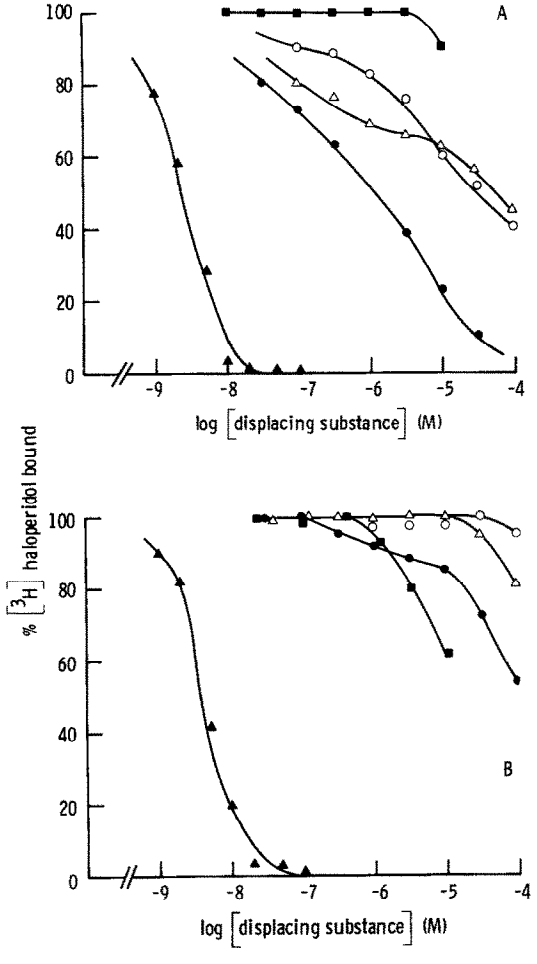

Fig. 3. Displacement of [^{3}H]haloperidol binding from antisera by various substances. [^{3}H]Haloperidol (approx. 1 nM) binding was determined as described to antiserum-1 (dilution 1/525) (A) or antiserum-2 (dilution 1/1575) (B) in the presence of different concentrations of (+)-butaclamol (■), domperidone (△), haloperidol (▲), mianserin (○), spiperone (●). The data are the means of duplicate experiments with points determined in triplicate.

Saturation analysis of [^{3}H]haloperidol binding to the antisera indicated high affinity saturable binding to each serum. Scatchard analysis indicated binding to a single class of sites in each case with slightly different affinities for [^{3}H]haloperidol [antiserum-1: K_d 0.78 ± 0.15 nM, B_{max} (serum diluted 1/1575) 28.6 ± 2.6 fmoles (mean ± S.D., three observations); antiserum-2: K_d 1.90 ± 0.63 nM, B_{max} (serum diluted 1/1575) 220 ± 100 fmoles (mean ± range, two observations)].

Partial purification of anti-haloperidol antibodies

Purification of anti-haloperidol antibodies from the antisera has been attempted using affinity chromatography. Affinity columns bearing haloperidol linked via a six or 10 carbon spacer arm were synthesised [P. G. Strange, unpublished results] and adsorption of anti-haloperidol antibodies from the antisera could be reproducibly achieved to either affinity column. Elution of the antibodies from the affinity columns could not, however, be achieved reproducibly despite considerable manipulation of conditions, e.g. use of hexyl or decyl spacer arm, varying the amount of haloperidol bound to the column, varying the eluting conditions (0.2 M glycine HCl, pH 2.8; 0.2 M glycine, pH 10; 0.2 M ammonium hydroxide; 0.2 M acetic acid; 5 M guanidinium hydrochloride; 100 μM haloperidol followed by extensive dialysis). It seems that the antibodies are too tightly bound to the column to be eluted with the conditions used.

The low but significant affinity of antiserum-1 for spiperone was therefore exploited and a spiperone–hexyl spacer–sepharose column was synthesised. Anti-haloperidol antibodies could be adsorbed from antiserum-1 to this column but complete adsorption was never achieved (Fig. 4). The bound antibodies could be eluted using 0.1 M acetic acid (pH 3) in good yield. By comparison of the applied antiserum and the eluted antibodies it was estimated that a purification of approx. 30-fold had been achieved. The eluted antibodies were unstable to freezing but could be stored at 4° in the presence of azide.

Displacement of [^{3}H]haloperidol binding by spiperone and domperidone was carried out in the unbound fraction and the eluted fraction from the affinity column. These were compared to the displacement seen in the whole antiserum. For both displacing substances (Fig. 5, Table 1) the affinity in the eluted fraction was increased relative to the antiserum and decreased in the unbound fraction.

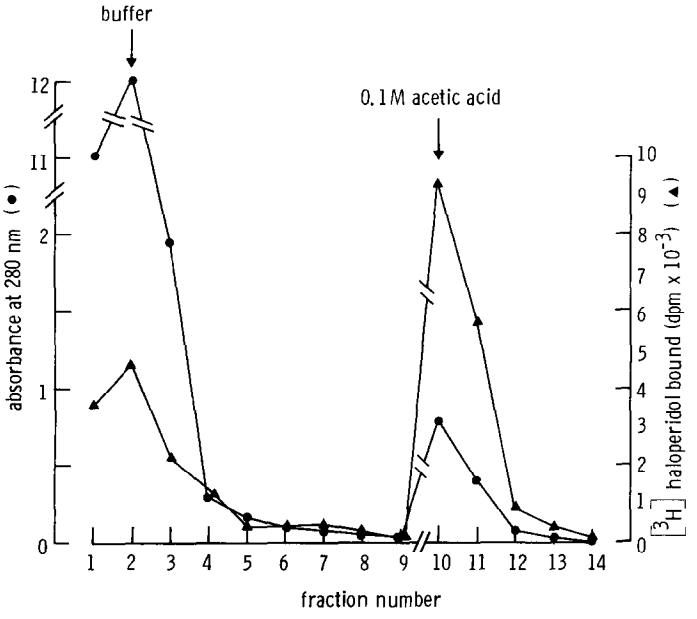

Fig. 4. Affinity chromatography fractionation of antiserum-1. Antiserum-1 was fractionated on a spiperone–sepharose column as described in the Methods section and [^{3}H]haloperidol binding (▲) and protein (absorbance at 280 nm) (●) determined.

The displacement curves in the eluted fraction conformed more closely to a single binding site model suggesting that the partially-purified fraction contains a less heterogeneous population of antibodies than the crude antiserum.

DISCUSSION

In this study we have prepared and characterized two antisera to haloperidol. Both antisera bind haloperidol with high affinity but differ in their selectivity for binding other substances as judged by the ability of these substances to displace [^{3}H]haloperidol binding. Antiserum-2 showed a low affinity for the substances tested so that it is fairly specific for haloperidol itself. Antiserum-1, however, showed a moderate affinity for the butyrophenone, spiperone, structurally related to haloperidol and the structurally less related domperidone. Whereas there is no evidence for heterogeneous populations of anti-haloperidol antibodies in antiserum-2, antiserum-1 does show some evidence for such heterogeneity. Displacement curves for spiperone and domperidone are flattened indicating multiple affinities for the

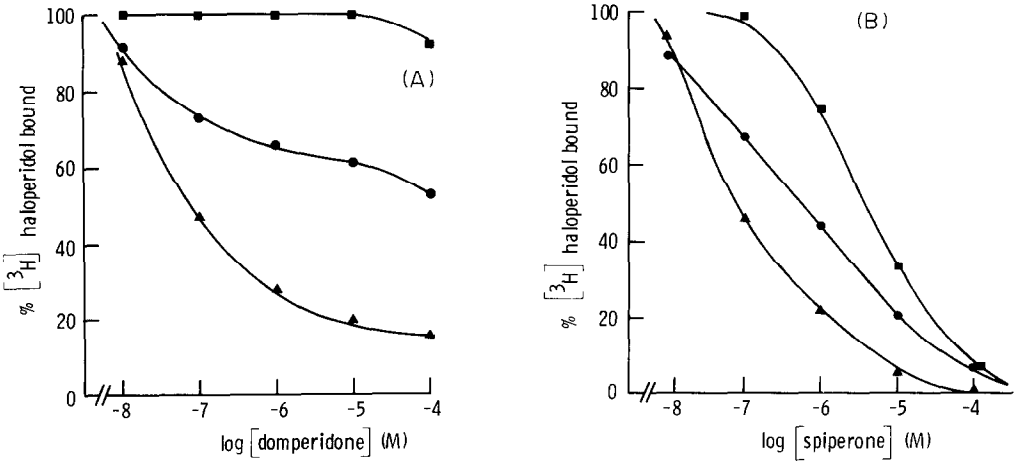

Fig. 5. Comparison of binding of domperidone and spiperone to antiserum-1 and partially-purified antibodies. Displacement of [^{3}H]haloperidol binding was determined as in Fig. 3 by domperidone (A) or spiperone (B) from antiserum-1 (dilution 1/105) (●), partially purified antibodies (dilution 1/26) (▲) or unbound material from affinity column (dilution 1/5) (■). The data are from single experiments (domperidone) or duplicate experiments (spiperone) with points determined in duplicate.

substances. This heterogeneity is further supported by the partial purification by affinity chromatography from antiserum-1 of a sub-population of antibodies with a high affinity for domperidone and spiperone.

Antisera have been raised by others to haloperidol coupled to albumin via the carbonyl group as described in the present study or via the hydroxyl group [2, 3]. Neither antiserum was characterised fully in terms of its binding specificity although the former antiserum resembled antiserum-2 of the present study in being fairly selective for haloperidol. The antiserum obtained to hydroxyl-coupled haloperidol in [2, 3] showed some ability to recognise spiperone as in the case of antiserum-1 of the present study.

It is of interest to compare the binding specificity of the antisera produced in the present study with that of the D_2 dopamine receptor which also shows a high ability to recognize haloperidol. In Table 1 the affinities of the two antisera from the present study for binding a range of ligands are compared with the affinities of the D_2 dopamine receptor for the same ligands. The specificity of antiserum-2 shows little resemblance to the specificity of the D_2 receptor, although it did show some stereoselectivity in binding the isomers of butaclamol. However, the affinity for binding the pharmacologically active (+)-butaclamol was about 10,000 times less than the affinity of the D_2 receptor for (+)-butaclamol. The specificity of antiserum-1, however, and particularly the specificity of the partially purified antibody obtained from this antiserum show some features in common with the D_2 receptor.

Thus haloperidol, spiperone and domperidone, (three D_2 receptor selective antagonists) are recognized well by the partially-purified antibody and although dopamine itself is recognized poorly, it seems likely that the recognition site of the antibody may share some structural features in common with the binding site of the D_2 receptor. The specificity shown by the antibody renders unlikely the possibility of any similarities to either serotonin ($5HT_2$) receptors or spirodecanone binding sites [4–6].

The similarities between the partially-purified antibody obtained here and the D_2 dopamine receptor raise the possibility that immunisation of animals with this antibody may elicit a second population of antibodies (anti-idiotypes) some of which may be anti-D_2 receptor antibodies. The use of this approach has been attempted for anti-spiperone antibodies raised in rabbits [7]. In order to test this possibility in the present system we have immunised mice with the partially purified antibody and fused spleen cells from the mice with NS-1 myeloma cells to produce hybridomas. Hybridoma supernatants were screened for antibodies that inhibited the binding of [^{3}H]-haloperidol to the serum from rabbit-1 and several positive supernatants were found. In half of the cases the supernatant also inhibited [^{3}H]spiperone binding to bovine caudate nucleus D_2 dopamine receptors. The hybridomas, however, stopped secreting antibody before cloning could be carried out. These results suggest that this is a fruitful approach to obtain anti-D_2 dopamine receptor antibodies as has been demonstrated successfully, for the case of the β-adrenergic receptor [8, 9] and nicotinic acetylcholine receptor [10].

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