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GUANINE NUCLEOTIDE EFFECTS ON AGONIST BINDING AT D_2 DOPAMINE RECEPTORS IN BOVINE ANTERIOR PITUITARY

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Abstract—[^{3}H]Spiperone binding to D_2 dopamine receptors on bovine anterior pituitary membranes is of high affinity, saturable and unaffected by guanine nucleotides. Antagonist displacement of [^{3}H]-spiperone binding is characterised by pseudo Hill coefficients close to one and is unaffected by guanine nucleotides. Agonist displacements show pseudo Hill coefficients less than one and agonist affinities are reduced by guanine nucleotides, although pseudo Hill coefficients are essentially unchanged. Agonist displacement curves may be treated in terms of two agonist binding sites with different agonist affinities, and in the presence of guanine nucleotides the two-binding site model remains the best description of the data.

The anterior pituitary gland offers an attractive tissue for studying the properties of D_2 dopamine receptors as the receptors are likely to be present on a single class of cell, the mammotroph cell [1]. $5HT_2$ receptors are likely to be absent from this tissue so that when using one of the most popular radioligands, [^{3}H]spiperone, for studying the receptors, only D_2 receptors should be labelled. The anterior pituitary offers the further advantage that enriched populations of individual cell types may be obtained for studying receptor mechanism [2].

Anterior pituitary D_2 receptors have been studied extensively using the ligand-binding technique [3–9]. From the effects of guanine nucleotides it has been suggested that the D_2 receptor interacts with a guanine nucleotide binding protein [3, 4] and this is likely to be essential for the post receptor mechanism. In the present report we describe studies on the effects of guanine nucleotides on bovine anterior pituitary D_2 receptors.

MATERIALS AND METHODS

Materials

All materials used were of the highest purity available and were obtained from suppliers given in [10] unless otherwise stated. [^{3}H]Spiperone (15.5–21.0 Ci/mmole) was from Amersham International, Amersham, U.K. Additional gifts of drugs are gratefully acknowledged; domperidone (Janssen Pharmaceutica, Beerse, Belgium), isomers of 5,6-dihydroxy-2-*N*,*N* di-*n*-propyl aminotetralin (Fisons Pharmaceuticals, Loughborough, U.K.), phenoxybenzamine (Smith Kline & French, Welwyn Garden City, U.K.). 6,7-Dihydroxy 2-aminotetralin and 6,7 dihydroxy 2-*N*-*n*-propyl amino tetralin were synthesized as described in [11]. α-Flupenthixyl chloride was synthesized using the procedure described in [12].

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METHODS

Preparation of anterior pituitary tissue

Whole bovine pituitaries were obtained from Nottingham City Abbatoir within 1 hr of slaughter and were transported in ice-cold sucrose (0.32 M). The anterior lobe was dissected out, the tissue weighed and homogenised in 6 vol of unbuffered sucrose (0.32 M) using a teflon–glass homogeniser (10 strokes, 0.18 mm radial clearance). The homogenate was filtered through two layers of cheesecloth and centrifuged (1700 *g*, 10 min). The supernatant was retained at 4° and the pellet was resuspended in 3 vol of sucrose (0.32 M) using the homogeniser (five strokes). The suspension was centrifuged (1700 *g*, 10 min) and the supernatant amalgamated with that from the previous centrifugation. The combined supernatants were centrifuged (126,000 *g*, 1 hr, 4°) and the pellet resuspended in HEPES–phosphate–saline buffer [13] containing dithiothreitol (0.1 mM) and pargyline (10 μM) at a protein concentration of approx. 17 mg/ml.

In some experiments anterior pituitary tissue was prepared as in [3]. Briefly, anterior pituitary tissue was homogenised in Tris buffer (50 mM, pH 7.7 at 25°, approx. 10 mg tissue/ml), filtered through cheesecloth and centrifuged twice (50,000 *g*, 10 min, 4°) with intermediate resuspension in fresh buffer. The final pellet was resuspended in Tris/ions buffer (Tris 50 mM, ascorbic acid 0.1%, NaCl 120 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, pH 7.1 at 37°) at a tissue concentration of approx. 20 mg (wet wt)/ml (approx. 2.5 mg protein/ml).

[^{3}H]*Spiperone binding assay*

D_2 dopamine receptors in the anterior pituitary membranes were assayed by [^{3}H]spiperone binding as described in [10]; pituitary membranes (300 μg protein approx.) were incubated with a given concentration of [^{3}H]spiperone (500 pM in displacement experiments) in HEPES–phosphate–saline buffer

[13] containing dithiothreitol (0.1 mM) and pargyline (10 μM) and other substances where stated in quadruplicate in a final volume of 1 ml of buffer at 25° for 30 min by which time equilibrium was achieved (data not shown). Assays were terminated by the addition of 5 ml of ice-cold buffer, rapid filtration under vacuum through GF/B glass fibre filters and two additional 5 ml buffer washes. Radioactivity was then determined by liquid scintillation counting.

In separate experiments [^{3}H]spiperone binding showed a linear dependence on protein added up to 450 μg. Specific [^{3}H]spiperone binding was defined as that displaceable by 3 μM (+)-butaclamol, by which concentration displacement of [^{3}H]spiperone binding had reached a maximum. Similar maximal displacements were seen with other compounds. In some cases the conditions were modified as follows. Some assays were conducted at 37° instead of 25° and the time of incubation was 15 min by which time equilibrium was attained [14].

Some assays were conducted according to conditions described in [3], using tissue prepared as described above. In this case binding assays were terminated by rapid filtration through GF/B glass fibre filters followed by three 5 ml washes of ice-cold Tris buffer (50 mM, pH 7.7 at 25°). In these experiments specific [^{3}H]spiperone binding was defined using 1 μM (+)-butaclamol, and the assay buffer used was Tris/ions buffer.

Data from displacement and saturation binding experiments were analysed using non-linear least-squares computer curve fitting as described in [10]. Kinetic data were analysed as in [15].

RESULTS

Characterisation of [^{3}H]*spiperone binding*

Association and dissociation kinetics for [^{3}H]-spiperone binding were determined and gave the following rate constants: k_{ass} $1.17 \pm 0.34 \times 10^{-5}$ $pM^{-1}\,sec^{-1}$ five experiments; k_{diss} $1.1 \pm 0.5 \times 10^{-3}$ sec^{-1} three experiments (mean ± S.D., n observations). In each case kinetic analysis suggested a homogeneous process and the ratio of rate constants gave an equilibrium dissociation constant of 91 pM.

Displacement of [^{3}H]spiperone binding by a series of antagonists (Fig. 1, Table 1) gave potencies in agreement with those described in other studies [3–9] and pseudo Hill coefficients were close to one. Among the substances tested were the putative irreversible antagonists phenoxybenzamine and α-flupenthixyl-chloride [12, 16, 17]. In separate studies (data not shown) we have shown that phenoxybenzamine acts as an irreversible antagonist of D_2 dopamine receptors in agreement with other published data [16, 17]. We have been unable to confirm that α-flupenthixyl chloride [12] acts irreversibly at D_2 receptors using the bovine anterior pituitary as the tissue source. Saturation analysis of [^{3}H]spiperone binding indicated generally a single class of sites although some slight indication of a small number of lower affinity sites was seen in some experiments. From experiments conducted under the standard conditions used here a B_{max} of 81 ± 19 fmol/mg protein and K_d of 112 ± 28 pM (mean ± S.E.M., five experiments) was seen in good agreement with the value derived kinetically. In parallel experiments Gpp (NH)p (0.1 mM) had little effect: B_{max} 91 ± 2 fmol/mg protein, K_d 174 ± 47 pM (mean ± S.E.M., three experiments) and in displacement experiments guanine nucleotides did not affect antagonist binding, e.g. for domperidone K_i (control) 5.1 ± 3.8 nM; +0.1 mM GTP 5.3 ± 4.0 nM (mean ± S.E.M., three experiments in parallel). These results are supported from observations of maximal [^{3}H]spiperone binding in displacement experiments: in no case was an effect of guanine nucleotides seen. Using the tissue preparation and assay conditions of [3], similar results were observed, B_{max} 91 ± 18 fmol/mg protein K_d 152 ± 34 pM (mean ± S.E.M., four experiments).

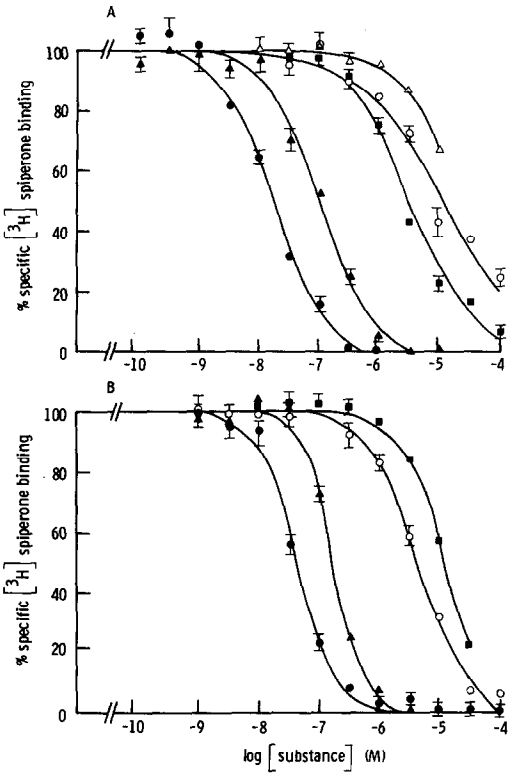

Fig. 1. Displacement of [^{3}H]spiperone binding from anterior pituitary D_2 receptors by antagonists. Specific [^{3}H]-spiperone (500 pM approx.) binding to anterior pituitary membranes was determined in the presence of different concentrations of A-domperidone (●), (+)-butaclamol (▲), sulpiride (■), phenoxybenzamine (○) or (−)-butaclamol (△). B-α-flupenthixol (●), α-flupenthixyl chloride (▲), β-flupenthixol (○), mianserin (■). The data are from single experiments and the curves are theoretical curves for equation 1 of [10]. Data points were determined in quadruplicate and the mean ± S.E.M. is plotted. Where no error bars are shown these were within the symbol.

Agonist displacement of [^{3}H]*spiperone binding*

Displacements of specific [^{3}H]spiperone binding by dopamine agonists (Fig. 2, Table 2) were characterised by pseudo Hill coefficients less than one in contrast to antagonists which gave pseudo Hill coefficients close to one. For apomorphine and dopamine, agonist affinities were greater when

Table 1. Antagonist binding to anterior pituitary D_2 receptors

Substance	K_i (nM)	pseudo Hill coefficient	No. of experiments
(+)-butaclamol	12.3 ∓ 3.5	1.1 ∓ 0.2	5
(−)-butaclamol	1620 ∓ 102	1.5 ∓ 0.2	3
domperidone	3.9 ∓ 4.9	1.2 ∓ 0.2	6
α-flupenthixol	6.4 ∓ 1.0	1.4 ∓ 0.2	3
β-flupenthixol	623 ∓ 48	1.0 ∓ 0.1	2
α-flupenthixyl chloride	41.5 ∓ 16.1	1.6 ∓ 0.4	5
mianserin	2140 ∓ 620	1.3 ∓ 0.1	3
phenoxybenzamine	1240 ∓ 540	0.8 ∓ 0.3	3
sulpiride	350 ∓ 56	0.9 ∓ 0.1	3

Displacement of [^{3}H]spiperone binding by the substances shown was carried out and K_i (dissociation constant) values and pseudo Hill coefficients were determined as described. Values are expressed as mean ∓ S.D. (three or more experiments) or mean ∓ range (two experiments).

assays were performed at 37° (rather than 25°) or using the conditions in [3]. Guanine nucleotides lowered agonist affinities in all cases with little change in pseudo Hill coefficient and the effects were maximal for concentrations of GTP or Gpp(NH)p of 100 μM (data not shown).

When the individual agonist displacement experiments were analysed as in Table 2 to give IC_{50} values and pseudo Hill coefficients, values were obtained which showed low inter-experimental variation. The data were also analysed using computer curve fitting to determine whether a one- or two-binding site model [10] was a better description of the data and under all conditions the two-binding site model was a better description. The parameters derived, however, showed some inter-experimental variation so we have not averaged the values obtained.

In parallel experiments, however, examining the effects of guanine nucleotides, these compounds tended to decrease the proportion of higher affinity sites and to alter the IC_{50} values for both sites, e.g. in one experiment with dopamine: control: IC_{50} (higher) 0.33 μM, IC_{50} (lower) 165 μM, % (higher) 41; +GTP (0.1 mM): IC_{50} (higher) 0.23 μM, IC_{50} (lower) 16.5 μM, % (higher) 24.

DISCUSSION

The properties of the D_2 dopamine receptors described here with [^{3}H]spiperone binding are in good agreement with those described by other workers in anterior pituitary [3–9]. Thus a single class of homogeneous antagonist binding sites is seen but agonist binding is more complex with pseudo Hill coefficients less than one for displacements. This pattern is similar to that described for bovine caudate nucleus D_2 receptors [10] and in fact there is a good correlation ($r = 0.96$) between potencies of ligands for binding to the caudate nucleus and anterior pituitary receptors. Thus the receptors in the two tissues are similar at the level of ligand binding.

The main purpose of this study was to investigate the effects of guanine nucleotides on anterior pituitary D_2 receptors in relation to receptor mechanism. In other studies on anterior pituitary D_2 receptors guanine nucleotides have been reported to weaken

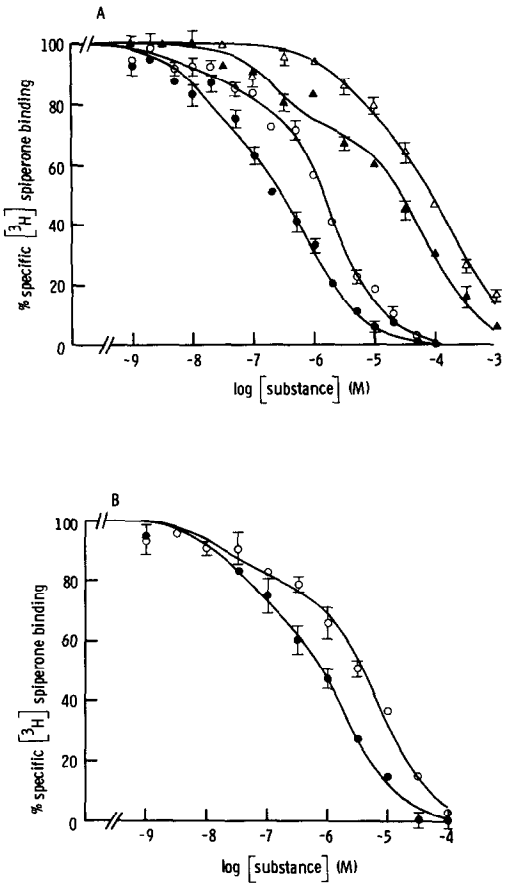

Fig. 2. Displacement of [^{3}H]spiperone binding to anterior pituitary D_2 receptors by agonists. [^{3}H]Spiperone (500 pM approx.) binding to anterior pituitary membranes was determined in the presence of different concentrations of A-apomorphine (●), apomorphine + 0.1 mM GTP (○), dopamine (▲), dopamine + 0.1 mM GTP (△), B 6,7-dihydroxy, 2-*N*-*n*-propyl aminotetralin (●), 6,7-dihydroxy-2-*N*-*n*-propyl aminotetralin + 0.1 mM GTP (○). The data are from single experiments and the curves are theoretical curves for a two binding site model (equation 2 of [10]). Data points were determined in triplicate and the mean ±S.E.M. is plotted. Where no error bars are shown these were within the symbol.

Table 2. Agonist binding to anterior pituitary D_2 dopamine receptors and effect of guanine nucleotide

		Control		+ guanine nucleotide (0.1 mM)		
	Condition	Corrected IC_{50} (nM)	n	Corrected IC_{50} (nM)	n	No. of experiments
Apomorphine	control	68 ± 15	0.63 ± 0.05	155 ± 23	0.69 ± 0.04	5
	37°	20 ± 1	0.69 ± 0.09	93 ± 8	0.72 ± 0.12	3
	as in [3]	27 ± 3	0.59 ± 0.04	188 ± 47	0.78 ± 0.07	2
(S)-5,6,dihydroxy, 2-*N*-*N* di-*n*-propyl aminotetralin	control	31 ± 8	0.50 ± 0.06	159 ± 39	0.55 ± 0.01	4
(R)-5,6,dihydroxy, 2-*N*,*N* di-*n*-propyl aminotetralin	control	520 ± 120	0.60 ± 0.04	1100 ± 200	0.67 ± 0.06	4
6,7,dihydroxy, 2 aminotetralin	control	74 ± 14	0.59 ± 0.02	282 ± 14	0.62 ± 0.01	3
6,7,dihydroxy, 2-*N*-*n*-propyl aminotetralin	control	75 ± 5	0.56 ± 0.03	406 ± 66	0.65 ± 0.07	3
dopamine	control	1800 ± 700	0.50 ± 0.03	6800 ± 800	0.60 ± 0.03	4
	37°	560 ± 170	0.50 ± 0.04	5800 ± 1900	0.65 ± 0.10	3
	as in [3]	600 ± 110	0.50 ± 0.03	12000 ± 4000	0.65 ± 0.04	2

Agonist binding was determined by displacement of [^{3}H]spiperone (500 pM approx.) binding and IC_{50} values (corrected for occupancy of receptors by [^{3}H]-spiperone) and pseudo Hill coefficients (n) were determined as described in [10]. Details of the conditions used are given in the experimental section. Data are given as mean ± range (two observations) or mean ± S.E.M. (three or more observations). The guanine nucleotide used was GTP except for assays at 37° where Gpp(NH)p was used.

agonist binding and increase pseudo Hill coefficients from values less than one to close to one although partial effects have also been reported [9]. Effects on antagonist binding, with guanine nucleotides causing increased antagonist affinity, have been reported in some studies [4, 9] but not in others [3, 7]. In the present study guanine nucleotides had little effect on antagonist binding and it was notable that for agonist binding an essentially parallel shift in the agonist displacement curves to lower affinities was seen with little alteration in pseudo Hill coefficient. The conditions used here were different from those used in other studies [3] where increases in pseudo Hill coefficient to one for agonists were seen in the presence of guanine nucleotides. In [3] a different tissue preparation method was used and assays were performed at 37°. Therefore firstly assays were performed under the normal protocol but with a 37° assay temperature and secondly tissue was prepared and assays performed exactly as in [3]. In both cases effects of guanine nucleotides (0.1 mM) on agonist affinity were found but little effect on pseudo Hill coefficient. Similar effects were also seen with 1 mM guanine nucleotide. Thus we are unable to observe alterations in pseudo Hill coefficient for agonist binding caused by guanine nucleotides and the results are similar to those described for brain D_2 receptors where the effect of guanine nucleotides is to lower agonist affinity with a roughly parallel shift in the agonist displacement curve [18–20].

The complex agonist binding curves and the effects of guanine nucleotides have been discussed extensively in terms of a model for coupling of receptor (R) and a guanine nucleotide binding protein (N) [3, 4, 9]. R and RN states have been proposed to exist in the control membranes and conversion of RN (higher agonist affinity) to R (lower agonist affinity) occurs with guanine nucleotides. In the present study agonist displacement curves may be described in terms of two binding sites but the effects of guanine nucleotides are complex leading to effects on the percentage of the sites (tending to lower the proportion of higher affinity sites) and the IC_{50} values for each site.

It has been suggested [4, 9] that the R and RN states may also have reciprocal higher and lower affinities, respectively, for antagonists. This has not been replicated in other published studies [3, 7] and in the present study no effects of guanine nucleotides on antagonist binding were seen. It has recently been suggested that guanine nucleotide effects on antagonist binding are due to residual dopamine in the preparation [7].

Thus, from the present studies it can be concluded that anterior pituitary D_2 dopamine receptors (R) couple with a guanine nucleotide binding protein (N). Complex agonist binding observed in control membranes may be related to R–N coupling as, whereas agonist binding is complex and affected by guanine nucleotides, antagonist binding is homogeneous and unaffected by guanine nucleotides. Effects of guanine nucleotides on agonist binding are complex and we did not see the simplification of agonist binding data reported in other studies even when replication of these was attempted. The reason for the differences between these findings and those

in [3, 4] is unclear. It cannot be due to detailed differences in tissue preparation methods or assay conditions between studies as we have replicated these exactly, still obtaining different results. Possibly it could relate to differences in the source of bovine material and animal feeding patterns between the U.S.A. [3, 4] and the U.K. (these studies). In relation to the models proposed and results obtained it is important to note that guanine nucleotide binding proteins are heterotrimers ($\alpha\beta\gamma$) whose dissociation is important for receptor function [21–23]. This may complicate the formulation of models to describe the mechanism of receptors linked to such guanine nucleotide binding proteins. Previously, models proposed have generally treated the guanine nucleotide binding proteins as non dissociating entities [3, 4, 9]. Modification of models to take into account the more complex nature of the guanine nucleotide binding proteins will be important in understanding the effects of D_2 dopamine receptors to inhibit adenylate cyclase [24] and inositol phospholipid metabolism [25].

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