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THE RELATIONSHIP BETWEEN THE OCCUPATION OF THE D-1 DOPAMINE RECEPTOR BY [^{3}H]PIFLUTIXOL AND THE ACTIVITY OF DOPAMINE-SENSITIVE ADENYLATE CYCLASE IN RAT STRIATAL MEMBRANES

S. FLEMINGER*
Department of Neurology, Institute of Psychiatry, London, U.K.

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Abstract—The relationship between occupation of the D-1 dopamine receptor by [^{3}H]piflutixol and inhibition of dopamine-sensitive adenylate cyclase has been studied. Experiments were performed in parallel; after the initial incubation to enable binding of [^{3}H]piflutixol, half the tubes were assayed for [^{3}H]piflutixol binding and the other half assayed for adenylate cyclase activity. The assay conditions for the two halves of the experiments were identical. (±)Sulpiride (3×10^{-5} M) was present in all tubes to mask drug binding to the D-2 receptor. The inhibition of dopamine- (10^{-3} and 10^{-5} M) sensitive adenylate cyclase with increasing concentrations of [^{3}H]piflutixol in the incubation mixture was compared to the saturation of specific [^{3}H]piflutixol binding with those same concentrations of [^{3}H]piflutixol. There was a linear relationship between receptor occupation by [^{3}H]piflutixol and inhibition of dopamine sensitive adenylate cyclase. In a second experiment dopamine was present during the initial incubation with [^{3}H]piflutixol. This resulted in a displacement of specific [^{3}H]piflutixol binding and, as a consequence, a reduction of [^{3}H]piflutixol's inhibition of dopamine-sensitive adenylate cyclase. In the absence of GTP in the initial incubation dopamine produced a greater reduction of [^{3}H]piflutixol's inhibition of dopamine adenylate cyclase than displacement of specific [^{3}H]piflutixol binding. In the presence of GTP in the initial incubation both displacement curves were shifted to the right, i.e. dopamine was less potent. However, under these conditions dopamine produced less inhibition of [^{3}H]piflutixol's inhibition of dopamine adenylate cyclase than displacement of specific [^{3}H]piflutixol binding. These results are interpreted as resulting from changes in D-1_{high} and D-1_{low} ratios as a result of incubation in the presence or absence of GTP.

Piflutixol is known to be an antagonist of the D-1 dopamine receptor [1]. The D-1 receptor is linked to stimulation of dopamine-sensitive adenylate cyclase [2]. [^{3}H]Piflutixol binds to the D-1 receptor [3, 4]. Therefore [^{3}H]piflutixol would also be expected to act as an antagonist at the D-1 receptor and might be used to study receptor occupation–activation relationships at this site. MacFarlane and Stump [5] demonstrated that it is possible to use antagonist radioligands to study occupation–activity relationships; they used the α-2 antagonist [^{3}H]yohimbine to inhibit the inhibition of platelet adenylate cyclase by adrenaline.

The series of experiments described in this paper were attempts to observe directly the inhibition of dopamine-sensitive adenylate cyclase of rat striatal membranes by [^{3}H]piflutixol, while, at the same time, observing the binding of [^{3}H]piflutixol to the membranes. The aim of the experiments was to investigate the relationship between the number of receptors left unoccupied by [^{3}H]piflutixol, and dopamine sensitive adenylate cyclase activity.

In one series of experiments a range of [^{3}H]piflutixol concentrations was used. By studying the saturation of specific [^{3}H]piflutixol binding it was possible to quantitively assess the relationship between receptor occupation and inhibition of dopamine-sensitive adenylate cyclase. In a second series of experiments a range of dopamine concentrations was used to displace [^{3}H]piflutixol from its binding site. The consequent inhibition of [^{3}H]piflutixol binding was compared to the reduction in [^{3}H]piflutixol's inhibition of dopamine-sensitive adenylate cyclase. The inhibition of [^{3}H]piflutixol binding by dopamine was carried out in the presence and absence of GTP. Sibley *et al.* [6] have demonstrated that the displacement of [^{3}H]flupenthixol binding by dopamine agonists is sensitive to the presence of guanine nucleotides. What are the functional consequences of the shift by GTP of the curve for dopamine's inhibition of [^{3}H]piflutixol binding?

Two observations suggested that the experiments might be feasible: (1) [^{3}H]piflutixol in the adenylate cyclase assay does not interfere with the competitive binding assay used to measure cyclic AMP (Table 1). (2) [^{3}H]Piflutixol has a slow rate of dissociation from its binding site. There is therefore little change in the amount of ligand binding to the tissue, in the face of a large drop in free ligand concentration, or as a result of competition for the receptor by a displacing drug, during the course of the 2 min incubation at 30° required for the adenylate cyclase assay (Table 2).

For the purpose of analysis the experiments described in this paper are essentially in two halves. The first half is the determination of specific [^{3}H]piflutixol binding using a standard radioligand

* Present address: Dr S. Fleminger, Department of Psychiatry, Institute of Psychiatry, Denmark Hill, London SE5 8AF, U.K.

Table 1. The standard curves for the cyclic AMP binding assay in the presence and absence of [^{3}H]piflutixol

Cyclic AMP concentration (pmol/L)	[^{3}H]Piflutixol −	[^{3}H]Piflutixol +
0.31	2091 (81)	2159 (47)
0.81	1388 (74)	1429 (18)
1.31	1015 (63)	1031 (41)
2.81	672 (21)	676 (24)

The results are expressed in cpm (counting efficiency constant at 45%). The results are the means (±1 SEM) of three separate standard curves. The standard curves were assayed in the presence or absence of [^{3}H]piflutixol. This was done by adding 0.01 mL of water, or 0.01 mL [^{3}H]piflutixol (4 nM, 6.75 Ci/mM) in water, to the incubation tubes before the start of the assay. In all other respects the standard protein binding assay to measure cyclic AMP was used.

Table 2. The effects of filtering the incubation tissue before or after the 2 min incubation at 30° on the binding kinetics of specific [^{3}H]piflutixol binding. A comparison of binding kinetics determined by Scatchard analysis or computer curve fitting

	K_d (nM)	B_{max} (pmol/g)	r
Scatchard analysis			
Before	0.69 (0.19)	72 (7)	0.943
After	0.70 (0.13)	78 (5)	0.942
Computer curve fitting			
Before	0.70 (0.18)	73 (8)	—
After	0.72 (0.14)	79 (4)	—

The results are the means (±1 SEM) of three separate experiments carried out on different occasions. The conditions for determining the saturation curves for specific [^{3}H]piflutixol binding, in the presence of 3×10^{-5} M (±)sulpiride, binding defined by 10^{-6} M *cis*-flupenthixol, are described in the text. The incubation tubes were filtered either at the start of the 2 min incubation at 30° (before), or at the end (after). The data from the saturation curves has been analysed by linear regression analysis of Scatchard plots or by the iterative computer curve fitting program designed to fit the data to a first order binding curve. r is the correlation coefficient of the linear regressions.

binding technique. The second is the determination of dopamine-sensitive adenylate cyclase activity in the presence and absence of [^{3}H]piflutixol. Samples were assayed in parallel, filtering some for analysis of [^{3}H]piflutixol binding and assaying the others for their cyclic AMP content.

MATERIALS AND METHODS

Chemicals. [^{3}H]Piflutixol (4-[^{3}H]*cis*-(*Z*)-piflutixol; 6.7–11.7 Ci/mmol), a gift from Dr J. Hyttel (Lundbeck, Copenhagen, Denmark); [^{3}H]cyclic AMP (ammonium salt; 25 Ci/mmol) from Amersham International (Amersham, U.K.); *cis*-flupenthixol from Lundbeck and Co. Ltd; (±)sulpiride from Delagrange; theophylline; dopamine hydrochloride; 2-mercaptoethanol; GTP; ATP (disodium salt); cyclic AMP (sodium salt); Tris maleate; EGTA and EDTA, all from the Sigma Chemical Co. (Poole, U.K.); charcoal (Norit GSX) from BDH Chemicals (Poole, U.K.).

Tissue preparation. Unless stated otherwise the following tissue preparation was employed: pooled rat (Bantim and Kingman 125 g ± 10) striata were homogenized in 100 volumes Tris maleate buffer (2 mM Tris maleate, 2 mM EGTA, pH 7.4) using 12 slow strokes of a teflon glass homogenizer. The suspension was centrifuged at 45,000 *g* at 4° for 15 min. Pellets were resuspended using four slow strokes in 100 volumes Tris maleate buffer, centrifuged at 45,000 *g* at 4° and resuspended in 20 volumes Tris maleate buffer.

Effect of [^{3}H]piflutixol on the assay of cyclic AMP. The effect of the presence of [^{3}H]piflutixol on the displacement of [^{3}H]cyclic AMP from cyclic AMP binding protein, by standard concentrations of cyclic AMP, was studied. The binding assay (see below) of standard concentrations of cyclic AMP was carried out in the presence of 0.01 mL of water or [^{3}H]piflutixol (4 nM).

The quantitative correlation of receptor occupation by increasing concentrations of [^{3}H]piflutixol and inhibition of dopamine-sensitive adenylate cyclase. Aliquots (0.10 mL) of tissue suspension were placed in glass incubation tubes with aliquots (0.05 mL) of water or 10^{-6} M *cis*-flupenthixol (final concentration; *cis*-flupenthixol being present in samples measuring non-specific binding of [^{3}H]piflutixol). Aliquots (0.05 mL) of (±)sulpiride (3×10^{-5} M final concentration) alone or containing [^{3}H]piflutixol (0.15–4 nM) were then added and the samples incubated for 20 min at 30°. This incubation enabled [^{3}H]piflutixol to bind to its receptor site. Some samples did not contain [^{3}H]piflutixol in order that adenylate cyclase activities in the absence of [^{3}H]piflutixol could be assayed.

After 20 min incubation samples were returned to ice and 0.75 mL of ice-cold M buffer (80 mM Tris maleate, 2 mM Mg_2SO_4, 10 mM theophylline, 0.2 mM EGTA, pH 7.4) with or without added dopamine (10^{-3} or 10^{-5} M final). Samples without dopamine were used for the assay of basal adenylate cyclase and [^{3}H]piflutixol binding, samples with dopamine were used for the assay of dopamine (10^{-3} or 10^{-5} M) stimulated adenylate cyclase. Aliquots (0.05 mL) of ATP (0.5 mM final) plus GTP (0.025 mM final) were then added to start the adenylate cyclase assay, and the tubes immediately returned to the shaking water bath at 30°.

Samples designated for the assay of [^{3}H]piflutixol binding were removed after 1 min. They were filtered over GF/C filters, washed twice with 5 mL of 50 mM Tris–HCl buffer, pH 7.4, 4°, and counted for radioactivity bound. After two min the remaining samples were removed from the water bath and placed in boiling water for 2.5 min. They were then assayed for their cyclic AMP content using the standard method (see below).

The saturation of specific [^{3}H]piflutixol binding to

the tissue, defined by 10^{-6} M *cis*-flupenthixol and in the presence of 3×10^{-5} M (±)sulpiride, with increasing [^{3}H]piflutixol concentrations, was assessed by analysis of the counts trapped on the filter papers. All data points were from samples assayed in triplicate. The data were subject to Scatchard analysis, or computer curve fitting using an iterative approach, to determine the K_d and B_{max} of specific [^{3}H]piflutixol binding to the tissue.

The percentage receptor occupation at each concentration of [^{3}H]piflutixol was then calculated:

$$\text{Receptor occupation } \% = \frac{B \times 100\%}{B_{max}}$$

where B was the amount of specific [^{3}H]piflutixol bound at each concentration (in pmol/g), and B_{max} is the maximum number of receptor sites (in pmol/g).

Dopamine-sensitive adenylate cyclase activity was determined by subtracting adenylate cyclase activities in the absence of dopamine (basal levels) from those in the presence of dopamine (10^{-3} or 10^{-5} M). Dopamine (10^{-3} or 10^{-5} M) sensitive adenylate cyclase activities were measured in the presence of the various concentrations of [^{3}H]piflutixol (0.15–4 nM), and in the absence of [^{3}H]piflutixol.

The percentage inhibition of dopamine-sensitive adenylate cyclase activity at each concentration of [^{3}H]piflutixol was then calculated:

$$\text{Inhibition of DSAC } \% = \frac{\text{DSAC}^o - \text{DSAC}^p}{\text{DSAC}^o} \times 100\%$$

where DSAC^o is the dopamine-sensitive adenylate cyclase activity observed without [^{3}H]piflutixol present, and DSAC^p is the dopamine-sensitive adenylate cyclase activity observed at each concentration of [^{3}H]piflutixol.

An initial experiment assessed the effects of the 2 min incubation at 30° on the kinetics of specific [^{3}H]piflutixol bound during the 20 min initial incubation. The same protocol was followed up until the point at which samples were returned to the water bath. For these experiments all samples had [^{3}H]piflutixol (0.15–4 nM) present, and dopamine (10^{-3} M final) was present in the aliquot (0.75 mL) of ice-cold M buffer (80 mM Tris maleate, 2 mM Mg_2SO_4, 10 mM theophylline, 0.2 mM EGTA, pH 7.4). After the addition of aliquots (0.05 mL) of ATP (0.5 mM final) plus GTP (0.025 mM final) some samples were immediately filtered over GF/C filters, washed twice with 5 mL of 50 mM Tris–HCl buffer, pH 7.4, 4°, and counted for radioactivity bound. The rest were returned to the water bath, 30°, incubated for 2 min and then filtered over GF/C filters, washed twice, and counted for radioactivity bound.

The experiment was repeated on four separate occasions, on each occasion all data points were the mean of triplicate estimations.

The inhibition of specific [^{3}H]*piflutixol binding by dopamine, in the presence and absence of GTP, and its effect on the inhibition of dopamine-sensitive adenylate cyclase.* These experiments compared the effect of dopamine (10^{-6}–10^{-4} M), present during the 20 min initial incubation with [^{3}H]piflutixol (1 nM), on the binding of [^{3}H]piflutixol with the effect of dopamine (10^{-6}–10^{-4} M), present during the same 20 min incubation with [^{3}H]piflutixol (1 nM), on the inhibition of dopamine-sensitive adenylate cyclase produced by that concentration of [^{3}H]piflutixol. The 20 min initial incubation with [^{3}H]piflutixol (1 nM) and dopamine (10^{-6}–10^{-4} M) was carried out in the presence or absence of GTP (0.025 mM). This was in order to determine the consequences of any shift of the curve of dopamine's displacement of specific [^{3}H]piflutixol binding, as a result of the presence of GTP, on the subsequent inhibition of dopamine sensitive adenylate cyclase.

Aliquots (0.10 mL) of tissue suspension were added to 0.75 mL of incubation buffer (80 mM Tris maleate, 2 mM Mg_2SO_4, 10 mM theophylline, 0.2 mM EGTA, pH 7.4) with or without dopamine (10^{-6}–10^{-4} M). In half the samples GTP (0.025 mM) was also present. Aliquots (0.05 mL) of (±)sulpiride (3×10^{-5} M final), alone or containing [^{3}H]piflutixol (1 nM) were added and the samples incubated for 20 min at 30°. In some samples the aliquots (0.05 mL) of (±)sulpiride (3×10^{-5} M) also contained *cis*-flupenthixol (10^{-6} M final) to measure non-specific binding of [^{3}H]piflutixol. This 20 min initial incubation allowed [^{3}H]piflutixol to bind to the membranes; in the samples with dopamine present the dopamine competed with the [^{3}H]piflutixol and resulted in inhibition of specific [^{3}H]piflutixol binding.

The samples were returned to ice and sufficient dopamine was added, in aliquots (0.10 mL) of incubation buffer (80 mM Tris maleate, 2 mM Mg_2SO_4, 10 mM theophylline, 0.2 mM EGTA, pH 7.4), to bring the final concentration in all samples to 10^{-4} M dopamine. However in some samples, to establish basal adenylate cyclase activity, no dopamine was present in either incubation. Aliquots (0.05 mL) of ATP (0.5 mM final) alone or containing GTP (0.025 mM final) were then added and the samples immediately returned to the water bath (30°). If GTP was present in the initial incubation then the aliquot contained ATP alone. If no GTP was present during the initial incubation then the aliquot contained ATP plus GTP.

Samples designated for the assay of [^{3}H]piflutixol binding were removed after 1 min. They were filtered over GF/C filters, washed twice with 5 mL of 50 mM Tris–HCl buffer, pH 7.4, 4°, and counted for radioactivity bound. After two min the remaining samples were removed from the water bath and placed in boiling water for 2.5 min. They were then assayed for their cyclic AMP content using the standard method (see below).

The 100% specific binding of [^{3}H]piflutixol was the binding observed in the absence of dopamine, specific binding being defined by the incorporation of 10^{-6} M *cis*-flupenthixol. The specific binding of [^{3}H]piflutixol, observed at each concentration of dopamine present in the incubation, was expressed as a percentage of this value.

Dopamine sensitive adenylate cyclase activity was determined by subtracting adenylate cyclase activities in the absence of dopamine (basal levels) from those in the presence of dopamine (10^{-4} M). Dopamine-(10^{-4} M) sensitive adenylate cyclase activities were

measured after the initial incubation with a single concentration of [^{3}H]piflutixol (1 nM) and various concentrations of dopamine (10^{-6}–10^{-4} M). Dopamine (10^{-4} M) sensitive adenylate cyclase in the absence of [^{3}H]piflutixol in either incubation was also measured; this was taken as being 100% dopamine sensitive adenylate cyclase activity. The inhibition of dopamine-sensitive adenylate cyclase activity by [^{3}H]piflutixol (1 nM) at each concentration of dopamine in the initial incubation was expressed as a percentage of the inhibition of dopamine-sensitive adenylate cyclase activity produced by [^{3}H]piflutixol (1 nM) in the absence of dopamine in the initial incubation. The experiment was performed on four separate occasions.

Assay of cyclic AMP by protein binding saturation. The method used was essentially that of Brown *et al.* [7]. Aliquots (0.01 mL) of the supernatant to be assayed, or of unlabelled cyclic AMP standards diluted in distilled water (5×10^{-8}–5×10^{-6} M), were put into glass incubation tubes kept on ice. Each tube contained 0.2 mL [^{3}H]cyclic AMP (approx. 15,000 dpm per 0.2 mL, sp. act. = 25 Ci/mmol) diluted in buffer (50 mM Tris–HCl, 8 mM theophylline, 6 mM 2-mercaptoethanol, pH 7.4). To each sample was added 0.1 mL of cyclic AMP binding protein, diluted 1:20 in buffer. The samples were mixed and left on ice for 90 min.

The unbound cyclic AMP was separated from bound cyclic AMP by the addition of 0.1 mL of a charcoal suspension (0.6 g bovine serum albumin plus 3 g charcoal in 30 mL of distilled water). The suspension was mixed and then centrifuged in a Sorvall multifuge at 4° at 2000 rpm for 20 min. Into plastic scintillation vials was placed 0.25 mL of supernatant and 4 mL of E.S. 299 (Packard). The vials were counted as for tritiated radioligand.

The standard curve of the counts bound versus the cyclic AMP concentration was plotted as $\log_{10}$ cpm versus $\log_{10}$ of the cyclic AMP concentration. The cyclic AMP concentration for each sample was read from this curve. It was necessary to subtract the concentration of radiolabelled cyclic AMP (approx. 3×10^{-8} M) to obtain the concentration of cyclic AMP transferred in the 0.01 mL aliquot.

RESULTS

Effect of [^{3}H]*piflutixol on the assay of cyclic AMP*

There is no effect of [^{3}H]piflutixol (0.01 mL, 4 nM solution) on the cpm of radioactivity measured for each standard cyclic AMP concentration in the cyclic AMP protein binding assay (Table 1).

The quantitative correlation of receptor occupation by increasing concentrations of [^{3}H]*piflutixol and inhibition of dopamine-sensitive adenylate cyclase*

The saturation curves of specific [^{3}H]piflutixol binding were the same whether samples were filtered at the beginning or end of the 2 min incubation (Table 2). There was good agreement between the K_d and B_{max} of binding derived from Scatchard analysis or computer curve fitting of the saturation plots of specific [^{3}H]piflutixol binding to the membranes (Table 2).

The presence of [^{3}H]piflutixol (4 nM) had no effect on basal adenylate cyclase activity (Fig. 1). Dopamine, 10^{-3} or 10^{-5} M, in the final incubation increased cyclic AMP formation over basal levels (dopamine-sensitive adenylate cyclase: 10^{-3} M dopamine = 13.6 ± 2.5; 10^{-5} M dopamine = 5.4 ± 0.8 pmol/2 min/2 mg wet wt).

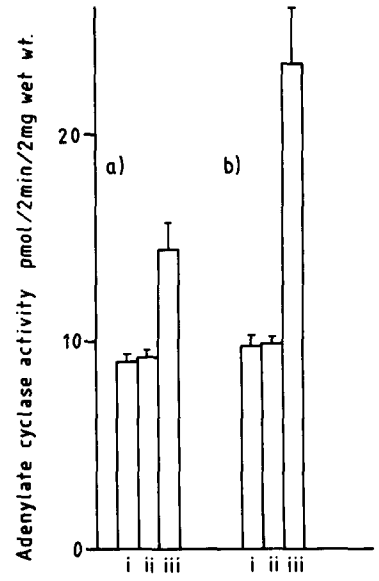

Fig. 1. Adenylate cyclase activity in striatal membranes in the absence and presence of dopamine (10^{-5} and 10^{-3} M) and [^{3}H]piflutixol (4 nM). (a) Dopamine 10^{-5} M. (b) Dopamine 10^{-3} M. (i) Basal adenylate cyclase activity. (ii) Basal adenylate cyclase activity in the presence of [^{3}H]piflutixol (4 nM). (iii) Adenylate cyclase activity in the presence of dopamine (10^{-5} or 10^{-3} M). Results are the means (±SEM) of three separate experiments.

[^{3}H]Piflutixol (0.15–4 nM) inhibited the increase in cyclic AMP formation produced by dopamine (10^{-3} or 10^{-5} M). The inhibition of dopamine-sensitive adenylate cyclase activity increased with increasing [^{3}H]piflutixol concentration. Figure 2 shows a typical experiment in which the inhibition of dopamine-sensitive adenylate cyclase is compared to the saturation of specific [^{3}H]piflutixol binding over the same concentrations. There is good agreement between the inhibition of dopamine-sensitive adenylate cyclase and the saturation of specific [^{3}H]piflutixol binding.

There was a close correlation between the percentage occupation of specific [^{3}H]piflutixol binding sites by [^{3}H]piflutixol (0.15–4 nM) and the percentage inhibition of dopamine- (10^{-3} or 10^{-5} M) sensitive adenylate cyclase by [^{3}H]piflutixol (0.15–4 nM) (Fig. 3a and b). Each plot is the combined results from four separate experiments. Regression analysis of all the results together, or averaging the results of the regression analyses of the separate experiments, gave the same result. There was an exact correlation between receptor occupation by [^{3}H]piflutixol and inhibition of dopamine- (10^{-3} M) sensitive adenylate cyclase (Table 3 and Fig. 3b). When 10^{-5} M dopamine was used to stimulate dopamine-sensitive adenylate cyclase then only 75–

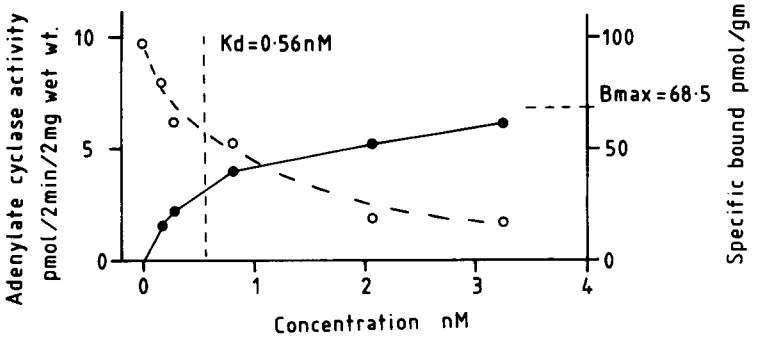

Fig. 2. Comparison of the inhibition of dopamine sensitive adenylate cyclase by [^{3}H]piflutixol (open circles), and the saturation of specific [^{3}H]piflutixol (0.15–4 nM) binding (closed circles) to the same striatal tissue preparation. Specific binding of [^{3}H]piflutixol, in the presence of 3×10^{-5} M (±)sulpiride, was defined by 10^{-6} M *cis*-flupenthixol. Dopamine (10^{-3} M) was used to stimulate adenylate cyclase activity. Results from a single experiment.

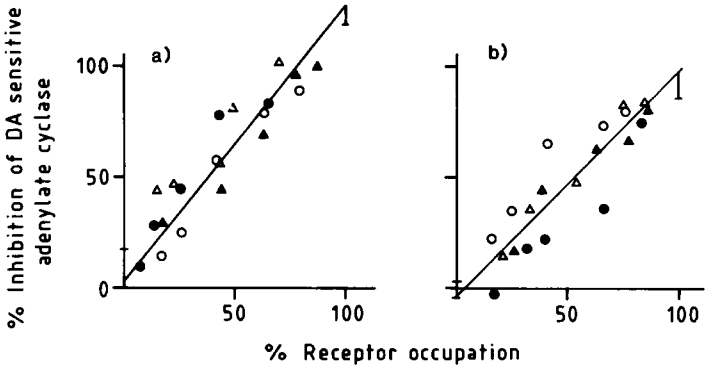

Fig. 3. The correlations between inhibition of dopamine sensitive adenylate cyclase by increasing concentrations of [^{3}H]piflutixol (0.15–4 nM) and saturation of specific [^{3}H]piflutixol binding sites. The points are the individual results from four separate experiments each using five concentrations of [^{3}H]piflutixol for the saturation curve. Dopamine [10^{-5} M (a) or 10^{-3} M (b)] was used to stimulate dopamine-sensitive adenylate cyclase.

80% occupation of specific [^{3}H]piflutixol binding sites, by [^{3}H]piflutixol, was necessary to produce full inhibition of dopamine-sensitive adenylate cyclase.

The inhibition of specific [^{3}H]piflutixol binding by dopamine, in the presence and absence of GTP, and its effect on the inhibition of dopamine sensitive adenylate cyclase

The presence of [^{3}H]piflutixol (1 nM) had no effect on basal adenylate cyclase levels (Fig. 4). GTP (0.025 mM) present in the 20 min incubation had a small, but not significant, effect on basal adenylate cyclase activity. GTP (0.025 mM) present in the initial incubation had no effect on dopamine-sensitive adenylate cyclase activity, or on the inhibition of dopamine-sensitive adenylate cyclase by [^{3}H]piflutixol (1 nM) when no dopamine was added to the 20 min initial incubation. Dopamine (10^{-4} M) produced the same stimulation of adenylate cyclase activity whether it was present throughout the experiment (12.2 ± 1.8 pmol/2 min/2 mg wet wt), or only present during the final 2 min incubation (11.6 ± 2.1 pmol/2 min/2 mg wet wt).

In the absence of GTP, dopamine (10^{-6}–10^{-4} M) present in the initial incubation with [^{3}H]piflutixol (1 nM) produces a greater percentage reduction of [^{3}H]piflutixol's inhibition of dopamine-sensitive adenylate cyclase than percentage displacement of specific [^{3}H]piflutixol binding (Fig. 5). In the presence of GTP (0.025 mM) in the initial incubation there is a shift, to the right, of the plots of both dopamine (10^{-6}–10^{-4} M) displacement of specific [^{3}H]piflutixol (1 nM) binding and dopamine (10^{-6}–10^{-4} M) inhibition of [^{3}H]piflutixol's (1 nM) inhibition of dopamine-sensitive adenylate cyclase (Fig. 5). Under these conditions dopamine produced a greater percentage displacement of specific [^{3}H]piflutixol binding than the percentage reduction of [^{3}H]piflutixol's inhibition of dopamine-sensitive adenylate cyclase.

DISCUSSION

It is likely that [^{3}H]piflutixol fails to interfere with the cyclic AMP binding assay because it binds avidly to the charcoal used in the assay to remove

Table 3. The results of linear regression of the correlation between D-1 receptor occupation by [^{3}H]piflutixol and inhibition of dopamine sensitive adenylate cyclase by [^{3}H]piflutixol in rat striatal membranes

Dopamine (10^{-6} M)	% Inhibition adenyl cyclase	Slope	% Receptor occupation	r
10	+4 (14)	1.24 (0.11)	77	0.929
1000	−4 (7)	1.02 (0.12)	102	0.895

The results are the values obtained from linear regression analysis of the plot shown in Fig. 3 of the correlation derived from four separate experiments performed on separate occasions. For each experiment the percentage receptor occupation of D-1 receptors by [^{3}H]piflutixol (0.15–4 nM) at five different concentrations of [^{3}H]piflutixol is plotted against the percentage inhibition of dopamine-sensitive adenylate cyclase produced by those same five concentrations of [^{3}H]piflutixol. The method for determining these values is described in the text. The values for the 'percentage inhibition of adenyl cyclase' are those given by the linear regression for the value of per cent inhibition of dopamine-sensitive adenylate cyclase when percentage receptor occupation is 0. The slope is the slope of the linear regression. The values in brackets for these parameters is their standard error given by the linear regression analysis. The 'percentage receptor occupation' is the percentage receptor occupation of D-1 receptors by [^{3}H]piflutixol when there is 100% inhibition of dopamine sensitive adenylate cyclase. It was the point at which the line of the linear regression crossed the line percentage inhibition of adenyl cyclase = 100. r is the correlation coefficient of the linear regressions.

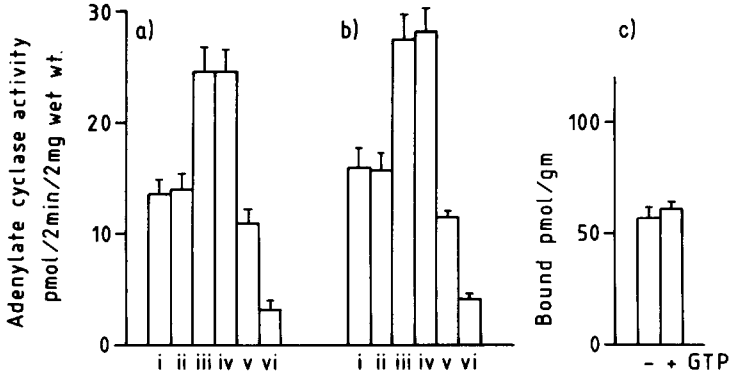

Fig. 4. (a and b) Adenylate cyclase activity in striatal membrane preparations in the presence and absence of dopamine (10^{-4} M) and [^{3}H]piflutixol (1 nM). Experiments were carried out in the absence (a) or presence (b) of GTP (25×10^{-6} M). (i) Basal adenylate cyclase. (ii) Basal adenylate cyclase in the presence of [^{3}H]piflutixol (1 nM). (iii) Dopamine-stimulated adenylate cyclase activity in response to dopamine (10^{-4} M) present only during the final 2 min incubation. (iv) Dopamine-stimulated adenylate cyclase activity in response to dopamine (10^{-4} M) present throughout the initial incubation and the final 2 min incubation. (v) Dopamine- (10^{-4} M) sensitive adenylate cyclase, in response to dopamine present only during the final incubation. (vi) Dopamine- (10^{-4} M) sensitive adenylate cyclase in the presence of [^{3}H]piflutixol (1 nM) throughout the experiment. (c) Specific [^{3}H]piflutixol (1 nM) binding in the absence (−) and presence (+) of GTP. Results are the means (±SEM) of three separate experiments.

free [^{3}H]cyclic AMP. A negligible amount of [^{3}H]piflutixol would be left in the supernatant to be counted. There was little change in the binding of [^{3}H]piflutixol to the tissue in the face of a large fall in free ligand concentration, or the introduction of a large concentration of dopamine that would be expected to displace the ligand, over a 2 min incubation at 30°.

It has therefore been possible to look directly at the increasing inhibition of dopamine-sensitive adenylate cyclase activity resulting from increasing concentrations of [^{3}H]piflutixol present in the incubation. Sulpiride was present in all assay tubes to mask any drug binding to the D-2 receptor. The results of the study are therefore not contaminated by D-2 receptor effects on dopamine sensitive adenylate cyclase [8].

There was a close correlation between the percentage of D-1 receptors occupied by [^{3}H]piflutixol and the inhibition, by [^{3}H]piflutixol, of dopamine-sensitive adenylate cyclase activity (Figs 2 and 3). There was a linear relationship between

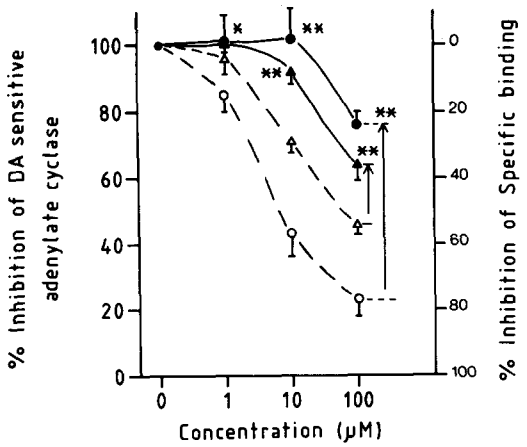

Fig. 5. The effect of GTP (25×10^{-6} M) in the 20 min initial incubation with various concentrations of dopamine (0, 1, 10 and 100×10^{-6} M) on the displacement of specific [^{3}H]piflutixol (1 nM) binding by dopamine during the initial incubation, or the inhibition, by dopamine, of [^{3}H]piflutixol's inhibition of dopamine- (10^{-4} M) sensitive adenylate cyclase measured immediately after the initial incubation. Triangles, inhibition of specific [^{3}H]piflutixol binding. Circles, inhibition of dopamine-sensitive adenylate cyclase. Open symbols and dotted lines, in the absence of GTP. Closed symbols and continuous lines, in the presence of GTP. Arrows indicate the shift of the curves in response to GTP. The 100% inhibition of specific [^{3}H]piflutixol binding was the binding observed in the presence of 10^{-6} M *cis*-flupenthixol. The 100% inhibition of dopamine-sensitive adenylate cyclase was the inhibition of dopamine sensitive adenylate cyclase by [^{3}H]piflutixol in the absence of dopamine. The results are the means (±SEM) of four separate experiments. (*) and (**)—significantly different from values in the absence of GTP ($P < 0.05$ and $P < 0.01$, respectively).

the percentage stimulation of adenylate cyclase by 10^{-3} M dopamine and the percentage of receptors left unoccupied by [^{3}H]piflutixol (Fig. 3). However, when 10^{-5} M dopamine was used to stimulate dopamine-sensitive adenylate cyclase then the regression line crossed the axis for 100% inhibition of enzyme activity when only 77% of specific [^{3}H]piflutixol binding sites were occupied by [^{3}H]piflutixol. This suggests that at least 20% of receptors need to remain unoccupied before 10^{-5} M dopamine is able to stimulate adenylate cyclase.

The conclusions from this study differ from those of Battaglia *et al.* [9] who suggested that there are spare D-1 receptors in rat striatum, i.e. that there may be 100% stimulation of dopamine-stimulated adenylate cyclase activity despite some D-1 receptors being occupied by antagonist. They used quite a different methodology, injecting rats *in vivo* with *N*-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ). This drug irreversibly blocks the D-1 receptor. The animals were killed at various time intervals after the injection and dopamine sensitive adenylate cyclase activity and [^{3}H]SCH 23390 binding to D-1 receptors assayed. They demonstrated that dopamine-stimulated adenylate cyclase activity recovered much more quickly that D-1 receptor numbers. They proposed that the effect was not due to non-specific changes in adenylate cyclase activity; there was no effect of the treatment on forskolin- or GTP-stimulated adenylate cyclase activity. They did not attempt to demonstrate that the effect could be replicated by using different concentrations of EEDQ, rather than different time points. This would have negated the criticism that their result is a reflection of the different times between injection of EEDQ and death, rather than a result of variable receptor occupation.

Our results are similar to those of Korf *et al.* [10] who demonstrated a linear relationship between occupation of [^{3}H]spiperone binding sites in striatum by [^{3}H]spiperone, *in vivo*, and reduction of striatal acetylcholine levels.

Dopamine is better able to stimulate adenylate cyclase ($K_m = 9 \times 10^{-6}$ M) than it is able to inhibit [^{3}H]piflutixol binding ($IC_{50} = 70$ and $>100 \times 10^{-6}$ M in absence and presence of GTP respectively; Fig. 5). Correcting the IC_{50} values to K_i values using the Cheng and Prussof [11] correction gives K_i values for dopamine inhibition of specific [^{3}H]piflutixol binding of 30 and $>43 \times 10^{-6}$ M respectively.

The finding of a discrepancy between the K_m of an agonist for stimulation of adenylate cyclase and its *Ki* for inhibition of radioligand antagonist binding is consistent with the results of Terasaki and Brooker [12]. They studied adenyl cyclase activity in rat glioma cells and observed that (−)-isoprenaline was 4000-fold more potent as a stimulator of cyclic AMP than as an inhibitor of [^{125}I]iodohydroxybenzylpindolol ([^{125}I]HYP), a β receptor antagonist, binding to the β-adrenergic receptor linked to adenylate cyclase activation. They concluded that this result reflected high coupling efficiency of agonist-induced adenyl cyclase activation. Occupation of only a small fraction of receptors by agonist is able to produce considerable stimulation of adenylate cyclase. Andersen *et al.* [13] studied [^{3}H]SCH 23390 binding and dopamine-stimulated adenylate cyclase, under identical assay conditions, in order to make direct comparisons between affinities of dopamine agonist and antagonist drugs in the two assays. They noted that there was a consistent discrepancy, most evident for agonists. Drugs were *more* potent at inhibiting [^{3}H]SCH 23390 binding than at stimulating or inhibiting dopamine stimulated adenylate cyclase.

The results from the receptor occupation study described in this chapter suggest that high coupling efficiency of agonist adenylate cyclase activation does not explain why the K_m of dopamine's stimulation of adenylate cyclase is less than its K_i for inhibition of specific [^{3}H]piflutixol binding. There appears to be a linear relationship between the number of receptors available for stimulation by dopamine and adenylate cyclase activation.

The likely explanation for the discrepancy between dopamine's affinity for inhibition of [^{3}H]piflutixol binding and its affinity for stimulation of adenylate cyclase is that there are two states of the D-1 receptor, $D\text{-}1_{high}$ and $D\text{-}1_{low}$ [14, 15]. [^{3}H]Piflutixol has equal high affinity at these two sites, while dopamine only has high affinity for the $D\text{-}1_{high}$ site. Dopamine stimulates adenylate cyclase through the $D\text{-}1_{high}$ site, rather than the $D\text{-}1_{low}$ site. Dopamine

is therefore more potent at stimulating adenylate cyclase ($D\text{-}1_{high}$) than it is at displacing [^{3}H]piflutixol binding from $D\text{-}1_{high}$ and $D\text{-}1_{low}$ sites.

Another possible explanation for the discrepancy is that dopamine only has to interact with a receptor for a fraction of the time to produce maximal stimulation of cyclase by that receptor. MacFarlane and Stump [5] proposed a similar mechanism to account for their observations of adrenaline inhibition of adenyl cyclase. The maximal response of the system at any one concentration of antagonist would be proportional to the numbers of receptors left unoccupied by antagonist. But, at any one time, while the system was producing a maximal response to dopamine not all receptors would be occupied by dopamine. A greater concentration of dopamine would therefore be required to produce a certain inhibition of radioligand antagonist binding than would be required to produce an equivalent percentage stimulation of adenylate cyclase.

For the IC_{50} experiments the concentration of 1 nM [^{3}H]piflutixol was chosen as a concentration producing about 70% inhibition of adenylate cyclase. This allowed dopamine to easily displace the [^{3}H]piflutixol from the D-1 receptor, yet meant that there was still plenty of inhibition of dopamine-sensitive adenylate cyclase by [^{3}H]piflutixol to be inhibited by dopamine.

The studies on the displacement of [^{3}H]piflutixol by dopamine, and the effect this has on the inhibition of dopamine-sensitive adenylate cyclase by [^{3}H]piflutixol, produced some interesting results. If the initial incubations were carried out in the absence of GTP then dopamine was more potent at reducing [^{3}H]piflutixol's inhibition of dopamine-sensitive adenylate cyclase than it was at displacing [^{3}H]piflutixol binding to D-1 sites. The receptors left unoccupied by [^{3}H]piflutixol, because they have been occupied by dopamine in the initial incubation, were slightly more effective at producing adenylate cyclase activation.

It is known that GTP shifts the displacement curve of dopamine against D-1 antagonist ligand binding to the right [16–18] as a result of GTP reducing high affinity displacement. When the initial incubation took place in the presence of 0.025 mM GTP the curve for the displacement of [^{3}H]piflutixol by dopamine was, as expected, shifted to the right. However the curve for dopamine's inhibition of the inhibition of dopamine-sensitive adenylate cyclase by [^{3}H]piflutixol is shifted even further to the right. Dopamine, in the presence of GTP, was less effective at inhibiting the inhibition of dopamine-sensitive adenylate cyclase by [^{3}H]piflutixol, than it was at displacing [^{3}H]piflutixol binding to D-1 receptors. The receptors left unoccupied by [^{3}H]piflutixol were less able to activate adenylate cyclase.

This was not a result of desensitization of dopamine-sensitive adenylate cyclase as a consequence of exposure to dopamine plus GTP; the adenylate cyclase response to 10^{-4} M dopamine, in the absence of [^{3}H]piflutixol, was the same whether GTP and dopamine (10^{-4} M) were present throughout the experiment, or only in the final 2 min incubation. The changes in response to GTP in the initial incubation therefore reflect changes in occupancy–activation relationships as a result of initial incubation with dopamine.

A possible explanation for this finding is as follows. In the absence of GTP in the initial incubation there is a relatively high percentage of $D\text{-}1_{high}$ sites remaining at the end of the initial incubation (perhaps 20%). Dopamine is able to displace [^{3}H]piflutixol from these sites with high affinity. In addition, because the $D\text{-}1_{high}$ site must be present for dopamine to stimulate adenylate cyclase during the following 2 min incubation, dopamine-sensitive adenylate cyclase will be preserved. In the presence of GTP during the initial incubation the majority of $D\text{-}1_{high}$ sites are converted to $D\text{-}1_{low}$ sites. There is therefore, as observed, a slight shift of dopamine's displacement curve against [^{3}H]piflutixol to the right (i.e. less displacement of [^{3}H]piflutixol at each concentration of dopamine). There is however a much greater reduction in dopamine-sensitive adenylate cyclase activity because of the paucity of remaining $D\text{-}1_{high}$ sites at which dopamine is able to act to stimulate adenylate cyclase.

Finally, the results indicate that it is not possible to ascribe the discrepancy between the K_m for dopamine's activation of adenylate cyclase, and its K_i for inhibition of [^{3}H]piflutixol binding to the D-1 receptor, to a hyperbolic occupation–activation relationship.

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