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IS THERE A CONNECTION BETWEEN HIGH AFFINITY ^{3}H-SPIPERONE BINDING SITES AND DA-SENSITIVE ADENYLATE CYCLASE IN CORPUS STRIATUM ?

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Abstract—The possibility of a connection between the high affinity ^{3}H-spiperone binding sites and the dopamine-sensitive adenylate cyclase system suggested by recent studies was investigated in corpus striatum. When measured under identical conditions (membrane preparation, incubation conditions, presence of dopamine), the affinity of spiperone for its binding site (1.5×10^{-10} M) was much higher than its affinity for inhibiting the dopamine sensitive adenylate cyclase (10^{-7} M). Phenoxybenzamine was found to block ^{3}H-spiperone binding irreversibly. Phenoxybenzamine (10^{-5} M) completely supressed ^{3}H-spiperone binding while the adenylate cyclase continued to be stimulated by dopamine (35 per cent of control stimulation) and inhibited by spiperone or haloperidol. The affinities of these neuroleptics for inhibiting the dopamine-sensitive adenylate cyclase were the same on control and phenoxybenzamine treated membranes. Consequently the ^{3}H-spiperone binding site has not to be occupied to allow inhibition of the dopamine-sensitive adenylate cyclase. Guanosine triphosphate reduced the affinities of dopamine, 3-{2-[*N*-(3-hydroxyphenylethyl)*N*-propylamino]ethyl}phenol (RU 24926) and serotonin for ^{3}H-spiperone binding sites and did not affect the affinity of 2-Br-α-ergocriptine. Since RU 24296 and serotonin neither stimulate nor inhibit the dopamine-sensitive adenylate cyclase, the effects of guanosine triphosphate on binding do not appear related to adenylate cyclase activation by agonists which is known to require guanosine triphosphate. Our experiments suggest that the high affinity ^{3}H spiperone binding sites are not related to the dopamine-sensitive adenylate cyclase.

Corpus striatum homogenates contain ^{3}H-spiperone binding sites considered to be dopaminergic [1, 2] and a DA-sensitive† adenylate cyclase which is inhibited by neuroleptics including spiperone [3, 4]. The affinity of spiperone for its binding sites is very high ($\simeq 10^{-10}$ M), whether measured in saline buffer or under adenylate cyclase conditions (this report); its affinity for inhibiting the DA-sensitive adenylate cyclase is much lower (10^{-7} M) [4]. This difference in affinity was also found with other butyrophenones [4]. There are even drugs such as benzamide derivatives [5, 6], domperidone [7, 8] and the *N*-diphenylethylamine derivatives RU 24926 (DA-agonist) [9] which have high affinities for ^{3}H-spiperone binding sites but are poorly active or inactive on DA-sensitive adenylate cyclase. Furthermore, a marked difference was found in the topographical [10] and subcellular [11] distributions of neuroleptic binding sites and DA-sensitive adenylate cyclase. Finally, after kainic acid lesions, most of the DA-sensitive adenylate cyclase activity disappears (85 per cent) whereas 60 per cent ^{3}H-spiperone binding remains [12]. All these results strongly suggest that ^{3}H-spiperone binding sites are distinct from the spiperone receptors mediating inhibition of the DA-sensitive adenylate cyclase.

However, a connection between the DA-sensitive adenylate cyclase systems and ^{3}H-spiperone binding sites has been suggested by several authors to account for GTP effects [13–15]. GTP reduces the affinity of ^{3}H-spiperone binding sites for DA, epinine, apomorphine and ADTN but not for bromocriptine and dopaminergic antagonists; DA, epinine, apomorphine and ADTN all stimulate adenylate cyclase activity of corpus striatum homogenates in the presence of GTP [16], whereas bromocriptine, a dopaminergic agonist at the pituitary receptor [17], does not [18]. Furthermore, when the DA-sensitive adenylate cyclase is destroyed by kainic acid lesions, GTP no longer affects the affinity of the remaining ^{3}H-spiperone binding sites for DA [14].

In order to support the hypothesis of a connection between part of the high affinity ^{3}H-spiperone binding sites and the DA-sensitive adenylate cyclase, we blocked completely and irreversibly the binding sites with phenoxybenzamine and tried to detect after this blockade modifications of the inhibition by spiperone of the DA-sensitive adenylate cyclase. As the results of these experiments did not indicate such a connection, we further analyzed the effects of GTP on ^{3}H-spiperone binding sites.

MATERIALS AND METHODS

Preparation of particulate fractions. Male Charles River rats of the Sprague–Dawley strain (200–300 g) were decapitated. Corpus striatum was dissected on a refrigerated plate and homogenized in 60 vol. 2 mM Tris-maleate, 2 mM EGTA, pH 7.2. The

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† Abbreviations used: DA, dopamine; 5-HT, serotonin; RU 24926, 3{2-[*N*-(3-hydroxyphenylethyl)*N*-propylamino]ethyl} phenol; bromocriptine, 2-Br-α ergocriptine; EGTA, ethylene glycol bis (β aminoethyl ether) -*N*, *N*' tetraacetic acid; ADTN, 2 amino-6,7 dihydroxytetralin.

homogenate was centrifuged at 47,000 g for 20 min and the pellet resuspended in the same buffer.

To study irreversible effects of phenoxybenzamine, corpus striatum was homogenized in 90 vol. of adenylate cyclase medium:25 mM Tris-maleate, pH 7.2, 0.5 mM ATP, 1 mM $MgSO_4$, 10 mM theophylline, 5×10^{-7} M GTP, 0.2 mg/ml creatine kinase and 10 mM creatine phosphate. This homogenate was incubated with or without phenoxybenzamine for 15 min at 30°C and then washed three times in 700 vol. of 2 mM Tris-maleate, 2 mM EGTA, pH 7.2.

For the GTP experiments, particulate fractions were prepared according to Creese *et al.* [19] except that homogenization was done with a teflon Potter–Elvehjem.

^{3}H-Spiperone binding. Tubes containing 500 μl adenylate cyclase medium (final composition given above), 100 μl ^{3}H-spiperone, 200 μl test drugs, and 200 μl particulate fractions (the protein concentrations are given in the legends to figures) were incubated for 40 min at 37°. In our experimental conditions, 30 min of incubation are needed to obtain equilibrium of the binding with the lowest ^{3}H-spiperone concentration used for Scatchard analysis. Membranes were then collected by filtration on GF/B glass fiber (Whatman), washed three times with 5 ml ice-cold, 50 mM phosphate buffer, pH 7.4 and the bound radioactivity measured. Specific binding was defined as the difference between ^{3}H-spiperone binding in the absence and in the presence of 5×10^{-6} M *d*-butaclamol. In the striatum, this specific binding was identical to that defined in the presence of 10^{-5} M ADTN.

When the effects of phenoxybenzamine treatments on ^{3}H-spiperone binding and DA-sensitive adenylate cyclase were compared, the binding was done in 100 μl (all volumes were therefore reduced 10-fold). We have verified that for ^{3}H-spiperone concentrations higher than 2×10^{-9} M, the specific binding to a given amount of particulate fractions (always less than 100 μg in 100 μl) was the same whether it was measured in 100 μl or in 1 ml.

For GTP experiments, ^{3}H-spiperone binding was done according to Creese *et al.* [19].

Adenylate cyclase assay. The reaction was allowed to proceed in 100 μl of the adenylate cyclase medium used to determine ^{3}H-spiperone binding. The reaction was started at zero time, and 30 min afterwards, 10 μl of 0.002 μCi ^{3}H-cAMP and 2 μCi α-^{32}P-ATP were added. The reaction was stopped at 40 min, as previously described [16]. ^{32}P-cAMP and ^{3}H-cAMP were isolated according to Salomon *et al.* [20]. This assay protocol was designed to measure the velocity of the reaction from 30 to 40 min after zero time for direct comparison with binding determinations performed 40 min after the reaction started.

Chemicals. ATP (disodium salt), dopamine were from Sigma; cyclic AMP, creatine kinase and creatine phosphate were from Boehringer Mannheim. Drugs were kindly donated by Rhône Poulenc (haloperiodol), Janssen Pharmaceutica (spiperone), Roussel-Uclaf (RU 24296), Sandoz (bromocriptine), Ayerst Research Laboratories (*d* and *l*-butaclamol), Smith, Kline & French (phenoxybenzamine).

Radiochemicals. ^{3}H-cyclic AMP (ammonium salt) 25 Ci/mmole, α-^{32}P-ATP (sodium salt) 10–20 Ci/mmole and ^{3}H-spiperone 25.6 Ci/mmole were purchased from NEN.

RESULTS

Irreversible blockade of ^{3}H-spiperone binding sites by phenoxybenzamine. Influence on DA-sensitive adenylate cyclase. In previous reports, ^{3}H-spiperone binding has never been measured in the incubation medium used for adenylate cyclase assay. We controlled that in this medium, ^{3}H-spiperone binds to one category of high affinity binding sites having a K_D similar to that usually reported (K_D 1.5 ×

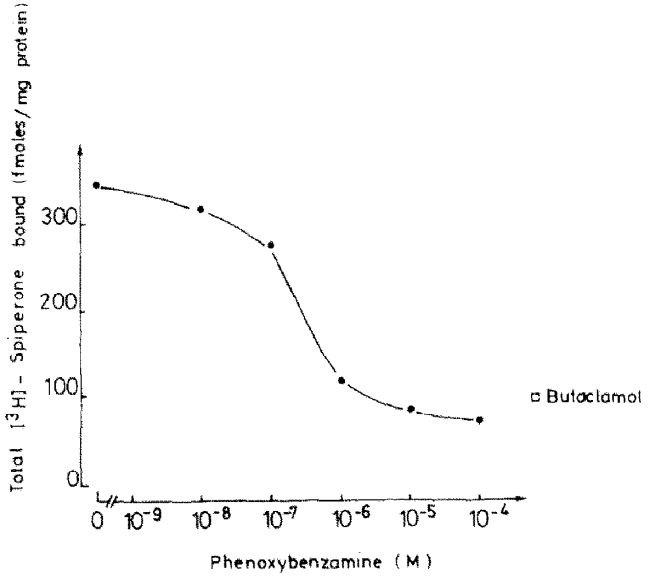

Fig. 1. ^{3}H-Spiperone binding displacement by phenoxybenzamine added directly in the assay. Corpus striatum homogenate (0.6 mg protein/ml) was used in this experiment. The ^{3}H-spiperone concentration was 2×10^{-9} M.

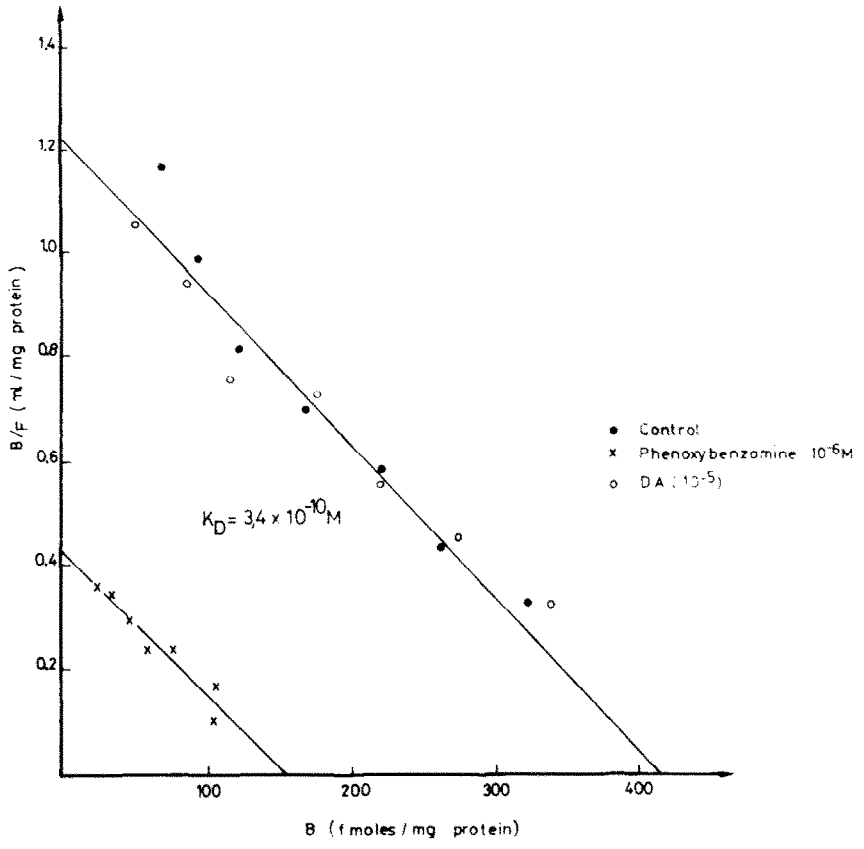

Fig. 2. Scatchard analysis of ^{3}H-spiperone binding, on control and phenoxybenzamine-treated membranes. Membranes were incubated with or without phenoxybenzamine 10^{-6} M or Da (10^{-5} M) and then washed as described under Materials and Methods. Phenoxybenzamine treatment reduced the total number of binding sites without changing their affinity (K_D = 3.4 × 10^{-10} M). The protein concentration was 0.18 mg/ml.

10^{-10} M) and maximal capacity of 400 fmoles/mg protein (data not shown).

Phenoxybenzamine, a well known irreversible ligand of a α-adrenergic receptors [21], interacts with the DA receptors coupled with an adenylate cyclase [22]. We observed that, when added in the binding assay, 10^{-5} M phenoxybenzamine is able to completely displace the specific binding of a saturating ^{3}H-spiperone concentration without affecting significantly non-specific binding (Fig. 1).

In order to know whether this blockade is irreversible, we compared ^{3}H-spiperone binding on membranes preincubated with or without 10^{-6} M phenoxybenzamine and extensively washed (see Materials and Methods). Total ^{3}H-spiperone binding was reduced and non-specific binding was not affected (data not shown). Scatchard plots are reported in Fig. 2. The presence of phenoxybenzamine during pre-incubation does not modify the affinity of ^{3}H-spiperone for its binding site but strikingly reduces the total number of specific binding sites. On the contrary, if we washed membranes preincubated with a reversible ligand which completely inhibits ^{3}H-spiperone binding sites during the pre-incubation (DA 10^{-5} M for example), we obtained a Scatchard plot which is superimposed with the control one (Fig. 2). Thus, the washing procedure completely eliminated the free ligand. Phenoxybenzamine therefore seems to be an irreversible blocking agent of ^{3}H-spiperone binding sites.

In agreement with Walton *et al.* [22], phenoxybenzamine treatment was also found to irreversibly block the maximal DA-adenylate cyclase stimulation (Figs. 3, 4) without significant effect on basal adenylate cyclase activity. In four experiments, the ratios between the basal adenylate cyclase activity of 10^{-5} M phenoxybenzamine treated membranes and control were 0.84, 1.2, 1.0 and 1.1.

Figure 3 compares the two irreversible blockades for different doses of phenoxybenzamine treated membranes. ^{3}H-spiperone binding and adenylate cyclase stimulation have been measured at saturating concentration of ^{3}H-spiperone (3.5×10^{-9} M) and DA (10^{-3} M), respectively, both on control and phenoxybenzamine treated membranes. Results are expressed in percentage of the maximal adenylate cyclase stimulation and total specific binding measured on control membranes.

Preincubation of the membrane with 10^{-5} M phenoxybenzamine completely suppressed ^{3}H-spiperone binding, whereas there was still 35 per cent of the control DA-adenylate cyclase stimulation. To completely block the maximal DA-adenylate cyclase stimulation, higher concentrations of phenoxyben-

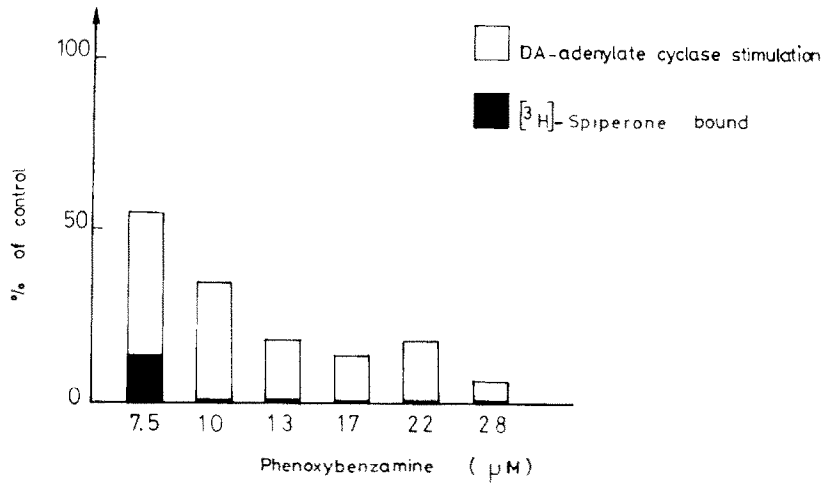

Fig. 3. Irreversible blockade of maximal DA-adenylate cyclase stimulation and specific ^{3}H-spiperone by binding treatment with different doses of phenoxybenzamine. The same membrane preparations were used to measure adenylate cyclase activities and ^{3}H-spiperone binding. DA and ^{3}H-spiperone concentrations were 10^{-3} M and 3×10^{-9} M, respectively (these are saturating concentrations). On control membranes, DA stimulated basal adenylate cyclase by 120 per cent and the total number of ^{3}H-spiperone binding sites was 330 fmoles/mg protein. The protein concentration in the assay was 0.65 mg/ml. DA-stimulations and total number of binding sites on phenoxybenzamine treated membranes were expressed in per cent of that found in control.

zamine were required (3×10^{-5} M). However, we cannot exclude that, after preincubation with 10^{-5} M phenoxybenzamine, there was still 5 per cent ^{3}H-spiperone binding, and that these 5 per cent are DA receptors responsive for the remaining 35 per cent adenylate cyclase stimulation. In this event, the coupling between the DA receptors occupancy and the DA-adenylate cyclase stimulation would have to be non linear and therefore, the apparent affinity of the DA to stimulate the adenylate cyclase should be lower after phenoxybenzamine treatment [23]. Figure 4 shows that this is not the case: the apparent affinity of DA is the same on control and phenoxybenzamine treated membranes (1.7×10^{-5} M). Therefore, even if 5 per cent ^{3}H-spiperone binding remains after treatment with 10^{-5} M phenoxyben-

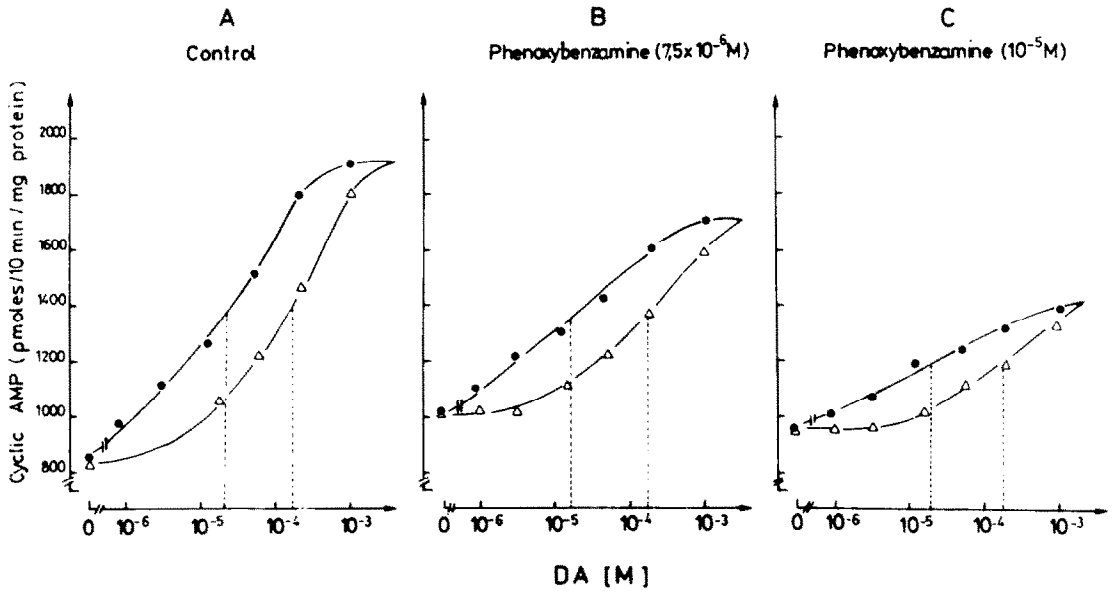

Fig. 4. Effect of phenoxybenzamine treatments on the affinities of DA and spiperone for the DA-sensitive adenylate cyclase system. The data for Fig. 5 were derived from the same experiment. Dose–response curves were based on experiments conducted in the absence (●) and presence (△) of spiperone 10^{-6} M, on control membranes (A) and membranes treated with 7.5×10^{-6} M (B) or 10^{-5} M (C) phenoxybenzamine. The apparent affinities for DA and spiperone were 1.7×10^{-5} M and 1.1×10^{-7} M, respectively (A, B, C).

Table 1. Effect of RU 24926 on basal and DA-sensitive adenylate cyclase

	Adenylate cyclase activity (pmoles cyclic AMP/10 min/mg protein)	
	Basal	+ DA (3×10^{-5} M)
Control	800 ± 30	1624 ± 50
RU 24926		
10^{-7}	824 ± 36	1608 ± 50
10^{-6}	776 ± 42	1576 ± 78
10^{-5}	750 ± 56	1604 ± 66

The protein concentration was 0.74 mg/ml. Values are the mean ± S.E.M. of triplicate determinations.

zamine, these sites cannot be DA receptors responsible for the remaining DA-adenylate cyclase stimulation.

By using an irreversible ligand, we thus demonstrated that the high affinity ^{3}H-spiperone binding sites are distinct from the DA receptors coupled with an adenylate cyclase. However, one could not be sure that the ^{3}H-spiperone binding site is not at all implicated in the inhibition of the DA-sensitive adenylate cyclase by neuroleptics. In order to test this point, we looked at the apparent affinity of spiperone to inhibit the DA-adenylate cyclase stimulation after partial (Fig. 4B) or complete (Fig. 4C) blockade of ^{3}H-spiperone binding sites by phenoxybenzamine treatments. These phenoxybenzamine treatments did not modify at all the ability of spiperone to inhibit the DA-sensitive adenylate cyclase (apparent inhibition constant = 1.1×10^{-7} M) (Fig. 4).

GTP effects on the interaction of dopaminergic ligands and 5-HT with ^{3}H-spiperone binding sites. So far it has been shown that GTP reduces the affinities of ^{3}H-spiperone binding sites for dopaminergic agonists which stimulate adenylate cyclase (DA, epinine, ADTN, apomorphine) but does not modify the affinity of bromocriptine, a dopaminergic agonist on several systems (inhibition of prolactin release [17], contralateral turning after 6-hydroxydopamine lesions [24], antiparkinsonian activity [25]), which does not stimulate the adenylate cyclase [26]. We have confirmed these results (data not shown).

Recently, a new compound RU 24926 has been found to be a dopaminergic agonist on several systems (inhibition of prolactin release, contralateral turning after 6-hydroxydopamine lesions, emesis in the dog, inhibition of cholinergic neurons in striatum [9, 27, 28]). We found that this drug was neither an agonist nor an antagonist of the DA-sensitive adenylate cyclase in striatum homogenates (Table 1). On the contrary, this drug has a high affinity for the ^{3}H-spiperone binding sites ($IC_{50} = 10^{-7}$ M) (Fig. 4). This affinity is about 30 times higher than that of DA ($IC_{50} = 3 \times 10^{-6}$ M) when measured in the same conditions (data not shown). It was thus of interest to know whether or not GTP will affect the affinity of RU 24926 for ^{3}H-spiperone binding sites.

Figure 5 shows that GTP reduces the affinity of RU 24926 for ^{3}H-spiperone binding sites. Note also that GTP changes the slope of the displacement curve as previously reported for the displacement of ^{3}H-dihydroalprenolol by isoproterenol in the presence of GTP [29]. The reduction of the potency of RU 24926 to inhibit ^{3}H-spiperone binding was 2.8-fold (Fig. 5) (2.6 ± 0.1; N = 3, mean ± S.E.M.), a figure which compares well with the reduction of the affinity of DA for ^{3}H-spiperone binding sites reported by others (about 3 fold) [13–15] and found by us (4.3 ± 0.6; N = 3; data not shown).

5-HT is unable to stimulate DA-sensitive adenylate cyclase. As shown in Fig. 5, GTP was also able to reduce the affinity of 5-HT for ^{3}H-spiperone binding sites. The reduction was lower (1.8 fold; 1.8 ± 0.2; N = 3) than for RU 24926 or DA.

GTP thus influences the affinity of ^{3}H-spiperone binding sites for some drugs which do not stimulate the adenylate cyclase.

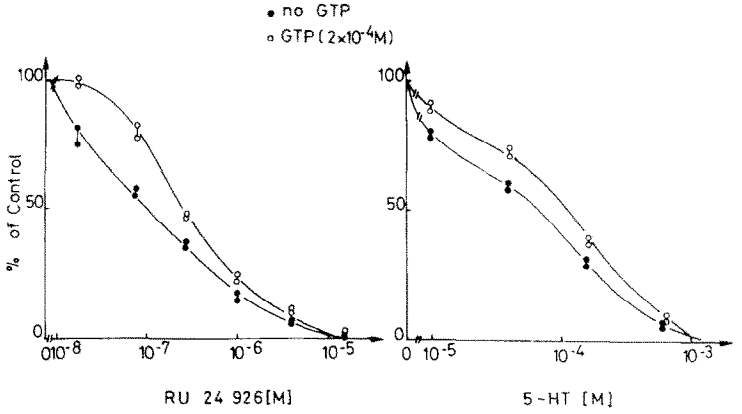

Fig. 5. GTP effects on ^{3}H-spiperone binding site properties. Specific ^{3}H-spiperone binding was displaced by RU 24926 and 5-HT in the absence and presence of GTP 2×10^{-4} M. Note that all drugs entirely displaced specific ^{3}H-spiperone binding. Control specific ^{3}H-spiperone binding was 300 fmoles/mg protein. Non-specific binding was 37.5 fmoles/mg protein. The ^{3}H-spiperone concentration was 10^{-9} M. The protein concentration was 0.2 mg/ml. Each point represents individual determination. This experiment was done three times (see text for the mean and S.D.).

DISCUSSION

It is generally admitted that the high affinity ^{3}H-spiperone binding sites are not the sites where neuroleptics bind to inhibit the DA-adenylate cyclase activation (see introduction to paper).

We have investigated the possibility of a relation between the high affinity ^{3}H-spiperone binding sites and the DA-sensitive adenylate cyclase system: the existence of such a relation was indeed strongly suggested by the previously described GTP effects [13–15] (see introduction to paper). One could imagine that a spiperone molecule has two subsites. The first one would have a high affinity ($\simeq 10^{-10}$ M) for a DA-binding site different from the DA-receptor coupled with an adenylate cyclase but located next to it. The DA-binding site would be the site labelled in binding experiments. The second subsite of the spiperone molecule would bind to the DA-receptor coupled with adenylate site with a low affinity, but only if the first site is bound. This would explain why neuroleptics have a much higher affinity in binding experiments than for inhibiting the DA-sensitive adenylate cyclase. In such an hypothesis, it will not be surprising that some drugs such as benzamine derivatives [5, 6], domperidone [7, 8] and RU 24926 [9] have high affinity for DA-binding sites and are inactive on DA adenylate cyclase. Furthermore, the GTP effects would be understandable. Such models involving multivalent ligand have recently been proposed [30, 31]. Phenoxybenzamine appeared to be a good tool to test this idea. We have shown that this compound blocks irreversibly ^{3}H-spiperone binding sites. After treatment of particulate fractions with 10^{-5} M phenoxybenzamine, there was a complete blockade of ^{3}H-spiperone binding sites, whereas the DA-sensitive adenylate cyclase was still activated (35 per cent of the control): this observation confirms that high affinity ^{3}H-spiperone binding sites are distinct from the DA-receptors coupled with an adenylate cyclase. Furthermore, under these conditions, spiperone always has the same affinity for inhibiting the adenylate cyclase (Fig. 5). According to this result, we conclude that high affinity ^{3}H-spiperone binding sites are not at all implicated in the inhibition of the DA-sensitive adenylate cyclase by neuroleptics.

We have observed GTP effects on the affinities of ^{3}H-spiperone binding sites for two drugs (RU 24926 and 5-HT) which do not stimulate the DA-sensitive adenylate cyclase. It is thus no longer possible to deduce from the effects of GTP the existence of a connection between part of the ^{3}H-spiperone binding sites and the DA-sensitive adenylate cyclase.

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