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THE CHARACTERIZATION OF [^{3}H]SULPIRIDE BINDING SITES IN PIG STRIATAL MEMBRANES

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Abstract—Pig striatal membranes have [^{3}H]sulpiride-binding sites similar to those identified in rat striatal membranes. The pharmacological profile indicates that this binding is to dopamine receptors. Agonist displacement of [^{3}H]sulpiride binding in pig striatal membranes is subject to guanine nucleotide regulation. This effect is mimicked by heat treatment. *N*-ethyl maleamide (20 μM) and dithioeryrthritol (3 mM) decrease agonist affinity for the [^{3}H]sulpiride-binding site in pig striatal membranes without significantly affecting maximal displacement.

The atypical neuroleptic, sulpiride, is an antagonist of dopamine in many systems. It thus antagonizes physiological responses such as the dopamine-induced inhibition of neuronal firing in the substantia nigra [1]. It inhibits behavioural responses such as 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN)-stimulated locomotor activity [2] and angiotensin-induced thirst [3]. Specific [^{3}H]sulpiride-binding sites have been detected in rat striatal membranes [4]. The location of these binding sites from lesion studies [5] and the pharmacological profile [4] strongly suggests that the sites are related to dopamine receptors. The [^{3}H]sulpiride binding sites are sodium-dependent, regulated by GTP and *N*-ethylmalemide (NEM) sensitive [6, 7].

In the present study we have examined the feasibility of using pig striatum to examine [^{3}H]sulpiride-binding sites. The pig is a readily available source of large amounts of striatal tissue and thus could be used for more comprehensive experimentation on the [^{3}H]sulpiride-labelled sites, if indeed these are related to dopamine receptors in this tissue. In a further set of experiments the organization of these sites, with respect to guanine nucleotides and sulphydryl modulation, has been examined. Although sulpiride does not inhibit the ability of dopamine to stimulate the striatal adenylate cyclase it has been proposed to act through an inhibitory cyclase system [6]. Furthermore, an essential SH group alkylated by NEM has been identified near the [^{3}H]sulpiride-binding site; this SH group was not protected from alkylation by guanine nucleotides [7] and it was therefore proposed to be on a site removed from the regulatory binding protein. The present study has examined the possibility of further sulphydryl groups associated with the inhibitory guanine-nucleotide regulatory protein linked to the [^{3}H]sulpiride-binding site.

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MATERIALS AND METHODS

Preparation of membranes. Rat striata were obtained from male Wistar rats killed by cervical dislocation. Pig striatal tissue was obtained from sows slaughtered at the local abbatoir. Striata were rapidly dissected out and placed on dry ice. They were stored at −20° for up to 2 months with no significant loss in subsequent binding.

Partially purified synaptic membranes were prepared from striatal tissue as described by Woodruff and Freedman [4]. The membranes were suspended in 50 mM Tris–Krebs' buffer (containing 50 mM Tris, 120 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$), pH 7.4 and stored at −20° for up to 2 weeks.

Binding assay. Membranes (150–200 μg protein) were incubated with 15 nM [^{3}H]sulpiride (diluted with unlabelled sulpiride to give a sp. act. of 26.2 Ci/mmole) in 50 mM Tris–Krebs' pH 7.4, in the presence and absence of drugs for 10 min at 37°. In those experiments, employing a sodium-free buffer, 120 mM choline chloride replaced the sodium in the buffer. Specific binding was defined by the addition of 1 μM (−)-sulpiride to half of the tubes. Bound and free ligand were separated by filtration through Millipore filters (HAWP 0025) following a 2-min period in ice. The filters were washed with 15 ml ice-cold buffer and bound radioactivity determined by liquid scintillation spectrometry. This procedure has been shown to retard dissociation of the ligand from the binding sites. The results were expressed as fmoles ligand bound per milligram protein, the amount of protein being determined by the method of Lowry *et al.* [8], using bovine serum albumin as the standard. This procedure is similar to that previously reported [4].

Heat treatment. The membranes were resuspended in Tris–Krebs' buffer (pH 7.4) and incubated at 50° for 4 min. The incubation was terminated by the

addition of ice-cold Tris–Krebs' pH 7.4, and membranes collected by centrifugation at 50,000 *g* for 15 min. The pellet was resuspended in Tris–Krebs' pH 7.4, and stored at −20°. Control membranes were prepared in the same fashion with the omission of the 50°-incubation step.

Protein modification. Membranes were preincubated with NEM (1 μM–10 mM) or dithiothreitol (DTT) (10 mM) for 30 min at 37°. The membranes were then collected by centrifugation at 50,000 *g* for 15 min, resuspended in 50 mM Tris–Krebs' pH 7.4 and [^{3}H]sulpiride binding assayed.

The effect of NEM (20 μM) or dithioeryrthritol (DTE) (3 mM) on antagonist- or agonist-displacement curves was assessed by preincubating the membranes with the protein-modifying agent for 30 min at 37°.

The sulfhydryl group oxidizing agent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (3 mM) was occasionally used to protect the membranes from DTE (3 mM) inactivation and in these cases DTNB was added prior to the addition of DTE.

Additive experiments. Control and heat-treated membranes were prepared in parallel as described above and the displacement of [^{3}H]sulpiride binding by ADTN was determined in the presence of guanylamidodiphosphate (Gpp(NH)p).

Materials. (−)[methoxy-^{3}H]sulpiride was obtained from New England Nuclear (60–80 Ci/mmole), (−)sulpiride was obtained from Ravizza, s.p. Milan. 2-Amino,6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene was supplied by Calbiochem-Boehring, San Diego, CA. All other drugs were from Sigma Chemical Co., St. Louis, MO.

RESULTS

[^{3}H]Sulpiride was bound to both rat and striatal membranes in a stereospecific saturable fashion. The equilibrium dissociation constant (K_d) and maximal binding capacity (B_{max}) were determined from computer-fit analysis of the saturation data [9]. The values for K_d were similar in the two species (6.8 nM for pig compared with 9.1 nM for rat) whilst the B_{max} values were significantly different, rat having a much higher binding capacity (426 ± 23 fmoles/mg protein compared with 231 ± 35 fmoles/mg protein in pig).

The specific saturable [^{3}H]sulpiride-binding component in striatal membranes from both species was sodium dependent. The absence of sodium from the incubation medium completely inhibited specific binding.

The effect of a series of dopamine agonists and antagonists on specific [^{3}H]sulpiride binding in rat and pig striatum was investigated and the results are shown in Table 1. Dopamine agonists and antagonists were active in inhibiting the binding, whilst noradrenaline and γ-aminobutyrate (GABA) were inactive. Antagonists of all classes were very active in displacing binding, agonists being in general less potent, although ADTN was potent in displacing sulpiride binding in both species. The displacement of [^{3}H]sulpiride from its binding site was stereospecific in that (−)sulpiride was more active than (+)sulpiride and (+)butaclamol was active whilst (−)butaclamol was inactive. The results in Table 1 demonstrate the similar potency of the compounds tested in displacing specific [^{3}H]sulpiride binding from rat and pig striatal membranes. The similarity is emphasized in Fig. 1 which shows the high degree of correlation between the IC_{50} values for drugs in displacing [^{3}H]sulpiride binding from the two membranes preparations.

Table 1. Pharmacological profile of [^{3}H]sulpiride binding to rat and pig striatal membranes

Drug	Pig IC_{50} (nM)	Rat IC_{50} (nM)
Spiroperidol	0.20	0.48
(+)Butaclamol	7.90	1.58
Zetidoline	7.50	6.00
cis-Flupenthixol	2.77	8.90
ADTN	21.60	11.58
Pergolide	84.00	25.00
(−)Sulpiride	32.00	31.60
trans-Flupenthixol	100.00	40.00
Apomorphine	190.00	125.00
Dopamine	451.00	398.00
(+)Sulpiride	5620.00	631.00
Ergometrine	1900.00	708.00

(−)Butaclamol, GABA, noradrenaline and nomifensine were relatively inactive ($IC_{50} > 10^{-5}$ M).

Drugs were incubated for 10 min at 37° with pig or rat striatal membranes in the presence of 15 nM [^{3}H]sulpiride as described in the Methods section. Maximal displaceable specific binding was defined by the addition of 1 μM (−)sulpiride. IC_{50} values were determined from full displacement curves (2–4 curves) of 5–7 observations in triplicate.

The effect of guanine nucleotides and heat treatment

The addition of Gpp(NH)p (100 μM) to pig striatal membranes decreased the ability of agonists but not

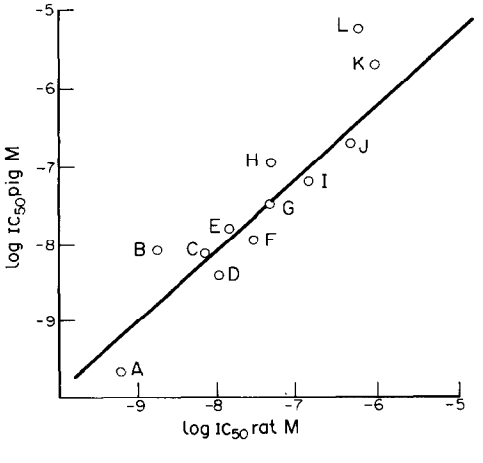

Fig. 1. Correlation between the IC_{50} for drugs on [^{3}H]-sulpiride binding in pig and rat striatal membranes. A, spiroperidol; B, (+)butaclamol; C, zetidoline; D, *cis*-flupenthixol; E, ADTN; F, pergolide; G, (−)sulpiride; H, transflupenthixol; I, apomorphine; J, dopamine; K, ergometrine; L, (+)sulpiride. The slope gradient of the graph is 1.05.

Table 2. The effect of Gpp(NH)p and heat treatment on agonist and antagonist displacement of specific [^{3}H]sulpiride binding in pig striatal membranes

	Control	IC_{50} (nM) [100 μM Gpp(NH)p]	Heated
Agonist			
Pergolide	84.0 ± 36	123 ± 63	—
ADTN	21.6 ± 2	173 ± 54*	302 ± 81*
Apomorphine	190.0 ± 77	974 ± 143†	—
Dopamine	451.0 ± 130	2787 ± 760*	—
Antagonist			
cis-Flupenthixol	2.7 ± 0.7	2.5 ± 0.8	—
(−)Sulpiride	27.0 ± 4	21.0 ± 4.0	30 ± 11

Drugs were incubated with pig striatal membranes for 10 min at 37° in the presence of 15 nM [^{3}H]sulpiride. Maximal displaceable binding was defined by 1 μM (−)sulpiride.

IC_{50} values were determined from at least 3 full displacement curves of [^{3}H]-sulpiride binding in the absence (control) and presence of 100 μM Gpp(NH)p and in heat-treated membranes. Statistical significance was determined using the unpaired Student's *t*-test:

* $P < 0.05$, † $P < 0.01$ from control values.

antagonists to displace [^{3}H]sulpiride (15 nM) binding. This effect was specific for guanosine triphosphate (GTP), guanosine diphosphate (GDP) and the stable GTP analogue Gpp(NH)p. Guanoside monophosphate (GMP), guanosine and ATP were ineffective although inosine triphosphate (ITP) reduced the affinity of agonists for the binding site less potently than guanosine nucleotides. This effect is similar to that reported previously [6]. A similar effect on agonist affinity for the binding site was produced by mild heat inactivation of the membranes (50° for 4 min). A comparison of heat treatment and guanosine nucleotide addition is shown in Table 2 and Fig. 2.

The effect of protein modification by sulfhydryl reagent

Preincubation of pig striatal membranes with NEM inhibited subsequent specific binding of [^{3}H]-sulpiride in a similar fashion to that reported for the rat [7]. The IC_{50} for NEM inhibition was 0.4 mM. Preincubation with DTT or its stable isomer DTE [10] had no effect on the binding.

Further investigation of the effects of sulhydryl-modifying agents demonstrated that preincubation with low concentrations (20 μM), significantly altered the displacement of [^{3}H]sulpiride binding by ADTN having no effect on sulpiride displacement (Fig. 3). The figure shows that DTE (3 mM) pre-

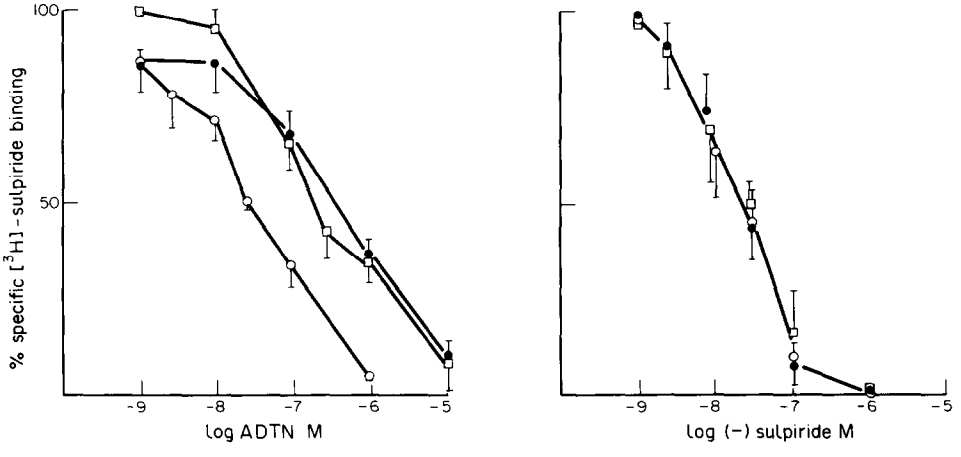

Fig. 2. The effect of heat treatment and Gpp(NH)p on ADTN and sulpiride displacement of [^{3}H]-sulpiride binding to pig striatal membranes. Pig striatal membranes were treated for 4 min at 50° and collected by centrifugation as described in the Methods. Subsequently [^{3}H]sulpiride binding (15 nM) was assessed in aliquots of the resuspended membranes in the presence of increasing concentrations of the agonist ADTN (a) or the antagonist (−)sulpiride (b). Alternatively, the effect of Gpp(NH)p (100 μM) on ADTN (a) or sulpiride (b) displacement on [^{3}H]sulpiride binding was assessed. Each point is the mean of at least 3 determinations S.E.M. in triplicate S.E.M. ○, control; ●, 100 μM Gpp(NH)p; □, heat treatment.

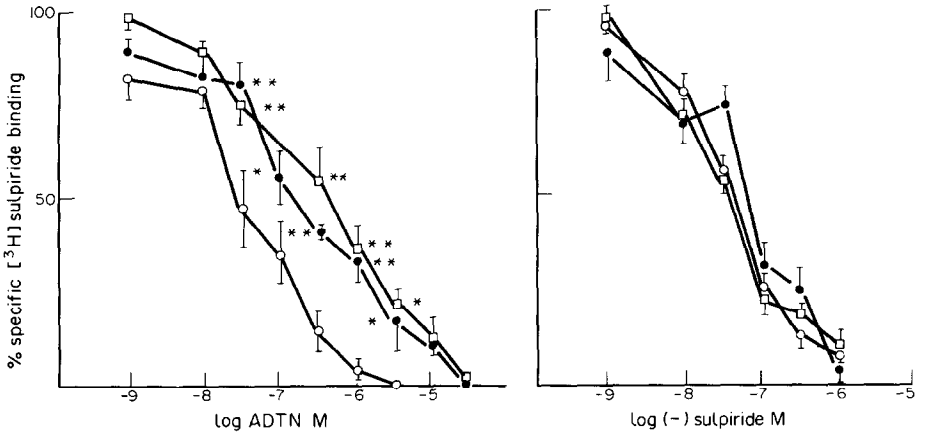

Fig. 3. The effect of NEM and DTE on ADTN and (−)sulpiride displacement of [^{3}H]sulpiride binding. Pig striatal membranes were incubated with 15 nM [^{3}H]sulpiride and either NEM (20 μM) or DTE (3 mM) and increasing concentrations of the agonist ADTN (a) or (−)sulpiride (b) for 10 min at 37°. Each point is the mean of at least 3 observations in triplicate. S.E.M. Statistical significance was determined using the Students' t-test: * $P < 0.05$; **$P < 0.01$. ○, control; ●, DTE; □, NEM.

incubation produced a similar effect. If the membranes were preincubated with DTNB, which stabilizes disulphide bonds, the effect of subsequent DTE treatment was lost. Thus in the presence of DTE (3 mM) the percentage inhibition of [^{3}H]sulpiride binding by 1 μM ADTN was 67 ± 3 compared with 92 ± 2% in control experiments ($P < 0.001$, N = (4) DTNB (3 mM) alone had no effect on the inhibition of [^{3}H]sulpiride binding by ADTN (86 ± 6%) but prior treatment with DTNB reversed the DTE effect (85 ± 6%).

Thus Gpp(NH)p, heat treatment and low concentrations of NEM all produced selective alterations in agonist affinity for [^{3}H]sulpiride binding. The possibility that these effects are at similar sites was tested by addition experiments. Table 3 demonstrates that the effects of the various treatments on ADTN displacement of [^{3}H]sulpiride binding was not additive.

Table 3. The effects of NEM, heat treatment and Gpp(NH)p on ADTN displacement of specific [^{3}H]sulpiride binding

Treatment	ADTN IC_{50} (nM)
Control	41 ± 7.5
Heated	191 ± 65*
20 μM NEM	241 ± 42‡
Heated + 20 μM NEM	182 ± 21‡
100 μM Gpp(NH)p	182 ± 42†
100 μM Gpp(NH)p + 20 μM NEM	221 ± 33‡

Pig striatal membranes were heated, 50° for 4 min as described in the Methods section. Following this treatment membranes were collected by centrifugation and the pellet resuspended. For experiments without heat treatment membranes were treated in the same way with the omission of the 50° incubation. [^{3}H]Sulpiride binding (15 nM) was assessed by incubation at 37° for 10 min with additions of NEM (20 μM) and/or Gpp(NH)p (100 μM) as indicated. Binding displaceable by ADTN was determined from full displacement curves and expressed as IC_{50} (nM). Results are mean ± S.E.M., N = 5, and statistical difference from control determined using paired Students' t-test, *$P < 0.05$; †$P < 0.02$; ‡$P < 0.002$.

DISCUSSION

The results presented demonstrate that pig striatal membranes possess specific [^{3}H]sulpiride-binding sites which are similar to those on rat striatal membranes. Both sites have similar kinetic properties, show Na^+ dependency, have an essential SH group on or near to the sulpiride-binding site and both sites show altered agonist affinity for GTP, GDP and Gpp(NH)p. The similarity of the [^{3}H]sulpiride-binding sites in the two species was further demonstrated by the good correlation of compounds in displacing [^{3}H]sulpiride from the sites. These results are somewhat in contrast to the observations of Creese *et al.* [11] who demonstrated differences in affinity for agonists and antagonists at [^{3}H]spiperidol-binding sites in different species. Sulpiride in particular, showed the largest species variation. However, the present results suggest that pig striatal membranes can be used to further study [^{3}H]sulpiride binding sites.

The involvement of guanine nucleotides in striatal [^{3}H]sulpiride-specific binding was further investigated in this species. Alterations in agonist affinity for the site by guanine nucleotides have been interpreted as evidence for dopamine receptors being linked to regulatory GTP-binding proteins [6, 12]. Mild heat treatment mimicked this action of guanine nucleotides, changing agonist affinity for [^{3}H]sulpiride binding, whilst antagonist affinity remained unchanged. This result is in keeping with suggestions that the guanine nucleotide-binding protein in thermolabile [13].

The presence of an essential sulphydryl group on or near to the [^{3}H]sulpiride-binding site was inferred from the susceptibility of specific binding to the alkylating agent, NEM, at millimolar concentrations [7]. Dopamine-agonist binding was sensitive to sul-

phydryl modification (but by low micromolar concentrations of NEM) and inhibition of binding by DTT suggested the involvement of a disulphide bridge [14]. The present study confirmed these observations. [3H]sulpiride binding being inhibited by NEM (IC_{50} 0.4 mM). However, in the presence of low (20 μM) concentrations of NEM which had no demonstrable inhibitory effect on [3H]sulpiride binding, there was a significant decrease in the affinity of the dopamine agonist, ADTN, for the binding site whilst antagonist displacement curves were unaffected. Similarly, although DTE had no effect on [3H]sulpiride binding it again decreased the affinity of the binding site for agonists, antagonist affinity remaining unchanged. These observations could be explained by the possibility that the high-affinity form of the receptor for agonists is stabilized by a disulphide bond.

Furthermore the effects of mild heat treatment, Gpp(NH)p and low concentrations of NEM on agonist (ADTN) affinity for the [3H]sulpiride-binding sites were not additive. These results could be interpreted to suggest that a thermolabile, guanine nucleotide, regulatory protein may be linked to the [3H]sulpiride-binding site in striatal membranes and a sulphydryl group may be involved in stabilizing the high-affinity GTP-binding protein–receptor complex.

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