Cation regulation differentiates specific binding of [^{3}H]sulpiride and [^{3}H]spiperone to rat striatal preparations

A. E. THEODOROU, M. D. HALL, P. JENNER, C. D. MARSDEN*, *University Department of Neurology, Institute of Psychiatry and King's College Hospital Medical School, Denmark Hill, London SE5, UK*

Sulpiride, a substituted benzamide drug, blocks cerebral dopamine receptors and is a clinically effective antipsychotic agent (Justin-Besancon et al 1967; Borenstein et al 1969). Although in many respects the central actions of sulpiride on dopamine function resemble those of typical neuroleptics, two important differences are apparent. Thus, sulpiride does not induce marked catalepsy in rodents and in vitro it does not block the dopamine stimulation of adenylate cyclase from dopamine containing brain areas (Laville 1972; Trabucchi et al 1975; Elliott et al 1977; Jenner et al 1978). These differences have been utilized to distinguish different classes of dopamine receptors. Indeed, it is currently believed that substituted benzamide drugs such as sulpiride are specific antagonists at D-2 dopamine receptors which are thought not dependent on linkage to adenylate cyclase (Kebabian & Calne 1979).

We have demonstrated previously that (±)-[^{3}H]-sulpiride is specifically bound to a limited receptor population in rat striatal preparations, and that this site may differ from that at which other neuroleptics act (Theodorou et al 1979). *cis*-Flupenthixol, which preferentially interacts with D-1 adenylate cyclase dependent receptors (Hyttel 1980) has a limited ability to displace (±)-[^{3}H]sulpiride.

We now examine the effect of monovalent and divalent cations contained in the incubation buffer on the specific binding of [^{3}H]sulpiride in comparison to that of [^{3}H]spiperone, a ligand which binds to both D-1 and D-2 receptors. We have demonstrated that the specific binding of [^{3}H]sulpiride is highly dependent on the presence of sodium ions, a feature not observed in the specific binding of [^{3}H]spiperone.

Female Wistar rats (Bantin & Kingman Ltd; 150 ± 10 g) were decapitated and the paired corpus striata dissected into ice-cold 50 mM Tris-HCl (pH 7·7). Tissue was usually used immediately, but in some experiments was stored at −20 °C for between 24–72 h. Striatal tissue was prepared according to Leysen et al (1978) such that the final membrane preparation was suspended in 50 mM Tris-HCl buffer (pH 7·7) containing 0·1 % ascorbic acid and 10 μM pargyline hydrochloride. The normal ionic content was (mM) NaCl 120, KCl 5, $CaCl_2$ 2 and $MgCl_2$ 1. In other experiments the tissue was suspended in cation-free buffer to which was added varying concentrations (mM) of NaCl (25–200), KCl (1–100), $CaCl_2$ (1–100) or $MgCl_2$ (0·5–10), either individually or in combination. All solutions were made using distilled and deionized water. Experiments were carried out using 1 ml aliquots of the final tissue preparation, containing 10 mg striatal tissue, at 37 °C for 10 min. After incubation separation of free and bound [^{3}H]sulpiride was carried out by rapid centrifugation and subsequent washing of the resulting pellet as previously described (Theodorou et al 1979). For [^{3}H]spiperone separation was achieved by vacuum filtration as described by Leysen et al (1978). Tissue pellets or filters were placed in 10 ml Instagel scintillatipn cocktail (Packard Instruments) and counted using a Packard 2425 liquid scintillation spectrometer at an efficiency of approximately 40 %.

Specific binding of (±)-[^{3}H]sulpiride (5–40 nM; 26·2 Ci mmol^{-1}; custom synthesized by the Radiochemical Centre) was defined using (−)-sulpiride (5×10^{-6} M; SESIF, France). The specific binding of [^{3}H]spiperone (0·125–4·0 nM; 20 to 26 Ci mmol^{-1}; Radiochemical Centre) was defined using (+)-butaclamol (5×10^{-6} M; Ayerst Laboratories). In most experiments a standard single non-saturating ligand concentration was employed namely 15 nM for [^{3}H]-sulpiride and 0·5 nM for [^{3}H]spiperone. Where definition of the changes occurring in the presence of sodium ions was carried out, a range of ligand concentrations was employed and the data was subjected to Scatchard analysis for the determination of the number of binding sites (B_{max}) and the dissociation constant (K_D).

In a typical experiment with standard ligand concentrations and carried out in the presence of the normal cation of the incubation buffer, the total binding for [^{3}H]sulpiride (15 nM) was 10436 ± 264 d min^{-1} of which 3132 ± 264 d min^{-1} were specifically displaced by (−)-sulpiride (5×10^{-6} M), representing 4·7 ± 0·4 pmol g^{-1} wet weight of tissue. For [^{3}H]spiperone (0·5 nM) total binding was 6940 ± 77 d min^{-1} of which 5764 ± 77 d min^{-1} were specifically displaced by (+)-butaclamol (5×10^{-6} M), representing 8·7 ± 0·1 pmol g^{-1} wet weight of tissue. To allow comparison of the effects of buffer cation composition on the specific binding of [^{3}H]sulpiride and [^{3}H]spiperone, data are subsequently expressed as a percentage of specific binding occurring in presence of the normal cation content (which is taken as 100 %).

The omission of the cation content of the incubation buffer caused an almost total loss of specific [^{3}H]-sulpiride binding, but only a 17 % decrease in specific [^{3}H]spiperone binding (Table 1).

Inclusion of NaCl (25–200 mM) restored specific [^{3}H]sulpiride binding to or above the level observed in the presence of the normal cation content of the incuba-

* Correspondence.

Table 1. The effect of cation composition of the incubation buffer on the specific binding of (±)-[^{3}H]sulpiride (15 nM; as defined using (−)-sulpiride 5×10^{-6} M) and [^{3}H]spiperone (0·5 nM; as defined using (+)-butaclamol 5×10^{-6} M) to rat fresh striatal preparations.

		Specific binding as percent of that occurring in the presence of normal buffer cation composition	
		[^{3}H]Sulpiride	[^{3}H]Spiperone
Normal cation composition (mM)		100	100
No cations		0†	83†
NaCl	25	99*	102*
	50	124*	108*
	100	151*	110*
	200	151*	102*
KCl	1	8	72
	5	16	75
	10	26	78
	20	12	80
	100	42	73*
$CaCl_2$	1	34	78
	2	45*	83
	5	8	74
	10	18	64*
	100	0	20*
$MgCl_2$	0·5	17	78
	1	3	81
	5	20	77
	10	15	62*

Results are expressed as the mean of at least 9 determinations carried out on at least 3 occasions.
Statistical analysis was carried out on the absolute values for specific binding in each individual experiment using a two-tailed Student's *t*-test and the data subsequently expressed as a mean percentage of specific binding occurring in the presence of normal buffer cation composition for all determinations.
* $P < 0.05$ compared with cation free buffer.
† $P < 0.05$ compared with normal buffer cation content.

tion buffer. This effect appeared specific for sodium ions. The inclusion of equimolar concentrations of KCl was only partially effective in restoring specific [^{3}H]sulpiride binding (Table 1). The inclusion of $CaCl_2$ caused a stimulation of specific [^{3}H]sulpiride binding at 1 or 2 mM, but higher concentrations were ineffective. $MgCl_2$ (0·5–10 mM) caused no significant increase.

The inclusion of NaCl (25–200 mM) similarly restored specific binding of [^{3}H]spiperone to the levels seen in the presence of the normal buffer cation content (Table 1). A low KCl content (1 mM) further reduced [^{3}H]-spiperone binding from that obtained in the absence of ions, and higher concentrations of KCl had similar effects. Concentrations of $MgCl_2$ and $CaCl_2$ up to 5 mM did not restore [^{3}H]spiperone binding and higher concentrations reduced it.

In the presence of incubation buffer containing NaCl (120 mM) the further inclusion of KCl, $CaCl_2$ or $MgCl_2$ in concentrations up to 5 mM did not alter specific binding of [^{3}H]sulpiride (Table 2). Each salt at a concentration of 10 mM decreased specific binding of [^{3}H]sulpiride. Similarly in the presence of NaCl (120 mM) the inclusion of KCl (1–10 mM) or $CaCl_2$ (1–10 mM) or $MgCl_2$ (0·5–10 mM) either did not alter specific [^{3}H]spiperone binding or reduced it.

Table 2. The effect of incorporation of KCl (1–10 mM), $CaCl_2$ (1–10 mM) and $MgCl_2$ (0·5–10 mM) on the specific binding of [^{3}H]sulpiride (15 nM: as defined using (−)-sulpiride 5×10^{-6} M) and [^{3}H]spiperone (0·5 mM; as defined using (+)-butaclamol 5×10^{-6} M) occurring in fresh rat striatal tissue incubates containing 120 mM NaCl.

		Specific binding as percent of that occurring in the presence of normal buffer cation composition	
		[^{3}H]Sulpiride	[^{3}H]Spiperone
Normal cation composition (mM)		100	100
NaCl	120	104	112
KCl	1	88	111
	5	88	98*
	10	64*	104
$CaCl_2$	1	90	104
	2	107	96*
	10	34*	49*
$MgCl_2$	0·5	87	108
	1	74	102
	10	41*	93*

Results are expressed as the mean of experiments carried out in triplicate on at least 2 occasions.
Statistical analysis was carried out on the absolute values for specific binding in each individual experiment using a two-tailed Student's *t*-test and the data subsequently expressed as a mean percentage of specific binding occurring in the presence of normal buffer cation composition for all determinations.
* $P < 0.05$ compared with incubation buffer containing 120 mM NaCl.

Freezing of striatal tissue at −20 °C for up to 72 h before homogenization did not alter specific [^{3}H]-sulpiride binding or [^{3}H]spiperone binding observed in the presence of normal buffer cation content. Removal of the cations from the incubation medium caused a dramatic loss of specific [^{3}H]sulpiride binding to previously frozen tissue, but this could still be restored by the inclusion of NaCl (25–200 mM). The specific binding of [^{3}H]spiperone to previously frozen tissue was slightly reduced by omission of cations from the incubation buffer (although on this occasion the 21% change did not reach statistical significance), and the inclusion of NaCl (25–200 mM) restored specific binding to normal (Table 3).

Using a range of ligand concentrations, we carried out Scatchard analysis of the specific binding of [^{3}H]-

Table 3. Comparison of the effect of NaCl (25–200 mM) on the specific binding of A [^{3}H]sulpiride (15 nM: as defined using (−)-sulpiride 5×10^{-6} M) and B [^{3}H]-spiperone (0·5 nM; as defined using (+)-butaclamol 5×10^{-6} M) to either fresh rat striatal preparations or to tissue previously frozen at −20 °C for up to 72 h.

	Specific binding as percent of that occurring in the presence of normal buffer cation composition	
(A) [^{3}H]Sulpiride	Fresh	Frozen
Normal cation composition (mM)	100	100
No cations	24†	0†
NaCl 25	60*	69*
50	99*	105*
100	94*	104*
200	85*	74*
(B) [^{3}H]Spiperone		
Normal cation composition (mM)	100	100
No cations	64†	79
NaCl 25	95*	98
50	98*	107
100	96*	103
200	99*	97

Results are expressed as the mean of two experiments carried out in triplicate. Preparations from fresh and frozen striata were assayed in parallel on each occasion. Statistical analysis was carried out on the absolute values for specific binding in each individual experiment using a two-tailed Student's *t*-test and the data subsequently expressed as a mean percentage of specific binding occurring in the presence of normal buffer cation composition for all determinations.
* $P < 0.05$ compared with cation-free buffer.
† $P < 0.05$ compared with normal buffer cation content.

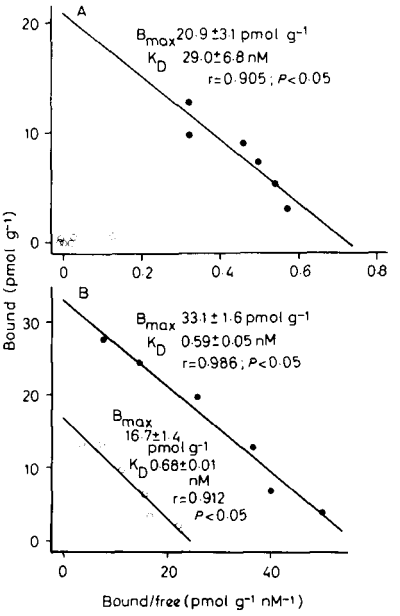

FIG. 1. Comparison by Scatchard analysis of the characteristics of specific binding of (A) [^{3}H]sulpiride (5–40 nM; as defined using (−)-sulpiride 5×10^{-6} M) and (B) [^{3}H]spiperone (0·125–4·0 nM; as defined using (+)-butaclamol 5×10^{-6} M) to fresh rat striatal preparations in the presence (—●—) and absence (—○—) of 120 mM NaCl in the incubation buffer. Ordinates: bound (pmol g^{-1}). Abscissa: bound/free (pmol g^{-1} nM^{-1}). Data was subjected to linear regression analysis ($r = $ at least 0·905, $P < 0.05$ except for [^{3}H]sulpiride binding in the absence of NaCl $r = 0.140$, $P > 0.05$). Kinetic constants were obtained from the regressed data.

sulpiride (5–40 nM) and [^{3}H]spiperone (0·125–4·0 nM) in the presence of incubation buffer containing no cations, or containing 120 mM NaCl. The omission of cations caused [^{3}H]sulpiride binding at all ligand concentrations to decrease to such a low and inconsistent level that kinetic constants could not be calculated (Fig. 1A). In the presence of 120 mM NaCl a straight line relationship was obtained on Scatchard analysis and the data showed B_{max} to be 20·9 ± 3·1 pmol g^{-1} wet weight of tissue and K_D 29·0 ± 6·8 nM. For [^{3}H]spiperone binding (Fig. 1B) the presence of 120 mM NaCl caused an increase in B_{max} to 33·1 ± 1·6 pmol g^{-1} wet weight of tissue from 16·7 ± 1·4 pmol g^{-1} wet weight of tissue observed on removal of cations but no change in K_D was apparent (0·68 ± 0·01 nM in the absence of sodium compared with 0·59 ± 0·05 nM in the presence of NaCl 120 mM; $P < 0.05$) (Fig. 1).

Substituted benzamide drugs, such as sulpiride, are distinguished from other neuroleptics by their differing pharmacological spectrum of activity (Jenner & Marsden 1979). These compounds exhibit an apparent specificity of action on cerebral dopamine receptors since they do not affect cerebral noradrenaline, 5-hydroxytryptamine, acetylcholine or GABA mechanisms (Spano et al 1978; Fjalland et al 1977; Jenner & Marsden 1979). It is probable that the specific binding of [^{3}H]sulpiride to striatal tissue represents an interaction with some form of dopamine receptor, a fact confirmed by the failure of other neurotransmitters to displace [^{3}H]sulpiride from its binding site (Theodorou et al 1979; unpublished data). The almost total dependence of specific binding of [^{3}H]sulpiride on the presence of sodium ions now suggests this site may differ in some respects from that at which [^{3}H]spiperone binds. This ionic effect appears specific for NaCl since KCl, $MgCl_2$ or $CaCl_2$ had little effect on [^{3}H]sulpiride binding. The action of these other ions may, however, occur at the same site as that of NaCl since in the presence of a maximally effective concentration of NaCl (120 mM), no additive changes were observed; indeed, in general, the inclusion of other ions tended to suppress the specific binding of [^{3}H]sulpiride in the presence of NaCl.

The interaction of a number of hormones and neurotransmitters with their receptors has been shown to be influenced by ionic factors, in particular by

divalent cations in systems linked to adenylate cyclase. Since sulpiride does not appear to act on adenylate cyclase-linked dopamine systems, it is perhaps not surprising that divalent cations exerted little effect on the specific binding of [^{3}H]sulpiride. However, in contrast to a recent study by Usdin et al (1980) we were unable to demonstrate any marked dependence of specific [^{3}H]-spiperone binding on the presence of divalent cations in fresh tissue homogenates. However, our findings are in agreement with the earlier studies of Clement-Cormier & George (1979) who, using frozen rat preparations, could demonstrate only slight cation effects on the binding of [^{3}H]haloperidol or [^{3}H]spiperone.

Several possible explanations are apparent for the specific requirement of sodium ions for [^{3}H]sulpiride binding. The effect of NaCl is not due to molarity changes, since equimolar concentrations of other ions did not have the same effect. Nor is it confined to the displacing agent used to define specific binding, (−)-sulpiride, for no specific binding of [^{3}H]sulpiride could be detected in the absence of sodium ions using (+)-butaclamol (5×10^{-6} M) to define specificity (unpublished data).

By analogy to other neurotransmitter systems, in particular GABA pathways, the sodium dependent binding of [^{3}H]sulpiride might be attributed to the presence of sodium-dependent uptake mechanisms (Iversen & Kelly 1975). Attempts to minimize uptake by freezing, however, suggest this is unlikely since specific binding was not decreased in previously frozen tissues, and residual binding still exhibited sodium dependence. In addition, other experiments have shown that specific [^{3}H]sulpiride binding is unaffected by the inclusion of reuptake blockers such as nomifensine or desmethylimipramine (10^{-9}–10^{-4} M) (unpublished data).

The effects of sodium on specific [^{3}H]sulpiride binding appear to be occurring at the transmitter receptor. It is possible that sodium ions act to hold the receptor in the preferred conformation for the binding of [^{3}H]sulpiride as has been previously suggested for the binding of opiate antagonists (Pasternak & Snyder 1975). Why this should be necessary for [^{3}H]sulpiride, however, which takes up a conformation closely resembling the preferred conformation of dopamine (Testa, personal communication), but not other neuroleptic ligands is unclear. Sodium ions may act by stabilizing the specific binding of [^{3}H]sulpiride to the receptor such that a normally extremely rapid dissociation, which perhaps cannot be detected by the present methodology, is slowed. The prevention of time-dependent degradation of binding has been proposed by Usdin et al (1980) to explain the effect of cations on [^{3}H]spiperone binding. In agreement with their finding we have shown NaCl to cause an apparent increase in B_{max} without changing K_D suggesting protection of the receptor complex from degradation. However, why the effect of sodium ions should be specific in this respect is puzzling. Thus, Usdin et al (1980) found all cations to be effective to varying degrees in augmenting [^{3}H]spiperone binding in this manner.

Alternatively, the specific effect of sodium may reflect the fact that this is the ion on which changes in normal conductance are dependent after activation of the dopamine receptor recognition site. The atypical action of sulpiride (and other substituted benzamides) might be explained if such compounds act at dopamine receptor ionophore sites controlling sodium conductance and not solely at the dopamine recognition site.

In conclusion, the specific binding of the D-2 receptor ligand [^{3}H]sulpiride to rat striatal preparations can be distinguished from that of [^{3}H]spiperone binding (which presumably binds to both D-1 and D-2 receptors) on the basis of a specific sodium dependency. This effect may signify an action at a site distinct from that at which other neuroleptics exert their effects, and may explain the atypical properties of substituted benzamide drugs.

This study was supported by the Medical Research Council, the Wellcome Trust and the Research Funds of King's College Hospital and the Bethlem Royal and Maudsley Hospitals. We thank SESIF, France Ltd. for generous supplies of compounds.

April 14, 1980

REFERENCES

Borenstein, P., Champion, C., Cujo, P. H., Gekiers, P., Olivenstein, C., Kramarz, P. (1969) Sem. Hop. (Paris) 45: 1301–1314

Clement-Cormier, Y. C., George, R. J. (1979) J. Neurochem. 32: 1061–1069

Elliott, P. N. C., Jenner, P., Huizing, G., Marsden, C. D., Miller, R. (1977) Neuropharmacology 16: 333–342

Fjalland, B., Christensen, A. V., Hyttel, J. (1977) Naunyn-Schmiedeberg's Arch. Pharmacol. 301: 5–9

Hyttel, J. (1980) Psychopharmacology 67: 107–109

Iversen, L. L., Kelly, J. S. (1975) Biochem. Pharmacol. 24: 933–938

Jenner, P., Marsden, C. D. (1979) Life. Sci. 25: 479–486

Jenner, P., Clow, A., Reavill, C., Theodorou, A., Marsden, C. D. (1978) Ibid. 23: 545–550

Justin-Besancon, L., Thominet, M., Laville, Cl., Margarit, J. (1967) C. R. Acad. Sci. (Paris) 265: 1253–1254

Kebabian, J. W., Calne, D. B. (1979) Nature (London) 277: 93–96

Laville, Cl. (1972) Lille Med. Actual. 17: Suppl. 3, 4–13

Leysen, J. G., Gommeren, W., Laduron, P. M. (1978) Biochem. Pharmacol. 27: 307–316

Pasternak, G. W., Snyder, S. H. (1975) Nature (London) 253: 563–565

Spano, P. F., Govani, S., Trabucchi, M. (1978) in: Roberts, P. J., Woodruff, G. N., Iversen, L. L. (eds) Adv. Biochem. Psychopharmacol. Volume 19 pp 155–165, Raven Press, New York

Theodorou, A., Crockett, M., Jenner, P., Marsden, C. D. (1979) J. Pharm. Pharmacol. 31: 424–426

Trabucchi, M., Langoni, R., Fresia, P., Spano, P. F. (1975) Life. Sci. 17: 1551–1556

Usdin, T. B., Creese, I., Snyder, S. H. (1980) J. Neurochem. 34: 669–676