DIFFERENTIATION OF SOLUBILIZED DOPAMINE RECEPTORS FROM SPIRODECANONE BINDING SITES IN RAT STRIATUM

H. GORISSEN, B. ILIEN, G. AERTS and P. LADURON
Department of Biochemical Pharmacology, Janssen Pharmaceutica, B-2340 Beerse, Belgium

Received 29 August 1980
Revised version received 30 September 1980

1. Introduction

Solubilization of dopamine receptors, in a native form, was first achieved in our laboratory using dog striatum and the mild detergent digitonin [1–3]. These results were confirmed by another group in both dog and human brain [4]. However, earlier attempts to solubilize dopamine receptors from rat striatum were unsuccessful, even though a similar experimental procedure was used [3,5]. Although the binding properties of dopamine receptors in striatal membrane preparations were identical in both animals, the discrepancy between the data from rat and dog remained unexplained [3]. This study was undertaken to solve the problem.

The results show that the solubilized dopamine receptors from rat striatum are masked by a high number of non-specific but displaceable spiperone binding sites. These binding sites have been already described in membrane preparations as non-stereospecific or spirodecanone sites (cf. fig.2) [6,7]. The use of a compound which is structurally related to spiperone, but is inactive on dopamine receptors enabled us to determine and characterize solubilized dopamine receptors from the rat striatum, by means of the charcoal assay method.

2. Materials and methods

Wistar rats were decapitated, mongrel dogs and cats were anaesthetized with pentobarbital; their brains were dissected-out and homogenized in 0.25 M ice-cold sucrose. After preparation of the microsomal fraction [8], which may be kept at −16°C, the extraction was performed at 0°C with 1% digitonin suspended in 4 vol. 0.25 M sucrose containing 10 mM sodium phosphate (pH 7.2) and 0.02% NaN_3, and supplemented by 1 mM EDTA, 1 mM EGTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF) to prevent proteolytic degradation. The radiochemical purity (95–98%) of the [^{3}H]spiperone (25.5 Ci/mmol and 23.6 and 25.7 Ci/mmol, respectively from IRE, Fleurus, Belgium and NEN, Boston, MA) was controlled by thin-layer chromatography on silicagel plates developed with benzene–ethanol–NH_4OH (85:10:0.5, by vol.). Incubations were at 0°C for 16 h. The Sephadex G-50 assay method was used as in [5]. The charcoal and ammonium sulphate techniques were as in [9]. The equilibrium dialysis was carried out using a Dianorm dialyser (Diachem, Zürich) equiped with pairs of 1 ml teflon cells, mounted with a Spectra Por 2 membrane (Spectrum Medical Industries, Los Angeles, CA). Specific binding was defined as the difference between the binding of [^{3}H]spiperone (2×10^{-9} M) with or without 10^{-6} M (+)-butaclamol.

3. Results and discussion

The failure of digitonin to solubilize dopamine receptors from rat striatum could be explained either by a proteolytic degradation, or by a non-appropriate detection by gel filtration. Therefore, in a first approach, using striatal dog preparations as reference system [2–4], extraction was in the presence of inhibitors of proteolysis and determinations of the specific binding sites were performed with different assay techniques.

To assess the specificity of each procedure, three different parameters (table 1) were considered: the

Table 1
[^{3}H]Spiperone binding measured using different assay methods with a solubilized preparation from dog striatum

	IC_{50}-value[a] (M)		Binding[b] (fmol/ml)		Buffer blank (% specific binding)
	(+)-Butaclamol	(−)-Butaclamol	Specific	Non-specific	
A. Sephadex G-50	3.0×10^{-8}	7.9×10^{-6}	357 ± 19	115 ± 11	3.4
B. Charcoal	3.4×10^{-8}	6.3×10^{-6}	505 ± 12	102 ± 1.3	2.2
C. $(NH_4)_2SO_4$	1.7×10^{-8}	4.0×10^{-6}	185 ± 4.2	47 ± 1.3	52
D. Dialysis	–	–	205 ± 26	2038 ± 50	22

[a] IC_{50}-values were determined from competition experiments using 7 concentrations (10^{-10}–10^{-4} M) of (+)- and (−)-butaclamol
[b] Binding was expressed as the mean value ± SEM (A, $n = 3$; B–D, $n = 4$)

stereospecificity (IC_{50}-values for (+)- and (−)-butaclamol) and the relative amounts of specific and non-specific binding. Table 1 shows that a similar stereospecific effect was obtained with the gel filtration, charcoal and ammonium sulphate methods; indeed the (−)/(+)-butaclamol IC_{50}-ratios ranged from 185–260, being thus similar to those found in the corresponding membrane preparations [3,10]. When one examines the specific *vs* non-specific binding, it is obvious that the equilibrium dialysis method suffered from higher non-specific binding [91% of the total). The ammonium sulphate method yielded the lowest amount of specific binding (only 37% of that obtained by the charcoal method). Moreover its buffer blank (binding measured when buffer replaced the soluble extract) was also high. The Sephadex G-50 and the charcoal methods yielded high specific binding, concomitant with low non-specific binding. The charcoal assay method may be considered as the most appropriate technique, since it gives a high yield, is easy to perform, is not time-consuming, and is quite reproducible. This method was therefore applied to solubilized extracts from rat striatum.

Fig.1 shows that the displacement of [^{3}H]spiperone by (+)-butaclamol in soluble extracts from rat striatum revealed a biphasic curve when the ligand–receptor complex was measured by the charcoal technique. Such a curve was never obtained after gel filtration (cf. also the (+)-butaclamol displacement curve in [5]). The first slope between 10^{-9}–10^{-6} M probably corresponds to the specific binding and the second slope between 10^{-6}–10^{-4} M to a non-specific but displaceable binding. Interestingly the inflection point at 10^{-6} M (+)-butaclamol was repeatedly found in different experiments. Contrary to (+)-butaclamol, spiperone was found to displace both types of bind-

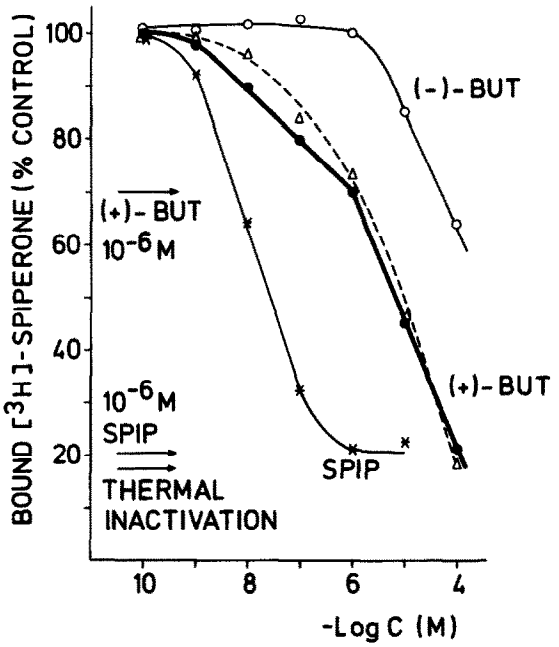

Fig.1. Competition curves of spiperone (SPIP) and the butaclamol (BUT) enantiomers for digitonin-solubilized preparations incubated with 2×10^{-9} M [^{3}H]spiperone. The full lines represent the results obtained using the charcoal assay method, the dashed line those obtained for (+)-butaclamol using the gel filtration method. Each point is the mean value of two determinations. Arrows indicate the blank values with 10^{-6} M (+)-butaclamol or spiperone and after thermal inactivation (56°C, 10 min). The IC_{50}-values for spiperone, (+)-butaclamol and (−)-butaclamol were, respectively, 2×10^{-9} M, 2.8×10^{-8} M and 10^{-5} M when (+)-butaclamol (10^{-6} M) was used as blank and of 1.4×10^{-8} M, 2.7×10^{-6} M and 1.4×10^{-4} M when spiperone (10^{-6} M) or thermal inactivation were used as blanks.

ing sites according a monophasic competition curve. In our earlier experiments on rat preparations the blank values (non-specific binding) were determined either by competition of 10^{-6} M spiperone [5] or after thermal inactivation [3]. In fact the use of (+)-butaclamol for the blank is preferable to the use of unlabelled spiperone, because the latter can also displace non-specific binding, presumably from the spirodecanone sites. For instance fig.1 shows that if 10^{-6} M spiperone is taken as blank, the apparent affinity of (+)-butaclamol (IC_{50}-value) is in the μM range, whilst it is ~10 nM when the non-specific binding is determined using 10^{-6} M (+)-butaclamol. The same argument is valid for the thermal inactivation which affects both specific dopaminergic and spirodecanone sites.

To prevent such non-specific binding we selected a compound, R 5260 (potent analgesic [11]) which possesses the spirodecanone moiety like spiperone (fig.2), but which is completely devoid of dopaminergic activity [3,11]. Both drugs were compared, in different binding experiments to benperidol (fig.3A,B), one of the most potent dopamine antagonists [3,11], which is a butyrophenone like spiperone

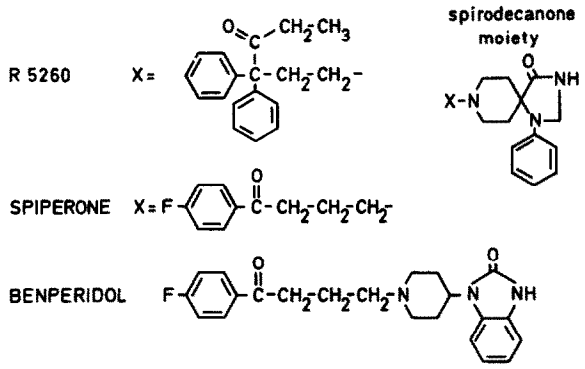

Fig.2. Comparison of the chemical structures of R 5260, spiperone (spiroperidol) and benperidol.

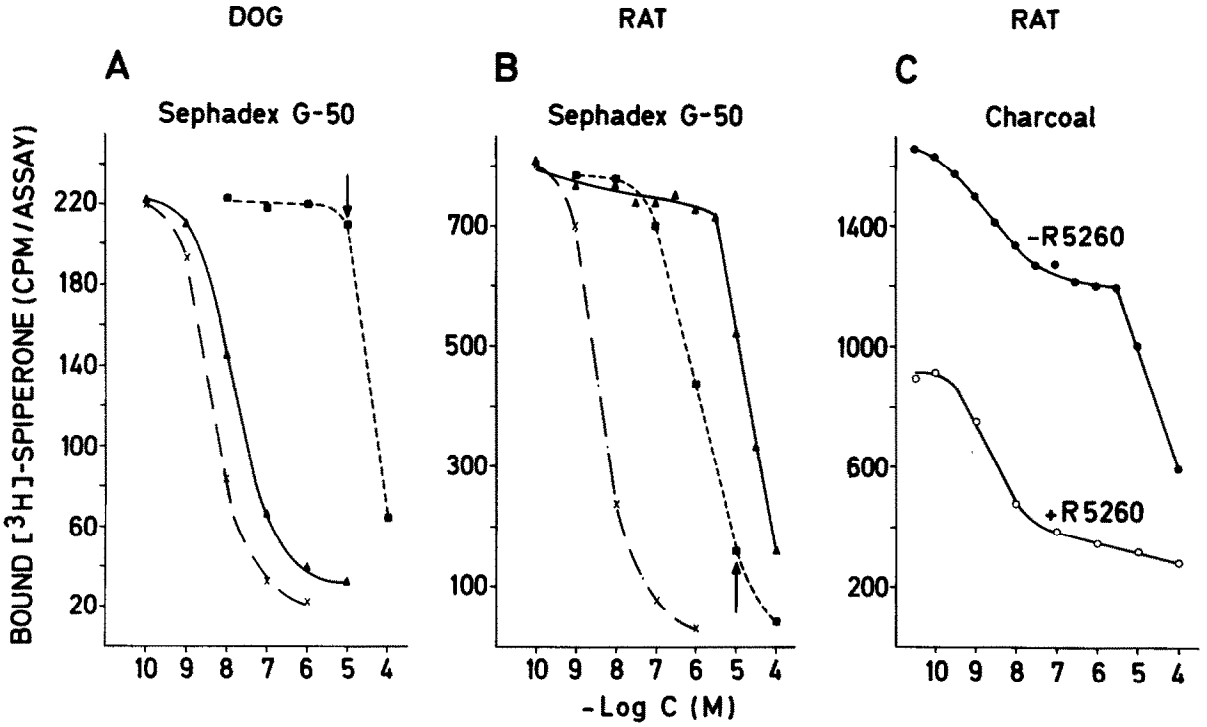

Fig.3. Competition curves of various drugs in solubilized preparations from dog and rat striata, after incubation with 2×10^{-9} M [^{3}H]spiperone. (A) Spiperone (–X– - -), benperidol (–▲–) and R 5260 (–■–) curves using striatal dog extracts (as in [2]) and the gel filtration technique. (B) Competition of the same drugs in the striatal rat extracts (as in [3,5]) using the gel filtration technique. An arrow indicates the displacement at 10^{-5} M R 5260. (C) Competition curves of benperidol using the charcoal assay in the presence or the absence of 10^{-5} M R 5260. The IC_{50}-values of benperidol were, respectively, 2.5×10^{-9} M (+ R 5260) and 5.8×10^{-9} M (– R 5260).

Table 2
[^{3}H]Spiperone binding[a] in striatal extracts from various species with or without R 5260 at 10^{-5} M

	IC_{50}-value[b] (M)				Binding (fmol/ml)			
	(+)-Butaclamol		(−)-Buta-clamol	Amino-tetralin derivative	Specific		Non-specific	
	− R 5260	+ R 5260			− R 5260	+ R 5260	− R 5260	+ R 5260
Dog	1.4×10^{-8}	0.8×10^{-8}	6×10^{-6}	2.2×10^{-7}	557	436	105	85
Cat	1.0×10^{-8}	1.7×10^{-8}	14×10^{-6}	2.8×10^{-7}	294	236	150	121
Rat	0.9×10^{-8}	0.9×10^{-8}	14×10^{-6}[c]	1.3×10^{-7}[c]	175	178	590	166

[a] Binding was determined in duplicate
[b] IC_{50}-values were determined by competition against [^{3}H]spiperone (2×10^{-9} M) using 7 concentrations (10^{-10}–10^{-4} M) of (+)- and (−)-butaclamol and the aminotetralin derivative
[c] Binding measured in the presence of R 5260 at 10^{-5} M

but does not possess the spirodecanone moiety (fig.2). Fig.3A shows that for solubilized striatal preparations from dog, assayed with the gel filtration method, spiperone and benperidol competed with [^{3}H]spiperone at very low concentrations, while R 5260 was inactive at 10^{-5} M. In contrast to this, solubilized preparations from rat striatum, measured with the same assay method, displayed a high affinity towards spiperone but a low one for benperidol, whereas R 5260 was found to compete with [^{3}H]-spiperone at 10^{-6} M (fig.3B). As shown in fig.3C the displacement curve of benperidol obtained in rat extracts by using the charcoal method, displayed a marked biphasic profile in the absence of R 5260. At 10^{-5} M this compound, used to block the spirodecanone sites, converts this competitition curve of benperidol into a classical monophasic displacement curve. Therefore the use of such a drug as R 5260 at an appropriate concentration, combined with the charcoal assay method, markedly improved the detection of the specific binding in solubilized preparations of rat striatum. This procedure was now applied to characterize the [^{3}H]spiperone binding sites in solubilized preparations from rat striatum, and then to compare them to the results obtained with other species.

Table 2 shows that (+)-butaclamol and the potent dopamine agonist (±) 2-(*N*,*N*-dipropyl)amino-5,6-dihydroxytetralin (ATL) revealed a high affinity in rat preparations, their respective IC_{50}-values being comparable to those already obtained in membrane preparations from rat and dog striatum [3,10], as in solubilized preparations from dog and cat. Table 2 shows also that R 5260 at 10^{-5} M did not markedly affect the IC_{50}-value of (+)-butaclamol in the different mammalian preparations. However, it was found to tremendously decrease the non-specific binding in rat preparations without altering the specific binding, whereas in cat and dog the low non-specific binding level remained almost unchanged, whilst the specific

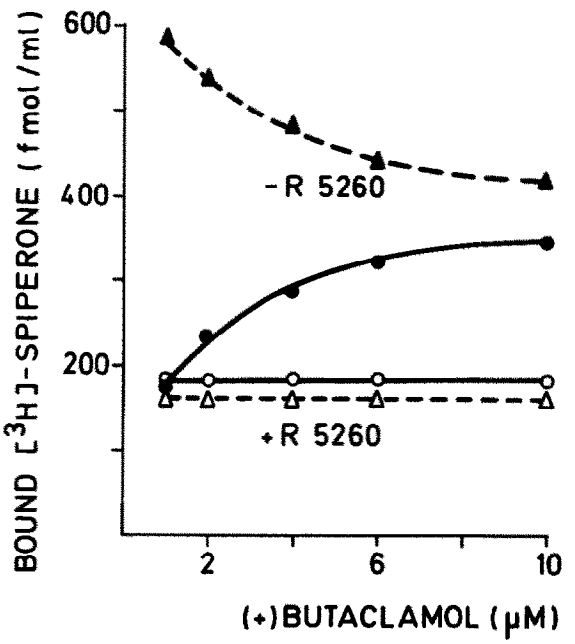

Fig.4. Variation of the apparent specific (——) and non-specific (– – –) [^{3}H]spiperone binding in the presence (○,△) or the absence (●,▲) of R 5260, with increasing (+)-butaclamol concentrations used for the determination of the blank values. Each point comes from (+)-butaclamol competition curves performed in duplicate with rat striatal solubilized preparations.

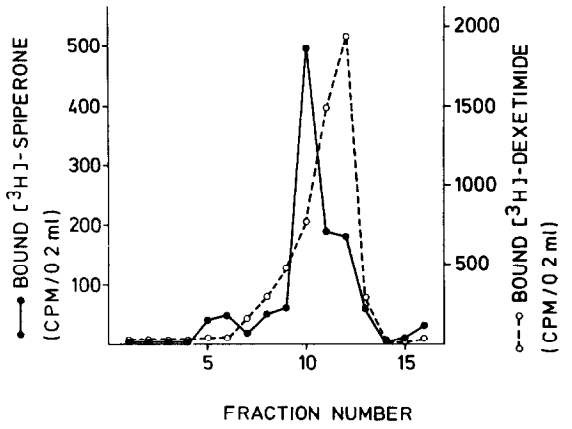

Fig.5. Gradient sedimentation profiles of dopamine and muscarinic receptors from a digitonin extract of rat striatum. Soluble extract (1 ml) was layered on a 15–30% sucrose gradient containing 10 mM sodium phosphate (pH 7.2), 0.02% NaN_3 and 0.03% digitonin, and then centrifuged at 152 000 × g (r_{av}) for 20 h with a SW40Ti rotor (Spinco) at 2°C. The dopamine receptor was measured in the presence of 10^{-5} M R 5260, using 2 × 10^{-9} M [^{3}H]spiperone (53 Ci/mmol, NEN) with 10^{-6} M (+)-butaclamol for the blank, while the muscarinic receptor did using 2 × 10^{-9} M [^{3}H]-dexetimide (17 Ci/mmol, IRE) with 2 × 10^{-7} M unlabelled dexetimide for the blank. The charcoal method was used for both receptor determinations.

binding decreased by ~20%. Consequently in rat preparations, the choice of an appropriate blank is of great importance, because of the high amount of non-specific binding. When R 5260 blocks these spirodecanone sites (fig.4) both the specific and the non-specific binding remained constant with (+)-butaclamol at 1–10 μM. In contrast, in the absence of this drug the apparent specific binding rose with increasing (+)-butaclamol concentrations, concomitantly the apparent non-specific binding decreased. Consequently, the use of R 5260 seriously improves the safety of measurement of the specific dopaminergic binding sites and must be strongly recommended for rat striatal extracts. Nevertheless when the spirodecanone binding is relatively low, for instance in cat and dog, the use of this compound is not necessary.

That more spirodecanone sites were found in the rat than in the corresponding preparations from dog is probably only the result of species variability. In addition to this difference between species, the amount of spirodecanone sites was much higher in the frontal cortex than in the striatum (cf. fig.1a,b of [6]); this repartition is incompatible with the regional distribution of dopamine receptors. A further difference between both sites is that a significant amount of spirodecanone sites, but not of dopamine receptors, was found in a soluble form when the procedure was carried out in the absence of the detergent (unpublished). Finally the spirodecanone sites are not relevant for any known pharmacological activity [7]. A similar phenomenon dealing with displaceable non-specific binding was recently reported in studies on β-adrenergic [12] and benzodiazepine [13] receptors. This problem also represented the main difficulty when studying the solubilization of serotonin receptors by lysolecithin (in preparation).

The solubilized dopamine receptor sites from rat striatum were further characterized by sedimentation gradient. Fig.5 shows the sedimentation profile of the specific [^{3}H]spiperone binding compared to that of the muscarinic receptor labelled by [^{3}H]dexetimide [14]. The position of both peaks is quite similar to that reported in the striatal dog preparations [2], which suggests an interspecies molecular homogeneity for dopaminergic sites.

From the above experiments, evidence is provided for the solubilization of a high-affinity dopamine receptor from rat striatum. If the non-specific binding can be reduced and if an appropriate assay method is used, solubilized receptors labelled by [^{3}H]spiperone may be identified as being of dopaminergic nature both by their binding and by their hydrodynamic characteristics.

That a similar dopaminergic macromolecular complex was extracted from the brain of different species (dog [2–4], human [4], cat and rat) using the same detergent, strongly favors the unitary concept of receptor systems. Since this macromolecular receptor complex has been extracted by a mild detergent, digitonin, it may belong to the class of the intrinsic bound membrane proteins [15]. These concordant facts from different studies on various species, are in discordant disagreement with reports on the solubilization of dopamine receptor sites from calf striatum [16,17], treated with high concentrations of salt (50–500% KCl). A similar discrepancy between the solubilization of the muscarinic receptor either by digitonin, or by salt (2 M NaCl) was recently resolved by demonstrating that the salt extraction resulted in an 'artefactual' solubilization of the muscarinic receptor [18]. This same question may be asked of the salt-solubilized dopamine receptor sites from calf striatum.

Acknowledgements

Part of this work was supported by a grant of IWONL and a fellowship to B. I. of the Fondation de l'Industrie Pharmaceutique pour la Recherche (Paris). We thank D. Ashton for his help in preparing the manuscipt.

References

[1] Gorissen, H. and Laduron, P. (1979) Arch. Int. Physiol. Biochim. 87, 414–415.
[2] Gorissen, H., Aerts, G. and Laduron, P. (1979) FEBS Lett. 100, 281–285.
[3] Gorissen, H. and Laduron, P. (1979) Nature 279, 72–74.
[4] Madras, B. K., Davis, A., Kunashko, P. and Seeman, P. (1980) in: Psychopharmacology and Biochemistry of Neurotransmitter Receptors (Yamamura, H. I. et al. eds) Elsevier/North-Holland, London, New York, in press.
[5] Gorissen, H. and Laduron, P. (1978) Life Sci. 23, 575–580.
[6] Leysen, J. E. and Gommeren, W. (1978) Life Sci. 23, 447–452.
[7] Howlett, D. R., Morris, H. and Nahorski, S. R. (1979) Mol. Pharmacol. 15, 506–514.
[8] Laduron, P. (1977) Int. Rev. Neurobiol. 20, 251–281.
[9] Gavish, M., Chang, R. S. L. and Snyder, S. H. (1979) Life Sci. 25, 783–790.
[10] Leysen, J. E., Niemegeers, C. J. E., Tollenaere, J. P. and Laduron, P. M. (1978) Nature 272, 168–171.
[11] Leysen, J., Tollenaere, J. P., Koch, M. H. J. and Laduron, P. (1977) Eur. J. Pharmacol. 43, 253–267.
[12] Pochet, R. and Schmitt, H. (1979) Nature 277, 58–59.
[13] Paul, S. M., Zatz, M. and Skolnick, P. (1980) Brain Res. 187, 243–246.
[14] Gorissen, H., Aerts, G. and Laduron, P. (1978) FEBS Lett. 96, 64–68.
[15] Singer, S. J. (1974) Ann. Rev. Biochem. 43, 805–833.
[16] Clement-Cormier, Y. C. and Kendrick, P. E. (1980) Biochem. Pharmacol. 29, 897–903.
[17] Clement-Cormier, Y. C., Meyerson, L. R. and McIsaac, A. (1980) Biochem. Pharmacol. 29, 2009–2016.
[18] Gorissen, H., Aerts, G. and Laduron, P. (1980) in: Drug Receptors in the Central Nervous System, Proc. EMBO Workshop, Rehovot (Balaban, M. ed) Wiley, London, in press.