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SOLUBILIZATION OF SEROTONIN RECEPTORS FROM RAT FRONTAL CORTEX

Brigitte Ilien, Hugo Gorissen and Pierre Laduron

Department of Biochemical Pharmacology, Janssen Pharmaceutica, B-2340 Beerse, Belgium

Brain serotonin receptors have been identified *in vitro* by measuring the binding of different radiolabelled ligands like LSD (1-3) and 5-HT (1,2,4). [^{3}H]-spiperone was also reported to label serotonergic receptors in the frontal cortex under *in vitro* as well as *in vivo* conditions (3) although it binds to dopamine receptors in the striatum (3,5). Further characterization of such binding sites needs solubilization (6). Previously, dopamine (7) and muscarinic receptors (8-10) were obtained in soluble form using digitonin whereas GABA receptors were extracted from rat brain with lysolecithin (11). We now report the solubilization by lysolecithin of [^{3}H]-spiperone binding from rat frontal cortex which retain the high affinity characteristics of the membrane serotonin receptor. This was made possible by using R 5573 in order to prevent the very high non-specific binding of [^{3}H]-spiperone to the spirodecanone (cfr. Fig. 1) sites (12,13).

MATERIALS AND METHODS

Wistar rats (± 150 g) were decapitated and the brains immediately removed. After dissection, frontal cortex, striatum or cerebellum were homogenized in 10 vol. ice-cold 0.25 M sucrose using a Duall homogenizer. The total homogenate was fractionated by differential centrifugation as described previously (14), except that for the frontal cortex and cerebellum, the M + L fraction was pelleted at 17,380 x g (10 min) instead of 30,890 x g for the striatum. The microsomal fraction (P) was suspended in 2 vol. ice-cold water and kept at -16°C before solubilization.

Solubilization procedures. a) Frontal cortex. The P fraction was treated with 0.25 % ice-cold lysolecithin (L-α-lysophosphatidyl choline from Egg Yolk-Type 1-No. L-4129-Sigma) suspended in 4 vol. 0.25 M sucrose containing 10 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA and 0.01 % NaN_3. The mixture was incubated for 15 min at 0°C and then centrifuged at 182,000 x g (r_{av}) for 60 min. The supernatant was carefully separated from the pellet and kept at 0°C before the binding assay. b) Striatum. Dopamine receptors from rat striatum were solubilized with 1 % digitonin (Serva) as already described for dog striatum (7). The buffer was supplemented with 1 mM EDTA and EGTA, 0.1 mM phenyl methyl sulfonylfluoride and 0.02 % NaN_3. The following operations were the same as those used for the frontal cortex.

Binding assays. a) Frontal cortex. Soluble extract (0.4 ml) was incubated at 0°C for 18 h with 10^{-9} M [^{3}H]-spiperone (spec. act. 53.4 Ci mmol^{-1}), 10^{-5} M R 5573, unless stated otherwise, and various concentrations of unlabelled drugs in 0.5 ml total vol. The radiochemical purity (95-98 %) of [^{3}H]-spiperone (23.6 and 53.4 Ci mmol^{-1}, NEN, Boston) was verified by thin-layer chromatography on silica-gel plates developed with benzene-ethanol-NH_4OH (90:10:0.5, v/v). Specific [^{3}H]-spiperone binding was defined as the difference between the total binding and the binding in the presence of 10^{-6} M pipamperone (see ref. 3). After incubation, 0.5 ml ice-cold saturated ammonium sulphate was added to the incubation mixture and then immediately filtered under vacuum through Whatman GF/B glass fiber filters which were washed twice with 5 ml ice-cold 50 % saturated ammonium sulphate. Then, the filters

were placed in vials with 10 ml of Insta-Gel II (Packard) and counted for radioactivity in a liquid scintillation spectrometer. b) Striatum. For the digitonin extract, the incubation conditions were similar except that 10^{-5} M R 5260 replaced R 5573 and that 10^{-6} M (+)-butaclamol was used for determining the non-specific binding. The ligand-receptor complex was separated from the free ligand by means of a charcoal procedure (11). 50 µl of an activated charcoal mixture (10 % charcoal and 2 % BSA in water) were added to 0.4 ml of the incubation medium and then centrifuged 3 min in a microfuge. The free [^{3}H]-spiperone was adsorbed and an aliquot of the supernatant counted for radioactivity.

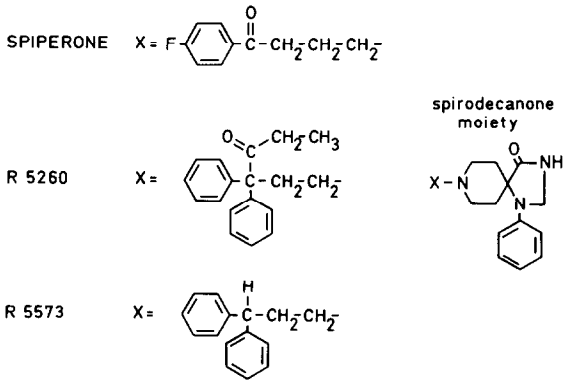

Fig. 1: Comparison of the chemical structure of spiperone (spiroperidol), R 5260 and R 5573.

RESULTS AND DISCUSSION

The major difficulty encountered throughout this work, was the occurrence of a very high non-specific binding. Indeed, in a preliminary experiment, the specific bound [^{3}H]-spiperone in a frontal cortex extract solubilized by lysolecithin represented about 10 % of the total binding (Fig. 2A).

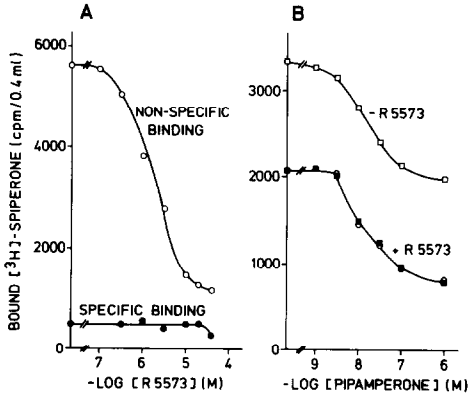

Fig. 2A: Competition of R 5573 for the binding of 2 nM [^{3}H]-spiperone (spec. act. 23.6 Ci mmol^{-1}) in lysolecithin extracts from rat frontal cortex. Non-specific binding sites (o——o) were obtained after displacement with 10^{-6} M pipamperone whereas specific binding sites (●——●) were taken as the difference between the total and the non-specific binding.

B: Inhibition curves of pipamperone on the binding of 1 nM [^{3}H]-spiperone (spec. act. 53.4 Ci mmol^{-1}) in a similar extract. The incubations were performed in the presence (■——■) or absence (□——□) of 10^{-5} M R 5573 or in the presence of 10^{-5} M R 5573 and of 10^{-7} M domperidone (o——o).

In these conditions, thus using 2 nM [^{3}H]-spiperone (spec. act. 23.6 Ci mmol^{-1}), reproducible results were quite difficult to obtain (the specific binding values taken from different experiments varied from 8 to 33 % of the total binding). This problem was solved by making two main improvements. First, non-specific binding was considerably reduced by adding to the incubation mixture, R 5573 (compound 23 of ref. 15), which has the same spirodecanone moiety as spiperone but does not compete for the serotonin receptor. Fig. 2A shows that R 5573 considerably reduces the non-specific binding without affecting the specific binding, even at 10^{-5} M. For the digitonin extract from rat striatum,

R 5260 was preferred to R 5573 (16). Secondly, the use of a lower concentration of [^{3}H]-spiperone (1 nM) but with a higher specific activity (53.4 Ci mmol^{-1}), also greatly increased the proportion of specific binding. Fig. 2B shows the displacement of [^{3}H]-spiperone with increasing concentrations of pipamperone, a serotonin antagonist (3). In the absence of R 5573, the specific binding represented nearly 40 % of the total binding. When 10^{-5} M R 5573 was present, the proportion of specific binding was reproducibly about 60 % (six independent experiments gave a mean value of 62 $\pm$ 2.8 % (S.E.M.) for the proportion of specific binding). The IC_{50}-values for pipamperone were quite similar in both conditions (2 and 1.4 10^{-8} M respectively). Note that the specific binding was not affected by domperidone, a potent and selective dopamine antagonist (17). In order to assess the nature of [^{3}H]-spiperone binding sites, a comparison of the inhibitory effects of pipamperone and (+)-butaclamol was performed in digitonin extracts from rat striatum and cerebellum and in lysolecithin extracts from rat frontal cortex and cerebellum.

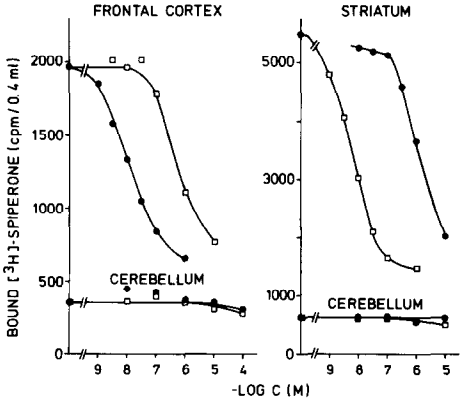

Fig. 3: Inhibition of [^{3}H]-spiperone binding by pipamperone (●——●) and (+)-butaclamol (□——□) in preparations solubilized by lysolecithin (frontal cortex and cerebellum) or digitonin (striatum and cerebellum)

Fig. 3 shows that when using optimal conditions for both preparations, pipamperone was about 100 times more potent in displacing [^{3}H]-spiperone binding in the frontal cortex than in the striatum whereas (+)-butaclamol, a selective dopamine antagonist (3), was 32 times less active in the frontal cortex than in the striatum. Interestingly, both drugs were without effects on both cerebellum preparations (18). The high potency of pipamperone and the low one of (+)-butaclamol or domperidone (Fig. 2B) in the frontal cortex already suggest a serotonergic nature for the solubilized [^{3}H]-spiperone binding sites. To confirm this, various compounds (agonists and antagonists), belonging to different chemical classes, were tested in both solubilized extracts, and then, their IC_{50}-values were compared to those obtained in membrane preparations.

Table 1. Inhibition of [^{3}H]-spiperone binding in solubilized and membrane preparations from rat frontal cortex and striatum

	Frontal cortex (A) IC_{50} (nM)		Striatum (B) IC_{50} (nM)		Ratio (B/A)	
	Soluble	Membrane*	Soluble	Membrane*	Soluble	Membrane*
5-HT (serotonin)	3,160	3,160	178,000	79,400	56	25
LSD	56.2	25.1	1,410	56.2	25	2.2
Dopamine	251,000	251,000	28,200	6,310	0.112	0.025
2-(N, N-dipropyl)amino-5, 6-dihydroxytetralin	158,000	79,400	178	126	0.001	0.001
Pipamperone	11.8	16.2	1,260	1,260	106	77
Mianserine	66	39.8	6,310	1,990	95	50
Spiperone	6.3	3.5	6.3	1.2	1	0.3
(+)-butaclamol	316	100	10.7	20	0.03	0.2
(-)-butaclamol	41,700	>10,000	8,910	>10,000	0.21	

* IC_{50}-values were taken from ref. (3).

Table 1 shows that the IC_{50}-values were similar in both membrane and soluble preparations. However, there was a marked difference in activity for most of these drugs between the frontal cortex and the striatum. For instance, the serotonin agonists (5-HT and LSD) and the serotonin antagonists (pipamperone, mianserine) were much more active in the frontal cortex than in the striatum. In contrast to this, 2-(N,N-dipropyl) amino-5,6-dihydroxytetralin, the most potent dopamine agonist (19), dopamine and (+)-butaclamol were weak inhibitors in the frontal cortex but much more active in the striatum. Spiperone revealed a high affinity in both systems. As a rule, the difference between both brain areas is slightly more pronounced in the soluble material than in the membrane preparations. Finally, other drugs, having different pharmacological activities (antimuscarinic, α and β adrenergic blocker, antihistaminic, minor tranquilizer and serotonin uptake blocker) were inactive at a concentration of 10^{-5} M. Table 1 shows also that the [^{3}H]-spiperone binding in the solubilized preparation from rat frontal cortex revealed a pronounced stereospecific effect: (+)-butaclamol competed at a concentration 130 times lower than (-)-butaclamol, the inactive enantiomer. The foregoing data provide evidence that the spiperone binding solubilized by lysolecithin from rat frontal cortex was of serotonergic nature. Such binding sites were found to be saturable (app. K_D 1.4 nM) and reversible. The low K_D, quite similar to that obtained for membrane preparations (20), indicates that, after solubilization, serotonin receptors retain their high affinity characteristics. Finally, most of the solubilization criteria were fulfilled: not sedimentable at 182,000 x g (r_{av}) for 60 min, much lower Svedberg Coefficient in comparison with that of membranes (in preparation) and lack of retention on GF/B filters before the ammonium sulphate precipitation.

In summary: the above results provide evidence that serotonin receptors from the rat frontal cortex can be solubilized by a lysolecithin treatment. The macromolecular complex obtained and labelled with [^{3}H]-spiperone retained the high affinity characteristics of the serotonin receptor in the original membranes; this receptor was not detectable in the cerebellar extract and differed markedly from the dopamine receptors solubilized by digitonin from rat striatum.

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