SOLUBILIZED SEROTONIN RECEPTORS FROM RAT AND DOG BRAIN

Selective labelling with [^{3}H]ketanserin

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1. Introduction

The ligands which are used to label brain serotonin receptors in in vitro binding assays do not all bind to the same site; [^{3}H]serotonin, for instance, is known to label serotonin-S_1 binding sites [1] whose physiological relevance is unclear. In contrast, in the rat frontal cortex, [^{3}H]spiperone was found to label serotonin receptors [2,3] which were called serotonin-S_2 receptor sites [1]. The physiological role of the S_2-receptors is better understood; they have been shown to mediate the antagonism of various serotonergic effects measured both in vitro and in vivo [1–4]. Recently, the serotonin-S_2 receptors were obtained in a solubilized form by means of lysolecithin [5]; the solubilized [^{3}H]spiperone binding sites from the rat frontal cortex retained the high affinity properties of the serotonin receptor in the original membrane [5,6]. However, this work was hindered by a high occurrence of non-specific binding.

Since [^{3}H]ketanserin, a new anti-serotonergic agent [7] was found to selectively label serotonin-S_2 receptors [4], we decided to re-examine the solubilization of serotonin receptors in rat as well as in dog brain using this ligand.

2. Materials and methods

Mongrel dogs were anaesthetized with pentobarbital and Wistar rats were decapitated; their brains were removed and the frontal cortex was dissected out and then homogenized in 0.25 M sucrose. A microsomal (P) fraction was prepared as in [5]. This membrane preparation was treated at 0°C for 15 min with 0.25% lysolecithin (L-α-lysophosphatidylcholine, 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. After centrifugation at 182 000 × g (r_{av}) for 1 h, 0.4 ml aliquots of the supernatant (which was taken as soluble extract) were incubated with 1 nM [^{3}H]spiperone (53.4 Ci/mmol, NEN) or with 1 nM [^{3}H]ketanserin (87.5 Ci/mmol, NEN) in the presence of various concentrations of unlabelled drugs, unless stated otherwise. After incubation (20 min at 30°C), the ligand–receptor complex was separated from the free ligand by means of a charcoal procedure [6,8]. An activated charcoal mixture 50 μl (10% charcoal and 2% BSA in water) were added to 0.4 ml of the incubation medium and rapidly centrifuged in a microfuge.

Finally, 0.2 ml aliquots of the supernatant were counted for radioactivity. Specific binding was defined as the difference between total binding and non-specific binding occurring in the presence of 10^{-6} M pipamperone.

3. Results and discussion

High non-specific binding represented a serious drawback when [^{3}H]spiperone was used to label solubilized serotonin receptors [5]. This problem was partially solved by selectively preventing [^{3}H]spiperone binding to the spirodecanone sites [5] or by changing the incubation conditions (30°C for 20 min) and the assay procedure (charcoal method [6]). Therefore, we decided to use these optimal conditions in the present study with both [^{3}H]ketanserin and [^{3}H]-spiperone as ligands.

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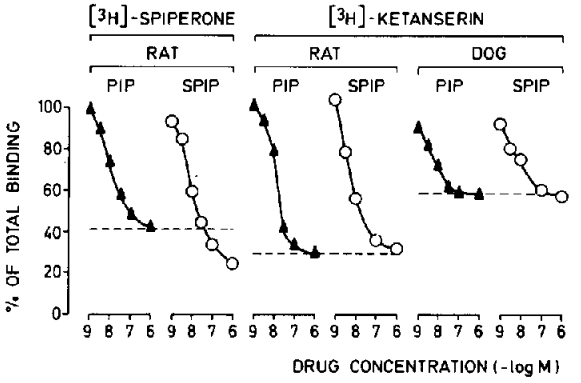

Fig.1. Inhibition of [^{3}H]spiperone and [^{3}H]ketanserin binding in soluble extracts from rat and dog frontal cortex. Both soluble extracts were prepared by means of the same solubilization procedure (section 2). Incubation was performed at 30°C for 20 min with 1 nM [^{3}H]ligand and in the presence of various concentrations of pipamperone (PIP) or spiperone (SPIP). Non-specific binding (– – –) was defined by inhibition at 10^{-6} M pipamperone.

Displacement curves of [^{3}H]spiperone and [^{3}H]-ketanserin binding were performed in soluble extracts of rat and dog. Fig.1 shows that spiperone and pipamperone (a potent serotonergic antagonist [3]) inhibited the binding of both [^{3}H]ligands at nanomolar concentrations. However, more [^{3}H]spiperone binding sites were displaced by unlabelled spiperone than by pipamperone or ketanserin; the latter drugs showed the same plateau-value at maximal inhibition (not shown). In contrast, pipamperone and spiperone inhibited to the same extent [^{3}H]ketanserin binding in solubilized extracts from rat or dog frontal cortex. These displaceable binding sites were not detectable in cerebellar extracts. Moreover, in a control experiment, we found that unlabelled ketanserin and methysergide (a potent serotonergic antagonist lacking α_1-adrenergic and H_1-histaminergic activities [7]) gave the same plateau-value as spiperone and pipamperone. The foregoing results clearly indicate that, in contrast to [^{3}H]spiperone binding [5], chemical recognition sites of the ligand structure do not seem to be involved in [^{3}H]ketanserin binding. Furthermore, pipamperone (a butyrophenone derivative lacking the spirodecanone moiety and chemically distinct from ketanserin) can be considered as a safe compound for defining non-specific levels of [^{3}H]ketanserin as well as of [^{3}H]spiperone binding.

Table 1 reveals that, under identical experimental conditions, [^{3}H]spiperone and [^{3}H]ketanserin label nearly the same amount of specific binding sites in the rat frontal cortex extract. A greater proportion of specific binding was detected in the rat extract with [^{3}H]ketanserin than with [^{3}H]spiperone (fig.1). However, in the dog, there was less specific [^{3}H]-ketanserin binding and relatively more non-specific binding.

Using a soluble extract from rat frontal cortex [^{3}H]ketanserin binding was saturable (fig.2) over the same range as membrane preparations [4]. Almost complete saturation was reached at 4 nM and under such conditions, the percentage of specific binding was still close to 50%. The Scatchard analysis provided a straight line suggesting the occurrence of a homogeneous population of solubilized binding sites; similar characteristics have already been described when using [^{3}H]spiperone [6]. K_d and B_{max}-values for [^{3}H]ketanserin binding were of 1.9 nM and 1.3 pmol receptor sites/g tissue, respectively. This K_d-value was very close to that reported for membrane preparations (K_d = 1.3 nM, measured in Tris–salt buffer [4]).

At 30°C, the solubilized binding sites remained

Table 1
Comparison of [^{3}H]spiperone and [^{3}H]ketanserin binding in soluble extracts from rat and dog frontal cortex

[^{3}H]Ligand (1 nM)	Animal	Total binding (fmol/ml extract)	Specific binding	
			fmol/ml extract	% Total binding
[^{3}H]Spiperone	Rat	144.9 ± 0.8	78.4 ± 3.4	54
[^{3}H]Ketanserin	Rat	130.0 ± 1.9	86.9 ± 3.2	67
[^{3}H]Ketanserin	Dog	133.9 ± 7.4	57.4 ± 5.2	43

Solubilization and incubation procedures were those in section 2. Specific binding was defined as the difference between the total binding and the binding obtained in the presence of 10^{-6} M pipamperone. The values presented are the mean of 3 independent determinations (± SD)

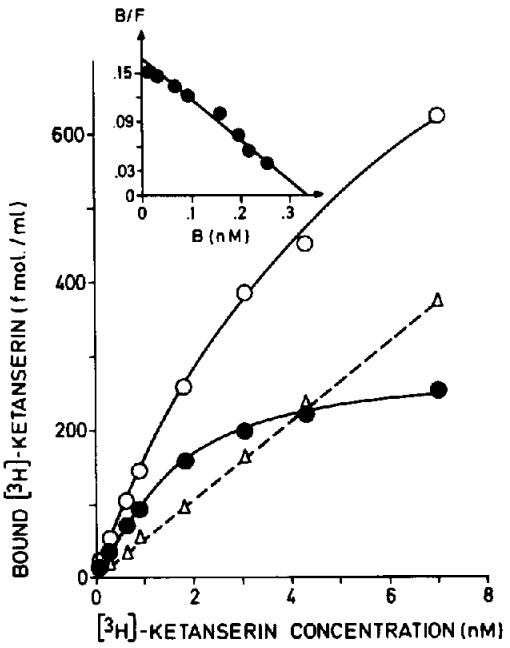

Fig.2. Concentration binding curves of [^{3}H]ketanserin binding in a soluble extract from rat frontal cortex. The assays were carried out for 20 min at 30°C with increasing concentrations of [^{3}H]ketanserin (0.11–7.02 nM) and the binding was measured by means of the charcoal technique. Specific binding (●——●) was defined as the difference between total (○——○) and non-specific binding (△– – –△) occurring in the presence of 10^{-6} M pipamperone. Inset: Scatchard plot of specific binding.

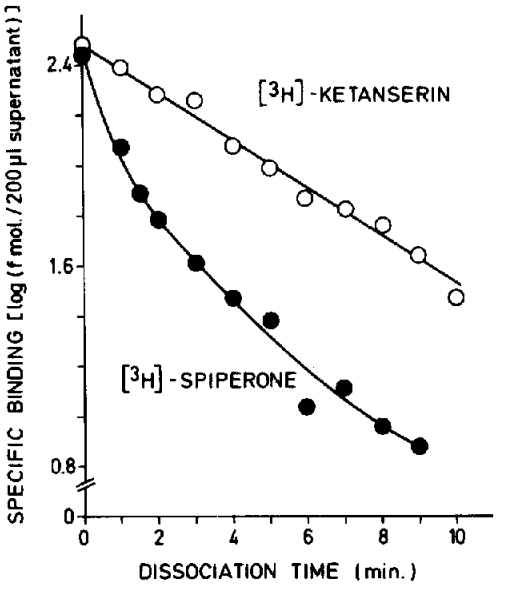

Fig.3. Time curves for dissociation at 30°C of specific [^{3}H]-spiperone (●——●) and [^{3}H]ketanserin (○——○) binding in a soluble extract from rat frontal cortex. Dissociation is presented as a first-order reaction plot of the release. [^{3}H]-Ligands were used at 1 nM; after 20 min at 30°C for reaching total association (specific binding values were 80 and 85 fmol/ml extract for [^{3}H]spiperone and [^{3}H]ketanserin binding, respectively), dissociation started by addition of 10^{-6} M pipamperone.

stable for ≥40 min [6]; the association rates of [^{3}H]-spiperone [6] and [^{3}H]ketanserin are relatively fast ($t_{1/2}$ ~ 1 min).

Some differences were found between both ligands with regard to their dissociation properties. Fig.3 shows that half the amount of specific [^{3}H]spiperone receptor complexes were dissociated after 2 min at 30°C; as reported for membrane preparations [9], the dissociation curve did not follow a first-order reaction plot but apparently involved a rapid and a slow component. This problem was not encountered with [^{3}H]-ketanserin in solubilized or membrane [4] preparations from rat frontal cortex. Indeed, [^{3}H]ketanserin was found to dissociate from the solubilized binding sites according to typical first-order kinetics. Moreover, its dissociation rate ($t_{1/2}$ = 7.5 min) was slower than that reported for [^{3}H]spiperone.

This observation added to the fact that the dissociation rate can be further reduced at low temperature, enabled us to compare the solubilization yield of ligand-free and of [^{3}H]ketanserin-bound receptors. Table 2 shows that 10-times more specific [^{3}H]-ketanserin binding sites were measured in the soluble extract when the membrane-bound receptors were labelled by [^{3}H]ketanserin before solubilization. About 40% of the membrane-bound receptors were solubilized by lysolecithin as the [^{3}H]ketanserin receptor complex, whilst only 4% of the original receptors were still detectable in the soluble preparations under post-labelling conditions. The pre-labelled then solubilized [^{3}H]ketanserin–receptor complexes were found to specifically dissociate when an excess of methysergide (10^{-6} M) was added to the soluble extract. Therefore, [^{3}H]ketanserin binding to the solubilized receptor sites retains the reversibility property even after the pre-labelling procedure. Furthermore, comparison of the solubilization yields in both pre- and post-labelling conditions allows a distinction to be made between extracted receptors which are accessible to, or inaccessible to, the ligand. This may be due to the presence of inhibitors which

Table 2
Solubilization of specific [^{3}H]ketanserin binding sites under pre- and post-labelling conditions

	Specific [^{3}H]ketanserin binding (fmol/mg protein)			Solubilization yield (%)	Recovery (%)	
	Starting membranes	Soluble extract	Remaining pellet		Specific binding	Proteins
Pre-labelling						
washed membranes	192.1	172.9	45.8	39.2	51.5	95.1
Post-labelling						
unwashed membranes	235.8	18.0	75.0	4.0	18.6	98.9
washed membranes	190.0	16.1	36.6	3.4	15.3	101.7

Microsomal membranes (P-fraction) were prepared from rat cortex. Pre-labelling conditions: P-fraction (1/20, tissue wt/vol.) was incubated in phosphate buffer (10 mM NaH_2PO_4, 0.01% NaN_3, pH 7.2) for 15 min at 37°C with 10^{-8} M [^{3}H]ketanserin in presence or absence of 10^{-6} M methysergide. After cooling, the incubation medium was centrifuged at 178 000 × g (r_{av}) for 45 min at 4°C. The resulting pellets (washed membranes) were suspended in 2 vol. ice-cold water and immediately submitted to the solubilization procedure described in Methods. Post-labelling conditions: Washed and unwashed microsomal membranes were first treated with lysolecithin (section 2). The supernatant (soluble extract) and the remaining pellet obtained after centrifugation at 182 000 × g (r_{av}) for 1 h were then incubated with 10^{-9} M [^{3}H]ketanserin (section 2) or with 10^{-6} M [^{3}H]ketanserin (see pre-labelling conditions) in presence or absence of 10^{-6} M methysergide, respectively. Specific binding was defined as the difference between total binding and non-specific binding occurring in the presence of 10^{-6} M methysergide. At each step of both experimental procedures, [^{3}H]ketanserin binding was immediately measured by filtration on GF/B filters (membrane preparation) or by the charcoal method (solubilized extract)

would become unmasked by the detergent treatment. Another possibility is that some receptor subunits dissociate during solubilization; this phenomenon could be prevented by labelling the receptor prior to its extraction.

In the soluble extract from rat frontal cortex, there was a good correlation (table 3) between the IC_{50}-values of various compounds for [^{3}H]spiperone and [^{3}H]ketanserin binding; serotonin antagonists revealed a much higher potency than dopamine antagonists or agonists and an α_1-antagonist. In addition, these IC_{50}-values were in good agreement with those determined for [^{3}H]spiperone [3] and [^{3}H]-ketanserin [4] binding to S_2-receptors in membrane

Table 3
Inhibitory potencies of various compounds for specific [^{3}H]spiperone and [^{3}H]ketanserin binding in soluble extracts from rat and dog frontal cortex

	IC_{50} (nM)		
	[^{3}H]Spiperone Rat	[^{3}H]Ketanserin	
		Rat	Dog
Ketanserin	10	7.9	4.2
Spiperone	6.3	6.3	7.3
Pipamperone	11.8	15.3	7.5
Methysergide	25.1	18.1	10
(+)-Butaclamol	316	331	178
2-(*N*,*N*-dipropyl) Amino-5,6-dihydroxytetralin	158 000	44 700	19 900
Prazosin	>10 000	105 000	28 900

Solubilization and incubation conditions for [^{3}H]ketanserin binding (1 nM) were described in section 2. IC_{50}-values for [^{3}H]spiperone binding (1 nM) were from [5,6]

preparations. The apparent affinities of these compounds for [^{3}H]ketanserin binding were similar in soluble extracts from rat or dog frontal cortex, although a slight increase in the inhibitory potencies of these drugs was observed in dog extracts.

The foregoing results provide evidence that the binding sites labelled by [^{3}H]ketanserin in soluble extracts from rat and dog frontal cortex are serotonergic, thus confirming our data with [^{3}H]spiperone [5]. However, since spiperone labels also dopamine receptors in lysolecithin extracts from rat striatum [6], the use of ketanserin certainly allows more accurate determinations of solubilized serotonin receptors in brain regions rich in dopamine receptors. Moreover, this ligand does not seem to label chemical recognition sites of ketanserin structure as spiperone did for spirodecanone sites. Under certain conditions, these non-specific binding sites could totally mask specific [^{3}H]spiperone binding sites [5,10].

This study demonstrates that [^{3}H]ketanserin specifically labels serotonin receptors in solubilized extracts from rat and dog frontal cortex. The main advantages of ketanserin when compared to spiperone are: its higher selectivity towards serotonin-S_2 receptors, its lower non-specific binding and its slower dissociation which follows a first-order kinetics. The latter property is probably essential to get a 40% solubilization yield in receptors which were labelled with [^{3}H]ketanserin before their extraction. In the rat frontal cortex, [^{3}H]ketanserin and [^{3}H]spiperone label an identical and homogenous population of solubilized serotonin receptors.

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