CHARACTERIZATION OF SOLUBILIZED DOPAMINE RECEPTORS FROM DOG STRIATUM

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1. Introduction

It is now accepted that neuroleptic drugs act by blocking dopamine receptors. Direct evidence of such an interaction has been provided by in vitro binding studies using [^{3}H]haloperidol [1,2] or dopamine agonists [3,4] as ligands. Recently spiperone was reported to be a very appropriate ligand, not only for in vitro binding assays [5] but especially for in vivo binding experiments [6,7], because of its very high affinity and slow dissociation rate [5]. Although [^{3}H]spiperone binding was found to be of serotonergic nature in the frontal cortex [8], there is no doubt that it binds to dopaminergic receptors in the striatum.

Preliminary experiments seemed to indicate that [^{3}H]spiperone binding sites can be solubilized from rat striatum [9]. However, the macromolecular complex thus obtained only revealed a high affinity for spiperone but not for other dopamine antagonists.

We now report the binding characteristics of solubilized receptor sites from dog striatum retaining the high affinity of membrane preparations.

2. Materials and methods

Mongrel dogs were anaesthetized with pentobarbital and their brains were removed. Striata were dissected, homogenized in 0.25 M ice-cold sucrose, then fractionated as in [10]. The resulting microsomal fraction (P) was suspended in 2 vol. ice-cold water and kept at −16°C before solubilization.

2.1. *Solubilization procedure*

P fraction was treated with digitonin (Serva) 1% suspended in 4 vol. sucrose 0.25 M containing 10 mM sodium phosphate buffer (pH 7.2) and 0.01% NaN_3. After gently stirring at 0°C for 15 min, the mixture was centrifuged at 120 000 × g (r_{av}) for 60 min. The supernatant was carefully separated from the pellet and stored at 0°C. It was then considered as the solubilized preparation (~2 mg protein/ml).

2.2. *Binding procedure*

Soluble material was incubated at 0°C for 16 h with [^{3}H]spiperone 2×10^{-9} M (spec. act. 23.6 Ci.mmol^{-1}, NEN) and various concentrations of unlabelled drugs in 0.5 ml total vol. Stereospecific [^{3}H]spiperone binding was defined as the difference between the binding in the presence of 2×10^{-7} M (−)-butaclamol and 2×10^{-6} M (+)-butaclamol [5]. A 0.1 ml aliquot of the incubation mixture was layered on a Sephadex G-50 medium column (13 × 0.5 cm) and eluted at 2–3°C with sodium phosphate buffer 10 mM (pH 7.2) containing 0.01% NaN_3. Fractions (4 drop) were collected in scintillation vials and then counted for the radioactivity.

2.3. *Sucrose gradient centrifugation*

Solubilized preparation (0.3 ml) incubated with [^{3}H]spiperone was layered on a 15–30% sucrose gradient containing 0.01% NaN_3, 10 mM sodium phosphate buffer (pH 7.2) and 0.03% digitonin (5 ml total vol. in a polyallomer tube). Centrifugation was at 2°C in a SW65Ti Spinco rotor (Beckman) 60 000 rev./min for 7 h. An aliquot of each fraction thus obtained was counted for the radioactivity.

In a second method, a 1 ml aliquot of the solubilized material was layered on a continuous sucrose gradient (13 ml total vol.) and centrifuged at 40 000 rev./min for 16 h in a SW40Ti Spinco rotor.

After centrifugation, all the fractions were assayed for stereospecific binding as above but using a higher concentration of [^{3}H]spiperone (10^{-8} M) and (+)-butaclamol (10^{-5} M).

2.4. *Protein and enzymatic assays*

Protein was estimated by the Lowry method [11]. β-Galactosidase (β-G) from *Escherichia coli* (Aldrich), malate dehydrogenase (MDH) from pig heart, lactate dehydrogenase (LDH) from rabbit muscle and catalase (CTL) from bovine liver (Boehringer) were assayed by different procedures [12]. Alkaline phosphatase (ALP) was detected by the hydrolysis of *p*-nitrophenyl phosphate [13].

2.5. *Muscarinic receptor assay*

Fractions of the soluble preparations were incubated with [^{3}H]dexetimide (spec. act. 17 Ci.mmol^{-1}, IRE, Fleurus) in the nM range with a 100-fold higher concentration of unlabelled levetimide or dexetimide, then submitted to the gel-filtration procedure [12].

3. Results and discussion

Figure 1 shows the competition of various neuroleptic drugs in the [^{3}H]spiperone binding using a solubilized preparation of dog striatum. First (+)-butaclamol (IC_{50} = 2.0 × 10^{-8} M) inhibited the binding at a concentration 300-times lower than (−)-butaclamol (IC_{50} = 5.5 × 10^{-6} M) indicating a stereospecific binding ratio comparable to that found in membrane preparations from the rat [5,8] and dog (this work). Secondly, the 7 drugs tested here which are all dopamine antagonists from different chemical classes (butyrophenone, diphenylbutylamine, thioxanthene, phenothiazine, procainamide and benzoquinolizine [14]) have IC_{50} values corresponding to those found in membrane preparations, which are themselves in agreement with their pharmacological and clinical potency [15]. Further evidence of the dopaminergic nature of these soluble binding sites was provided by the result that dopamine agonists like (±)-2-(*N*,*N*-dipropylamino-5,6-dihydroxytetralin (IC_{50} = 4.8 × 10^{-7} M) and lysuride (IC_{50} = 1.3 × 10^{-8} M) also competed at very low concentrations. The tetraline derivative was demonstrated to be a very specific dopamine agonist [16] by displacing spiperone from the binding sites in the striatum but not in the frontal cortex [8].

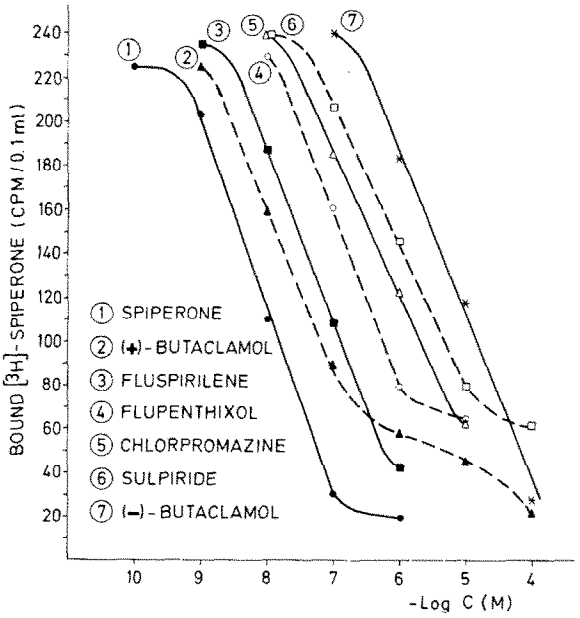

Fig.1. Competition of dopamine antagonists belonging to various classes of drugs with [^{3}H]spiperone in solubilized dog striatum preparation.

In contrast, many other drugs like anticholinergics, analgesics, β-blockers, minor tranquilizers and antiserotoninergics were without effect or very weak inhibitors of [^{3}H]spiperone binding in the soluble preparation.

As shown in fig.2, by increasing the concentrations of [^{3}H]spiperone, the stereospecific binding using solubilized preparation was found to be saturable in the 10^{-8} M range, whereas the non-specific binding (measured in the presence of 2 × 10^{-6} M (+)-butaclamol) increased linearly up to 25 nM. Scatchard analysis gave an app K_d ~ 4.8 nM, a value higher than that obtained with the corresponding membrane preparation (app K_d = 0.25 nM). Therefore as found for other receptors [17] the extraction by detergent leads to a decreased affinity, probably by means of conformation changes resulting from the loss of environmental lipids. The amount of active receptors in solution was ~18% when estimated from the Scatchard analysis (B_{max} soluble = 0.30 pmol/mg protein or 2.7 pmol/g tissue and B_{max} membrane = 0.77 pmol/mg protein or 15.4 pmol/g tissue).

The [^{3}H]spiperone stereospecific binding was reversible (fig.3). Association in the presence of

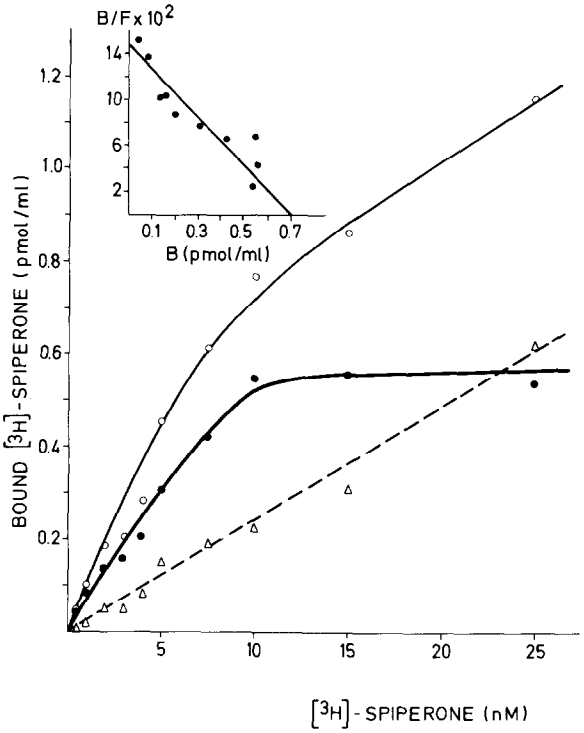

Fig.2. Binding curves at 0°C using a solubilized preparation with increasing concentrations of [^{3}H]spiperone: (–●–●–) stereospecific binding; (○—○) total binding and (△- -△) non-specific binding. The inset shows the Scatchard analysis.

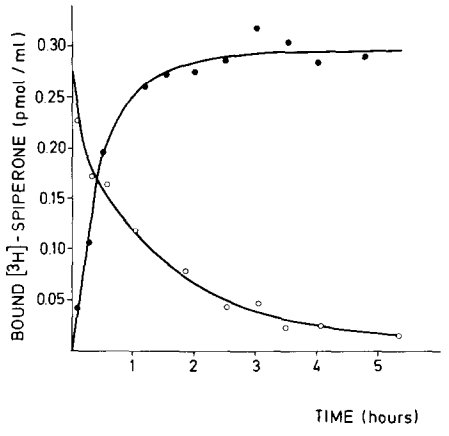

Fig.3. Association curve (–●–●–) for the stereospecific [^{3}H]spiperone binding in the presence of 2×10^{-9} M labelled drug and dissociation curve (○—○) in the presence of a 1000-fold excess of unlabelled drug at 5°C.

2×10^{-9} M [^{3}H]spiperone was faster ($t_{1/2}$ ~20 min at 5°C) than dissociation in the presence of a 1000-fold excess of unlabelled drug ($t_{1/2}$ = 49 min at 5°C). The release of [^{3}H]spiperone showed a first order kinetic profile ($k_{diss} = 2.4 \times 10^{-4}$ s^{-1}) and was not composed of two straight lines (log C drug-receptor complex versus time) as in membrane preparations of rat striatum [5].

Figure 4 shows that heat denaturation of dopamine receptors was strongly increased after digitonin extraction as compared to membrane preparations. Such a phenomenon was also reported for brain muscarinic receptors [12]. In addition, cerebellum extracts did not reveal heat inactivation indicating that only non-specific binding sites were detected in this brain region.

The foregoing results clearly show that although some differences exist between soluble and membrane preparations with respect to their kinetics and the thermal inactivation, this does not contradict the existence of active dopamine receptors in the soluble state.

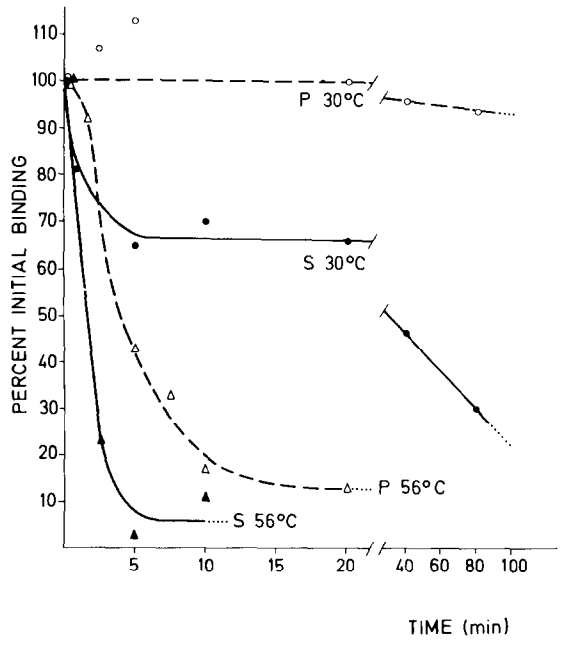

Fig.4. Comparison of the thermal sensitivity at 30 and 56°C for (——) solubilized (S) and (- - - -) membrane (P) preparations from dog striatum.

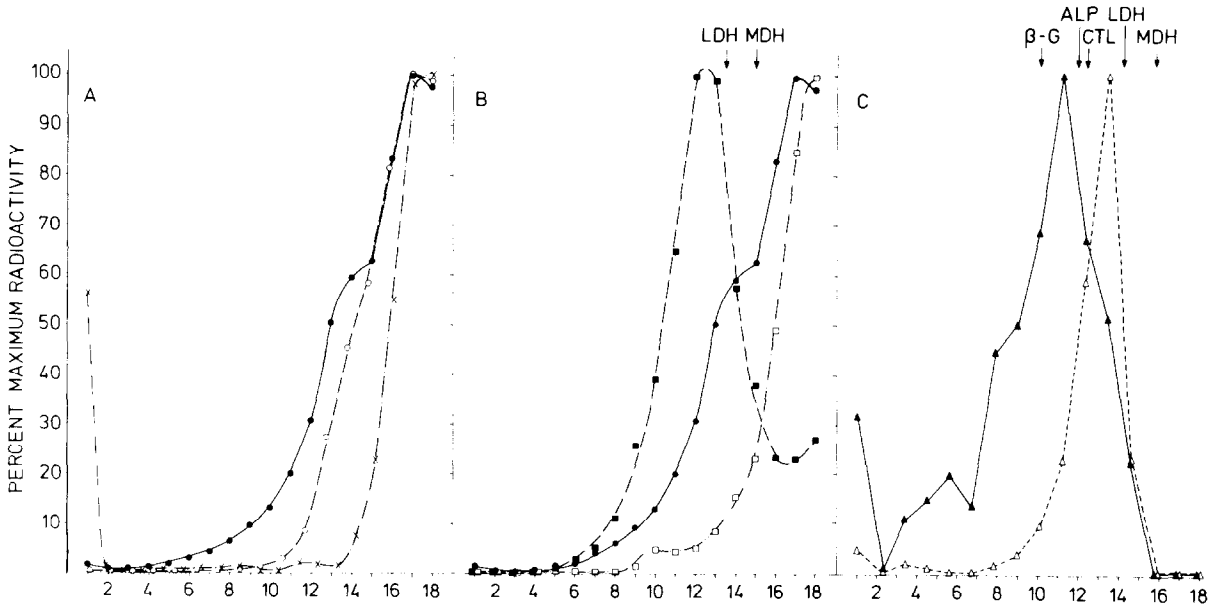

Fig.5(A) Sedimentation profiles of [^{3}H]spiperone in 15–30% sucrose gradient after centrifugation at 260 000 × g (r_{av}) for 7 h with a SW65Ti rotor using a solubilized preparation incubated with 2 × 10^{-9} M of the ligand in the absence (–●–●–) and presence (○–○) of 2 × 10^{-6} M (+)-butaclamol. Control experiment with a membrane preparation (–×–×–) incubated with 2 × 10^{-9} M [^{3}H]spiperone. 100% represents >2000 dpm/0.2 ml. 5(B) Sedimentation profile of [^{3}H]spiperone (–●–●) compared to those of [^{3}H]dexetimide (2 × 10^{-9} M) incubated with a solubilized preparation in the presence of a 100-fold excess unlabelled levetimide (■–■) and dexetimide (□–□). 100% represents >1200 dpm/0.2 ml. 5(C) Sedimentation profiles of the stereospecific binding determined after centrifugation at 200 000 g (r_{av}) for 16 h with a SW40Ti rotor. Fractions were incubated with 10^{-8} M [^{3}H]spiperone (▲—▲) without and with a 1000-fold excess of (+)-butaclamol or with 10^{-8} M [^{3}H]dexetimide (△- -△) in the presence of 10^{-6} M levetimide and dexetimide. 100% represents for the [^{3}H]spiperone distribution ~250 dpm/0.1 ml and for the [^{3}H]dexetimide distribution about 4000 dpm/0.1 ml. Marker enzymes: malate dehydrogenase (MDH), lactate dehydrogenase (LDH), catalase (CTL), alkaline phosphatase (ALP) and β-galactosidase (β-G).

Throughout this work, special attention was paid to the criteria of solubilization. One of those consists in the sedimentation properties of solubilized binding sites through sucrose gradient.

Figure 5A shows that different sedimentation profiles were obtained using either solubilized or membrane preparations; indeed the binding sites associated with the membranes were only detected in the bottom of the tube (fraction 1) while the labelling of soluble material appeared as a shoulder, at a distance ~1/3rd from the top of the tube (fractions 11–15). Such a shoulder profile for the solubilized binding sites which was quite reproducible (4 independent runs from different dog striata) disappeared when a 1000-fold excess of (+)-butaclamol was added to the incubation mixture (fig.5A).

When compared to the sedimentation profile of the muscarinic receptor (9 S) which is the same in rat [12] and dog brain, the solubilized binding sites labelled by [^{3}H]spiperone had apparently a lower S value, thus indicating a smaller molecular size (fig.5B). However, in contrast to [^{3}H]dexetimide which very firmly labels muscarinic receptor [12], [^{3}H]spiperone was found to dissociate from the macromolecular complex during the centrifugation; therefore fig.5C shows that the real migration of soluble dopamine receptors was only detectable when the binding assay was performed after the centrifugation run. The dopaminergic sites possessed a mean S value which was higher than catalase [11.3 S) and lower than β-galactosidase (16 S) and may be definitively considered as a larger molecular entity than the mus-

carinic receptor (~9 S) [12]. Comparison of the distribution profiles of both solubilized receptors from dog striatum also suggests that dopamine sites were markedly more heterogeneous than the muscarinic receptor sites. Alkaline phosphatase (ALP) a marker enzyme of the microsomal fraction [18] also extracted from the plasma membrane behaved in the gradient as a different entity. Indeed this phosphatase enzyme (mol. wt ~190 000 [19]) appeared between the muscarinic and dopamine receptors approximately where catalase would appear. Such results further substantiate the molecular dispersal [20] of enzymes and binding sites extracted from the same kind of membranes.

Besides the other criteria of solubilization the non-sedimentation of the soluble fraction at 260 000 × g (r_{av}) centrifugation for 7 h and the absence of lamellar structures [21] in electron microscopic examination [22] allow the molecular dispersal of the soluble digitonin extract to be assessed.

The foregoing results demonstrate that dopamine receptors from dog striatum solubilized using digitonin. The [^{3}H]spiperone binding was found to be very stereospecific in competition experiments using butaclamol enantiometers. Moreover, these binding sites retained their relative affinity for the drugs belonging to the principal classes of dopamine antagonists and also for true dopaminergic agonists like lysuride and (±)-2-(*N*,*N*-dipropyl)amino-5, 6-dihydroxytetralin. Furthermore, the good correlation of the inhibition of [^{3}H]spiperone binding between membrane and soluble preparation may be extended to a pharmacological test, such as the antagonism of apomorphine-induced emesis in dog (in preparation).

The soluble binding sites may associate with and dissociate from the ligand and are saturable with app K_d in accordance with a high affinity. They are also very specific for striatum because extraction from other brain areas like cerebellum and frontal cortex (in preparation) did not allow the detection of specific dopaminergic activity.

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