CHARACTERIZATION OF DIGITONIN-SOLUBILIZED MUSCARINIC RECEPTOR FROM RAT BRAIN

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1. Introduction

The presence of muscarinic receptors in mammalian brain has been extensively demonstrated by binding experiments using various radioactive ligands (cf. [1]). Amongst those, [^{3}H]dexetimide appeared to be a very appropriate ligand because of its pronounced stereospecificity, its very high affinity and its slow dissociation properties [2–4]. The solubilization of muscarinic receptors from mammalian brain has been successfully achieved by treating tissue with high salt concentration (NaCl, 2 N) [5–7] or with the natural detergent digitonin [2]. In these studies, the use of [^{3}H]propylbenzilycholine mustard, [^{3}H]atropine and [^{3}H]dexetimide as ligands, with the aid of equilibrium dialysis and nitrate cellulose filtration techniques made it possible to isolate the ligand–receptor complex. Although various biochemical properties have been determined for salt and detergent extracts a characterization of the muscarinic receptor by means of sedimentation centrifugation has never been described.

We now report some biochemical and pharmacological properties of a digitonin-solubilized complex from rat brain which was isolated by means of an improved gel-filtration technique [8,9] and sedimentation gradient using either [^{3}H]dexetimide or [^{3}H]-levetimide as ligands. The receptor protein revealed a single peak (9 S) after centrifugation and was identical to the membrane preparation in its affinity for various drugs.

2. Materials and methods

Male and female Wistar rats (± 250 g) were decapitated and the brains were immediately removed. The forebrain was rapidly dissected and placed in ice-cold sucrose (0.25 M). The tissue was then homogenized and fractionated as in [10]. The resulting microsomal fraction (P) was suspended in 1 vol. ice-cold water and kept at −16°C.

2.1. *Solubilization procedure*

Digitonin (Serva) suspension, 1 vol 2%, in phosphate buffer (NaH_2PO_4–Na_2 HPO_4, 20 mM, pH 7.2) containing 0.02% NaN_3, was added to aliquots of the P fraction. The mixture was gently agitated for 15 min and centrifuged at 120 000 × *g* for 1 h. The supernatant, henceforth called the solubilized preparation (about 2 mg protein/ml), was carefully removed using a Pasteur pipette without disturbing the pellet. The whole procedure was carried out at 0°C.

2.2. *Binding procedure*

Solubilized preparations were incubated with [^{3}H]-dexetimide (spec. act. 17 Ci/mmol, IRE, Fleurus) in the nM range and various concentrations of unlabelled drug in total vol. 0.5 ml. Specific [^{3}H]dexetimide binding was defined as the difference between the binding in the presence of levetimide (L-benzetimide) and dexetimide (D-benzetimide) at concentrations which were 100-times higher than the labelled ligand. In some cases [^{3}H]levetimide (spec. act. 19 Ci/mmol, IRE) was also used as radioactive ligand. A 0.1 ml aliquot of the incubation mixture was placed on the top of a Sephadex G-50 Medium (Pharmacia) column (13 × 0.5 cm) [8,9] which was carefully cooled to 2–3°C. The elution was carried out using a constant rate pump system (13 drops/min) with cold phosphate buffer, 10 mM, pH 7.2, containing 0.1% digitonin.

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Four-drop fractions were collected in counting vials and the radioactivity was measured in a Packard liquid scintillation spectrometer.

2.3. *Sucrose gradient centrifugation*

After incubation with [^{3}H]dexetimide 0.2 ml soluble extract (~2 mg protein/ml) was layered on the top of a gradient of 5–20% sucrose buffered with 10 mM sodium phosphate, pH 7.2, and supplemented by 0.03% digitonin. It was then centrifuged at 2–3°C in a SW 65 Ti rotor (Spinco, Beckman) at 40 000 rev./min for 16 h.

2.4. *Protein and enzymatic assays*

Protein was estimated by the method in [11]. Dopa decarboxylase (DDC) and dopamine-β-hydroxylase (DβH) from bovine adrenal medulla were estimated as in [12]. β-Galactosidase (βG) from *E. coli* (Aldrich), malate dehydrogenase (MDH) from pig heart, lactate dehydrogenase (LDH) from rabbit muscle and catalase (CTL) from bovine liver (Boehringer) were assayed according to different procedures [13–16]. Finally the acetylcholinesterase activity was detected in rat brain extracts by hydrolysis of *S*-acetylthiocholine [17].

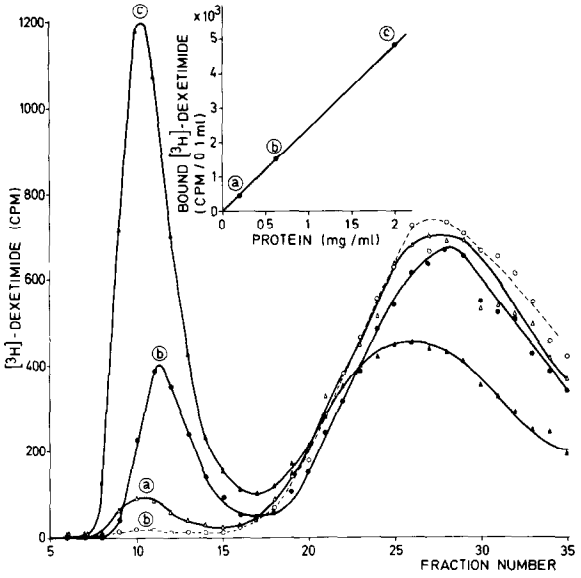

Fig.1. Gel-filtration elution patterns of solubilized preparations at various protein concentrations (a, b, c) after incubation with [^{3}H]dexetimide (10^{-8} M) in the presence of 10^{-6} M levetimide (——) or dexetimide (- - -). The inset shows the linear relationship of bound [^{3}H]dexetimide with increasing protein concentration.

3. Results and discussion

As shown in fig.1 a good separation between the [^{3}H]dexetimide bound macromolecular complex (first peak) and the free radioactive drug (second peak) was obtained with the gel-filtration procedure. After incubation in the presence of unlabelled drug, the first peak disappeared entirely, indicating that the [^{3}H]-dexetimide binding is displaceable. Moreover the bound [^{3}H]dexetimide was not affected by incubation with 10^{-5} M neostigmine bromide, a powerful inhibitor of acetylcholinesterase. By comparing the number of binding sites in the microsomal fraction and in the solubilized preparation, the extraction yield was found to vary from 5–12%.

The [^{3}H]dexetimide binding to solubilized preparations was stereospecific (fig.2A) as has been found for membrane preparations [2,4]. Indeed the displacement curve obtained for the active enantiomer of benzetimide (dexetimide, IC_{50} 1.4×10^{-9} M) markedly differed from that with the inactive enantiomer (levetimide, IC_{50} 5.6×10^{-6} M). This 4000-fold difference between optical isomers is quite compatible with that obtained in membrane preparations. Further evidence of stereospecificity was produced using [^{3}H]-levetimide. First the binding value obtained with the inactive ligand was markedly lower than that with the active ligand. Secondly the displacement curves for both enantiomers of benzetimide were similar and very flattened (fig.2B), indicating the non-specific nature of this binding.

In addition, a good correlation was obtained between the IC_{50} values of various muscarinic agonists and antagonists, in the soluble and membrane rat brain preparations (table 1). The competition curves of agonists with the digitonin-solubilized preparation were monophasic as was also observed for the salt-soluble extract [7]. From the foregoing experiments it is clear that the solubilized [^{3}H]dexetimide–receptor complex was undoubtedly of a muscarinic nature, because all the drugs tested revealed the same relative affinity in solubilized or membrane preparations [4] and in the pharmacological test [18].

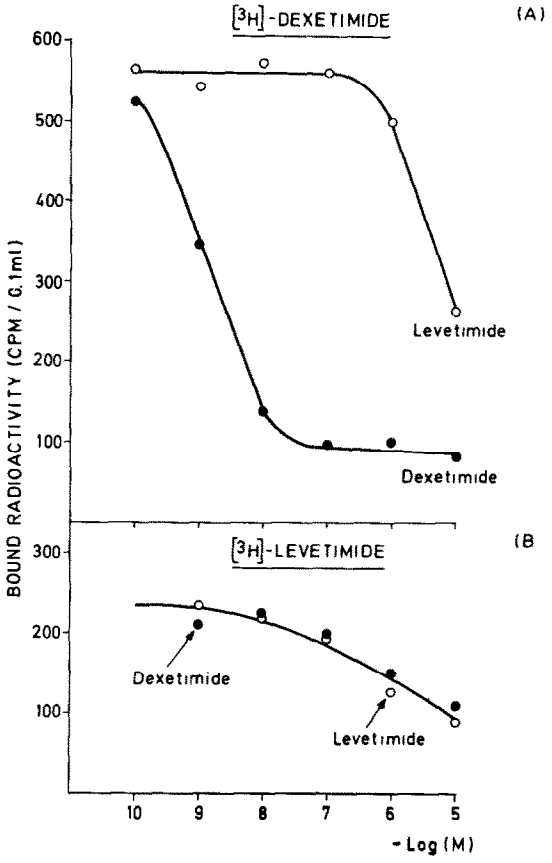

Fig.2. Inhibition curves of both enantiomers of benzetimide using (A) [^{3}H]dexetimide (2×10^{-9} M) and (B) [^{3}H]levetimide (2×10^{-8} M) in solubilized preparations.

Figure 3 shows that the [^{3}H]dexetimide binding is saturable in the same range as the membrane preparation [4]. The Scatchard analysis gave a linear relationship indicating a single class of binding site. Further the dissociation constant ($K_d = 5 \times 10^{-10}$ M) obtained using solubilized preparation is closely related to that measured with the corresponding membrane preparation ($K_d = 6.5 \times 10^{-10}$ M).

Interestingly, the soluble preparation displayed a much higher sensitivity to thermal inactivation than the membrane preparation [4]. Indeed after 5 min

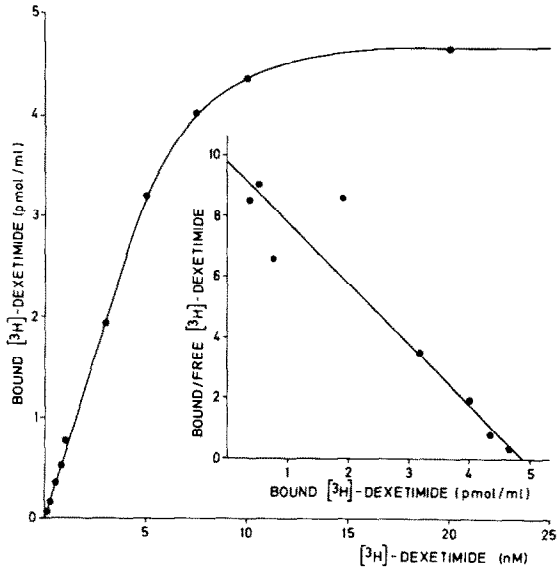

Fig.3. Saturation curve and Scatchard plot of the [^{3}H]dexetimide binding in a solubilized preparation.

Table 1
Comparison between the IC_{50} values of [^{3}H]dexetimide (2×10^{-9} M) binding using soluble and membrane [3,4] preparations and the ED_{50} values of antisecretory activity in the pilocarpine-test in rats [18]

	Binding IC_{50} (M)		Pharmacological activity ED_{50} (mg/kg)
	Soluble	Membrane	
Dexetimide	1.4×10^{-9}	2.8×10^{-9}	0.02
Atropine	2.8×10^{-9}	6.8×10^{-9}	0.12
Trihexyphenidyl	7.9×10^{-8}	2.1×10^{-8}	0.50
Levetimide	5.6×10^{-6}	6.5×10^{-6}	> 2.5
Oxotremorine	5.6×10^{-6}	6.5×10^{-6}	–
Carbamylcholine	3.5×10^{-5}	3.2×10^{-4}	–

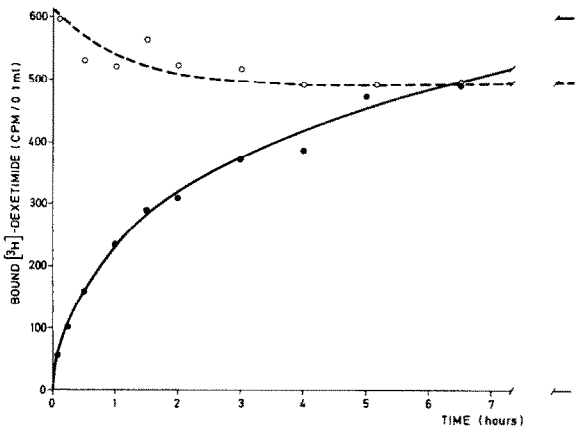

Fig.4. Time course of [^{3}H]dexetimide binding (——) at 0°C in a solubilized preparation and its dissociation (- - -) in the presence of a 100-fold excess of unlabelled dexetimide (2×10^{-7} M).

incubation at 56°C, only 17% of the original binding capacity was found whereas under the same conditions, 95% was still present in the membrane preparation.

The time course of association at 0°C (fig.4) indicated that 83% of the maximal binding value was reached after incubation at 0°C for 7 h, but the dissociation rate in the presence of a 100-fold greater concentration of unlabelled drug was noteworthy slow. Under these experimental conditions the binding of [^{3}H]dexetimide with soluble muscarinic receptor revealed an extremely slow dissociation rate, a property which makes this ligand very appropriate for further ultracentrifugation experiments.

Figure 5A shows that when a solubilized preparation was incubated with [^{3}H]dexetimide and then submitted to sedimentation through a sucrose gradient a high peak of radioactivity appeared approximately in the middle of the tube, indicating the presence of a [^{3}H]dexetimide-bound macromolecular complex. In contrast to this the radioactivity remained at the top when the incubation was performed in the presence of an excess of unlabelled dexetimide. A different distribution pattern (fig.5B) was obtained for the acetylcholinesterase, indicating that [^{3}H]dexetimide macromolecular complex was not associated with the enzyme. Moreover the enzyme activity was completely inhibited by 5×10^{-6} M neostigmine bromide without affecting the [^{3}H]dexetimide binding.

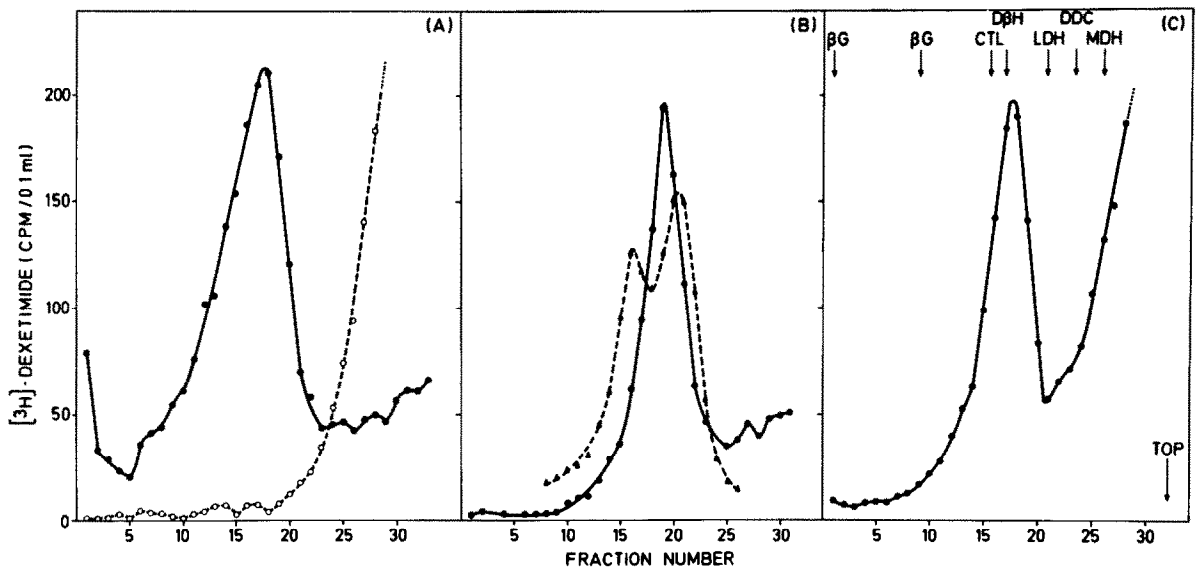

Fig.5. (A) Distribution profile of [^{3}H]dexetimide after centrifugation of a solubilized preparation incubated with 2×10^{-9} M of the ligand in the presence of 2×10^{-7} M (-●-●-) levetimide and (-○-○-) dexetimide. (B) Sedimentation of bound [^{3}H]dexetimide (-●-●-●-) compared to that of acetylcholinesterase (-▲-▲-). (C) Sedimentation profile of the soluble [^{3}H]dexetimide macromolecular complex and various marker enzymes.

In order to estimate the sedimentation coefficient of the [^{3}H]dexetimide–receptor complex, the distribution profile of various marker enzymes was determined after centrifugation in a sucrose gradient. Figure 5C shows that the [^{3}H]dexetimide–receptor complex sedimented in an area located between DβH and LDH. From the known sedimentation coefficient of the different marker enzymes [19–23], one can estimate that the sedimentation coefficient of the muscarinic receptor labelled with [^{3}H]dexetimide is ~9 S, a value which is in agreement with ~200 000 mol. wt. A more precise measure cannot yet be made because, as emphasized [19–21,24,25], macromolecular complexes extracted from membranes were often asymmetric structures and were also associated with large amounts of lipids, two parameters affecting the physico-chemical and particularly the hydrodynamic properties of biomacromolecules. Furthermore, the 9 S digitonin-solubilized muscarinic receptor appeared to have a more complex structure than that obtained by the NaCl 2 N extraction (30 000 mol. wt) [5,6].

Therefore, the present experiments show that [^{3}H]-dexetimide is a particularly appropriate ligand for the isolation of muscarinic receptors. Indeed its properties enabled us to characterize by means of gel-filtration as well as by sedimentation centrifugation a digitonin-solubilized macromolecular complex which is endowed with all the characteristics found in the original membrane preparation.

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