PARTIAL PURIFICATION AND CHARACTERIZATION OF A MUSCARINIC ACETYLCHOLINE RECEPTOR FROM RAT CEREBRAL CORTEX

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Received May 1,1974

Summary: Atropine binding protein has been partially solubilized from synaptosomal membrane fractions of rat cerebral cortex. Muscarine but not tubocurarine interferes with atropine binding. Gel filtration experiments revealed the existence of two proteins (MW $\sim$ 33,000 and $\sim$ 70,000, respectively) with atropine binding activity. Both proteins have isoelectric points between $\sim$ 4.8-5.0. A rapid filtration method has been devised for measurement of atropine binding.

Acetylcholine (ACh) exerts its physiological function through specific binding to receptor proteins localized in synaptic membranes (1). Two types of acetylcholine receptors (AChR) are known (2): the nicotinic AChR (nAChR) and the muscarinic AChR (mAChR). The former is subject to stimulation by nicotine and blocade by curare, whereas the latter is stimulated by muscarine and inhibited by atropine. The common approaches in studies of receptors have so far been: i.) measuring physiological responses brought about by binding of ligand to the receptor (e.g. muscle contraction (3), change of membrane permeability (4)), and ii.) isolation of the receptor molecule for direct biochemical studies (5-8). Both approaches have been successfully applied in the study of the nAChR from electroplax of electrical fishes (6-8) and from guinea pig brain (9). However, the mAChR has been studied mostly at the physiological (10,11) and not at the molecular level. The first attempts to extract mAChR from smooth muscle membranes was made by Takagi et al. (12) and by De Robertis and Fiszer (13).

The neurophysiological studies by Krnjević and Phillis (10), by Curtis and Ryal (11) as well as the recent autoradiographic studies by Hiley and Burgen (14) have shown that the neurons of mammalian cerebral cortex possess mAChR.

The main problems to be solved by a successful purification procedure are: to release the mAChR from the synaptic membrane to which it is bound similarly to the acetylcholine esterase (AChE, EC 3.1.1.7) and nAChR; and to separate mAChR from AChE. Contamination with AChE presents difficulties in binding studies on mAChR with cholinergic ligands since AChE binds many of these ligands (e.g. atropine (15)). This communication describes an attempt to isolate and characterize mAChR from rat cerebral cortex.

Materials and Methods

[^{3}H]-Atropine (289 mCi/mmole) was purchased from The Radiochemical Centre, Amersham. Phospholipase A_2 was purified from venom of Naja nigricollis by Dr. L. Wahlström, University of Uppsala.

Measurement of binding of [^{3}H]-atropine: Samples containing 0.5-1.0 mg/ml protein were incubated for 1 hour with 3 ml Ringer solution (116 mM NaCl, 2.1 mM KCl, 1.8 mM $CaCl_2$, 3 mM Na_2HPO_4, pH = 7.2) which contained either 10^{-7} or 10^{-8} M [^{3}H]-atropine. A Sartorius membrane filter (pore size: 0.45 µM) was washed with 15 ml ice-cold distilled H_2O after which the sample was rapidly filtered. The filter was rinsed with an additional 15 ml H_2O and then placed in 10 ml Brays solution and counted in a Beckman scintillation spectrometer. Binding was also measured by equilibrium dialysis against 10^{-6}, 10^{-7}, and 10^{-8} M [^{3}H]-atropine as described by Farrow and O'Brien (16).

Preparation of synaptosomal membranes: Synaptosomes were prepared according to Whittaker (16). Male rats (Sprague Dawley) were decapitated and the cerebral cortices homogenized in 10 vol. ice-cold 0.32 M sucrose. The homogenate was centrifuged at 1,000 g for 10 min (Servall rotor SS34) and the supernatant was subjected to a further centrifugation (10,000 g, 10 min Servall SS34 rotor). The resulting crude mitochondrial pellet was resuspended in 0.32 M sucrose, layered on a discontinuous sucrose gradient (0.6, 0.8, 1.0, 1.2, 1.4 M sucrose), and centrifuged in SW-40 rotor for 2 hours at 50,000 g. The fractions obtained were subjected to solubilization experiments.

Protein was determined according to Kalckar (18). Acetylcholinesterase

and ATPase were assayed as described by Ellman et al. (19) and Asami et al. (20), respectively.

Treatment of the crude mitochondrial pellet with purified phospholipase-A_2 was carried out by incubating 3 ml of the resuspended crude mitochondrial pellet (0.1 M phosphate buffer, pH = 7.4 ∿ 10 mg/ml protein) with 0.5 mg enzyme. The reaction was stopped after 15 minutes by adding 20 mg/ml EDTA. The resulting mixture was layered on a discontinuous sucrose gradient containing 5 mg/ml EDTA and centrifuged at 50,000 g for 2 hours.

Results:

Fig. 1 shows the relationship between bound [^{3}H]-atropine and protein

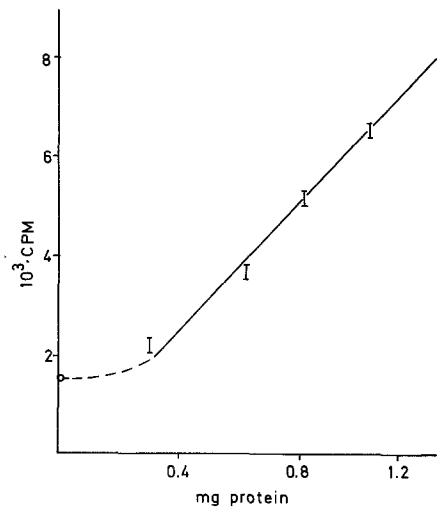

Fig. 1. Filter assay of [^{3}H]-atropine binding. Dependence of bound atropine on the amount protein used.

content of the sample. In the range of 0.5-1 mg/ml protein there is a linear dependence of binding on the amount of protein. The filtration method was preferred to equilibrium dialysis because of its speed and good reproducibility (5 %). The results obtained with equilibrium dialysis showed a good agreement with those of the filtration technique.

The fractions obtained by subcellular fractionation of the cerebral

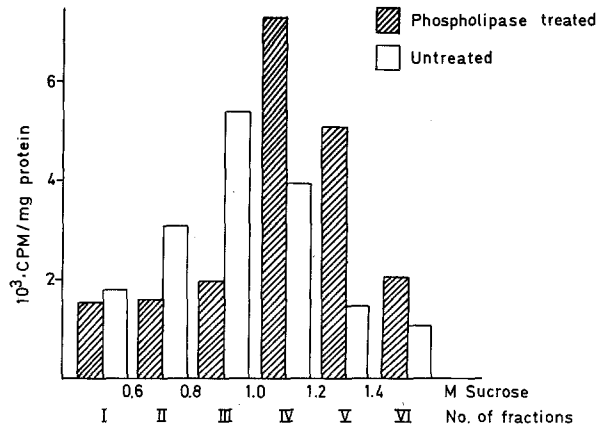

Fig. 2. Distribution of specific activity of mAChR on the discontinuous gradient when a) crude mitochondrial pellet □ and b) phospholipase A_2 treated crude mitochondrial pellet ▨ was layered on the sucrose gradient

Table 1. Purification of mAChR

Fraction	Specific binding capacity 10^3 cpm/mg	Remaining activity % mAChR	AChE	ATPase
Homogenate	0.22	100	100	100
Crude mitochondrial pellet	0.91	21.0	29.2	23.2
Fraction III of sucrose gradient	1.54	2.6	4.17	4.0
2M NaCl supernatant (after use of 0.5 M NaCl)	11.9	0.18	0.04	0.12

cortex were analyzed by means of marker enzyme studies (Na^+, K^+ ATPase, AChE, and LDH) as well as by electron microscopy. The results (Fig. 2, Table 1) show that mAChR occurs mostly in synaptosomal membrane fractions. This observation is in excellent agreement with the findings of Farrow and O'Brien (16). Fraction III of the sucrose gradient was richest in mAChR and was subjected to subsequent attempts to solubilize the receptor.

Solubilization by salts: Release of the mAChR occurs at NaCl concentrations

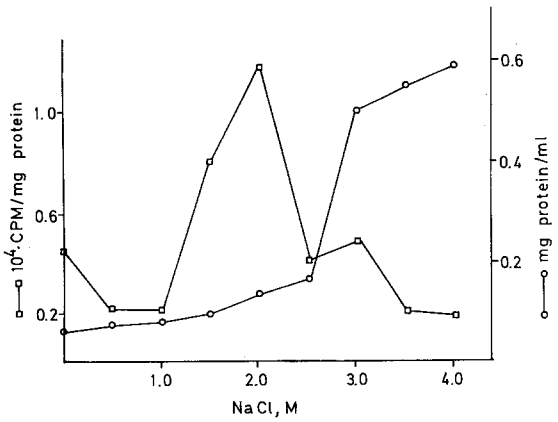

Fig. 3. Release of protein from synaptosomal membrane fraction (III) at various concentrations of NaCl. -O- O- Specific binding activity of the supernatant as a function of NaCl concentration applied for solubilization. 2 ml of fraction III has been diluted to 12 ml and incubated with various amounts of NaCl for 2 hours, 4°C. The mixture was then centrifuged at 100,000xg for 60 min and the binding capacity of the supernatant was determined.

higher than 1.5 M (cf. Fig. 3). As was shown earlier (21) AChE is released at relatively low salt concentration. We made use of this difference between mAChR and AChE and separated them by applying 1.0 M NaCl and then 2 M NaCl in a two step elution procedure. Urea in 1, 2, and 4 M concentrations was either unsuccessful in releasing mAChR or denatured the protein. The salts were removed by dialysis before binding was measured.

Solubilization by detergents: Two nonionic detergents, Triton-X 100 and Tween 20, were tested. Fig. 4 shows the effect of increasing Triton concentrations. Triton was removed from the solution with 1 g/ml Bio-Beads SM-2 (Bio-Rad Lab., Richmond, Calif.) (22) and checked at 275 nm. However, the detergent remaining bound to the membrane is inhibitory for the atropine binding. Similar results were obtained with Tween. A series of experiments was performed by varying the amount of protein at constant (0.5 and 1 % v/w) levels of Triton without producing an increase of the release of mAChR.

Treatment with phospholipase A: Application of phospholipase A_2 (free from proteolytic activities) on the crude mitochondrial pellet results in a shift of the peak of mAChR activity to higher density in the sucrose gradient. The

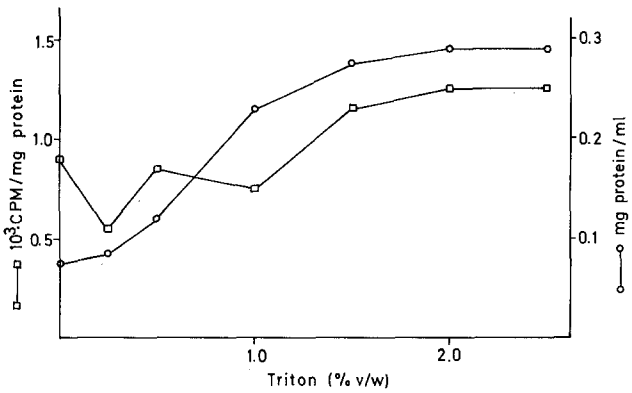

Fig. 4. Release of protein and specific activity of supernatant as a function of Triton X-100 concentration. The incubation mixture used contained 2 ml of fraction III and various amounts 5 % v/w Triton X-100 and Ringer solution to 12 ml final volume. The mixture was centrifuged at 100,000xg for 60 min and the binding capacity of the supernatant was determined.

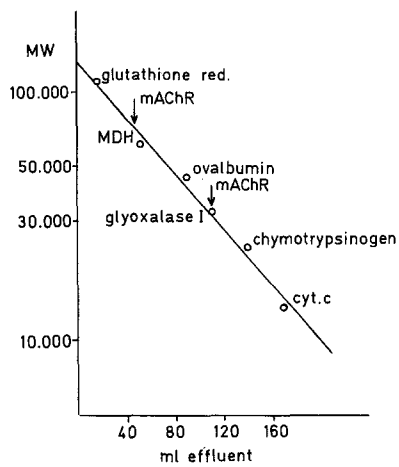

Fig. 5. Gel filtration of mAChR solubilized by Triton X-100 on Sephadex G-100 gel. A column with 920 mm length and 15 mm Ø was used. The eluting buffer 50 mM phosphate pH = 7.2. Since one of the mAChR activities was eluted at the same effluent volume as glyoxalase I from yeast it was necessary to check whether glyoxalase itself binds atropine. This test gave negative result.

new pattern shows an enrichment of the receptor in fraction IV instead of fraction III as shown in Fig. 2.

The result of the molecular weight determination is depicted on Fig. 5. Two species are found with atropine-binding ability, one at ∿ 33,000 and the other about 70,000 MW. The results of determination of the isoelectric point

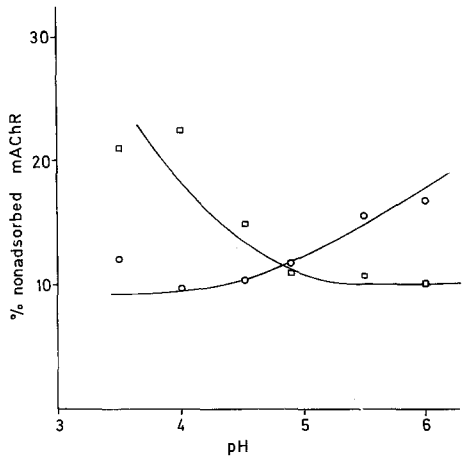

Fig. 6. Adsorption of mAChR on CM and DEAE Whatmann cellulose of various pH values. 3 ml gel was equilibrated either with 10 mM Na acetate or with 10 mM Tris/Cl buffer to give the desired pH. 10 ml buffer was used to wash out the non adsorbed mAChR.

by adsorption studies on ion exchangers equilibrated at different pH values are shown in Fig. 6. For both species the isoelectric point was between 4.8-5.0.

The specificity of the binding was checked by adding muscarine, tubocurarine, and physostigmine in 10^{-5}, 10^{-6}, and 10^{-7} M concentrations to the incubation mixture containing [^{3}H]-atropine and the mAChR fraction. Muscarine interferred with the atropine binding whereas tubocurarine and physostigmine had no effect. Acetylcholine could not be tested directly since the preparation was contaminated with AChE. Binding of atropine to the purified AChE was about 100 times less than to the fractions containing mAChR as calculated on the basis of bound atropine/mg protein.

Discussion:

The mAChR, or at least a subunit of it, is located on the surface of the synaptic (probably postsynaptic) membrane and can be released partially by a high salt concentration, similarly to AChE. The Cl^{-} ions applied exert a chaotropic effect.

It has been shown that the nAChR is part of a proteolipid complex (23).

The present results suggest that also the mAChR binds detergents very tightly. These could not be removed by dialysis nor by Bio-Beads SM-2 adsorption, and inhibited the binding of atropine to the receptor. Triton itself is also able to bind atropine.

Morphological studies by Cedergren et al. (24) have shown that phospholipase A_2 leaves the synaptic regions intact. The present results suggest that the binding ability was also retained after phospholipase A_2 treatment.

The observation that the mAChR peak is shifted to higher densities upon phospholipase A_2 treatment can be explained by the non-uniform distribution of the receptor in the plasma membrane and synaptic membrane parts of the synaptosomes. The mAChR seems to occur in the denser synaptic regions.

The two species of $\sim$ 33,000 and 70,000 molecular weight, respectively, found by gel filtration experiments might represent monomer and dimer forms of the receptor. The molecular weight of 70,000 also could fit with a monomer of the tetrameric AChE (25) which has a molecular weight of $\sim$ 260,000. No hydrolytic activity of the 70,000 MW species could be detected with acetylthiocholine as substrate. However, the monomers of an oligomeric enzyme need not possess enzymatic activity. The appearance of atropine binding capacity in the lightest fraction of the sucrose gradient might represent either extracellular or easily washed out receptor.

For further characterization of the mAChR it will be necessary to include additional purification steps such as ion exchange chromatography and affinity chromatography on columns prepared with cholinergic ligands.

Acknowledgements

The authors want to express their gratitude to Dr. Edith Heilbronn for helpful discussions. We would like to thank Dr. Bengt Mannervik and Professor Lars Ernster for their continuous interest and help. We thank Mrs. Cecilia Roos (FOA) for the electronmicroscopic studies.

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