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Is the acetylcholine releasing protein mediatophore present in rat brain?

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Mediatophore is a protein purified from the nerve terminal membranes of *Torpedo* electric organ. It confers to artificial membranes a calcium-dependent mechanism that translocates acetylcholine. When similar reconstitution experiments are applied to rat brain synaptosomal membranes they reveal the presence of mediatophore activity with properties close to those described for the *Torpedo* protein (extractability, sensitivity to calcium, and effect of the drug cetiedil). The acitivity was more abundant in synaptosomal membranes than in mitochondrial or myelinic membranes and in cholinergic areas as compared to cerebellum.

Acetylcholine release; Synaptosome; Proteoliposome; Proteolipid; (Rat brain)

1. INTRODUCTION

Mediatophore is a protein that has been purified from the nerve terminal plasma membrane of *Torpedo* electric organ. When this protein is inserted in the artificial membrane of liposomes containing acetylcholine (ACh), they become able to release the transmitter in response to a calcium influx. The rate of ACh release was found to be proportional to the amount of mediatophore incorporated into the membrane; a functional assay of the protein was consequently developed and used to purify it. The mediatophore has a molecular mass of about 200 kDa as deduced from its Stokes radius and sedimentation coefficient. This doughnut-shaped molecule is built up by the association of 15 kDa subunits; no other subunit has been identified until now [1]. More recently it has been found that the ACh translocating properties of the mediatophore were blocked by the drug cetiedil that inhibits the release of ACh from stimulated synaptosomes or electric organ [2]. A polyclonal antibody against *Torpedo* mediatophore was recently prepared and used to study the localization of the protein in the neurites of motor end-plates of *Torpedo* muscles [3]. This antibody did not cross-react with rat proteins and we could not easily answer the question often raised as to whether or not mediatophore is found in mammalian nerve terminals. Without the help of the antibody we decided to apply to mammalian brain fractions the mediatophore extraction protocol and find out if we could reveal the activity in rat synaptosomal membranes. A comparison of different brain areas would also give an indication of the localization in cholinergic regions.

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2. MATERIALS AND METHODS

2.1. *Preparation of synaptosomes and presynaptic membranes from rat brain*

Male, 200 g Wistar rats were anesthetized with ether and killed. Synaptosomes were prepared essentially as described by Whittaker [4,5]. With minor modifications the procedure was applied to rat cortex [6]. Pure synaptosomes were first prepared and a presynaptic membrane fraction derived from the synaptosomes was then obtained. The three fractions A (myelin), B (synaptosomes), and C (mitochondria) were half diluted in an isotonic solution (NaCl 136 mM, KCl 3.6 mM, $MgCl_2$ 1.2 mM, glucose 5.5 mM, Tris buffer, pH 8) and centrifuged at 41000 × *g* for 30 min. The pellets were osmotically shocked in 5 ml H_2O per g original tissue and the membranes collected by centrifugation at 41000 × *g* for 30 min. The presynaptic membranes derived from the synaptosomes were further purified on a

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gradient made of 1 M sucrose (17 ml) and 0.5 M sucrose (12 ml). The presynaptic membranes were retained at the 1 M interface while the intraterminal mitochondrial elements were recovered in the pellet, after 2 h at 25000 rpm in the SW28 Beckman rotor.

When different brain regions were compared for their proteins and mediatophore activity we had in a few experiments extracted directly the crude mitochondrial fraction P_2 prepared from different areas (caudate plus putamen, cortex, cerebellum). In all cases the homogenate (5–10%) in 0.32 M sucrose was first centrifuged at 1000 × *g* for 15 min to sediment the nuclear P_1 fraction and the supernatant collected to sediment the P_2 fraction at 10000 × *g* for 30 min.

2.2. *Mediatophore extraction*

The procedure described for electric organ membranes was adapted to rat brain membrane fractions. For fractions A, B and C we worked on the membranes derived from 4.5 g tissue. For the purified synaptosomal membrane, the amount of tissue fractionated was doubled, while for P_2 pellets, fractions derived from 0.3 to 0.6 g tissue were sufficient. The alkaline extraction of membranes was done in 10 or 20 mM Tris buffer, pH 11, plus 10 mM NaCl. The volume of buffer was adjusted to get a final pH between 9.7 and 10, due to the buffering capacity of the membranes. After 20 min at 15°C the extract was centrifuged at 41000 × *g* for 1 h and the supernatant dialysed for 3 h against 10 l of distilled water (14000 kDa cut-off). The material was then lyophilized and the powder extracted in chloroform-methanol. For each mg of protein to be extracted we moisturized the powder with 250 μl H_2O and added 5 ml chloroform-methanol 1:1. The denatured proteins were centrifuged at 41000 × *g* for 1 h and the supernatant containing the mediatophore collected and dried under N_2. For a mediatophore assay the solution corresponding to 0.35 g tissue (P_2 fraction) or 0.7 g tissue (A, B, C or purified membranes) was sufficient. For protein separations an additional delipidification step was necessary and the amount of starting material doubled. The interface water-emulsion delipidification procedure was used [7]. In this case the chloroform-methanol extraction was performed in the proportion of 50 μl H_2O and 1 ml chloroform-methanol per mg of lyophilized protein extracted. After centrifugation at 41000 × *g* for 1 h the supernatant was emulsified by shaking it in 2 vols of water and centrifuged at 12000 × *g* for 10 min. The interface contains the delipidified proteins, while lipids remain in the organic phase as previously described. The interface was dried under a stream of N_2.

2.3. *Preparation of ACh containing proteoliposomes*

ACh containing proteoliposomes were prepared as described in [8,9]. The release of ACh was monitored with the choline-oxidase chemiluminescent procedure [10,11]. Transmitter release was induced by the successive additions of ionophore A23187 and calcium.

2.4. *Proteins*

Proteins were estimated using the method of Lowry et al. [12]. The addition of 1% SDS solubilizes hydrophobic materials [13]. Polyacrylamide gel electrophoresis was carried out according to Laemmli [14] in a 7.5 to 18% acrylamide gradient. The mediatophore was solubilized in the sample SDS buffer, brought at pH 10, and boiled for 5 min with 5% β-mercaptoethanol.

3. RESULTS

3.1. *Mediatophore activity of rat synaptosomal membranes*

With minor modifications, the method described by Gray and Whittaker [4] and Whittaker [5] was applied to rat cerebral cortex and used to purify pinched-off nerve endings from that tissue [6].

The purity of the synaptosomal fraction (fraction B) is illustrated in fig.1a at a low magnification. A negatively stained synaptosome is shown (inset fig.1b) for comparison with presynaptic membrane ghosts purified from the osmotically disrupted synaptosomes (fig.1b).

The purified presynaptic membranes were submitted to the mediatophore extraction procedure previously developed for *Torpedo* electric organ presynaptic membranes [1,7]. The rat membrane extract was mixed with synthetic lecithin to prepare proteoliposomes containing ACh. The release of transmitter from these particles was tested in response to a calcium influx generated by the successive additions of calcium ionophore A23187 and calcium. ACh release was monitored using the choline-oxidase chemiluminescent procedure [10,11] and a typical curve is shown in fig.2A. This calcium-dependent ACh release was blocked in the presence of the drug cetiedil (fig.2A) which has been shown to inhibit the translocation of ACh in *Torpedo* electric organ [2] and from rat brain synaptosomes [15]. Inhibition of rat brain mediatophore activity was half maximum at about 12 μM (fig.2B).

ACh release from proteoliposomes was then used to evaluate the mediatophore content of different membrane fractions. Table 1 compares mediatophore activity in extracts of the membrane fractions, A (essentially myelinated axons), B (synaptosomes) and C (essentially mitochondria). The activity was maximum in the synaptosomal membrane extract. When the mediatophore extraction procedure was applied to these three fractions, the highest proteolipid protein amount was obtained from fraction B. Considering that fraction A contains axonal membranes but also disrupted synaptosomes, while fraction C may contain some

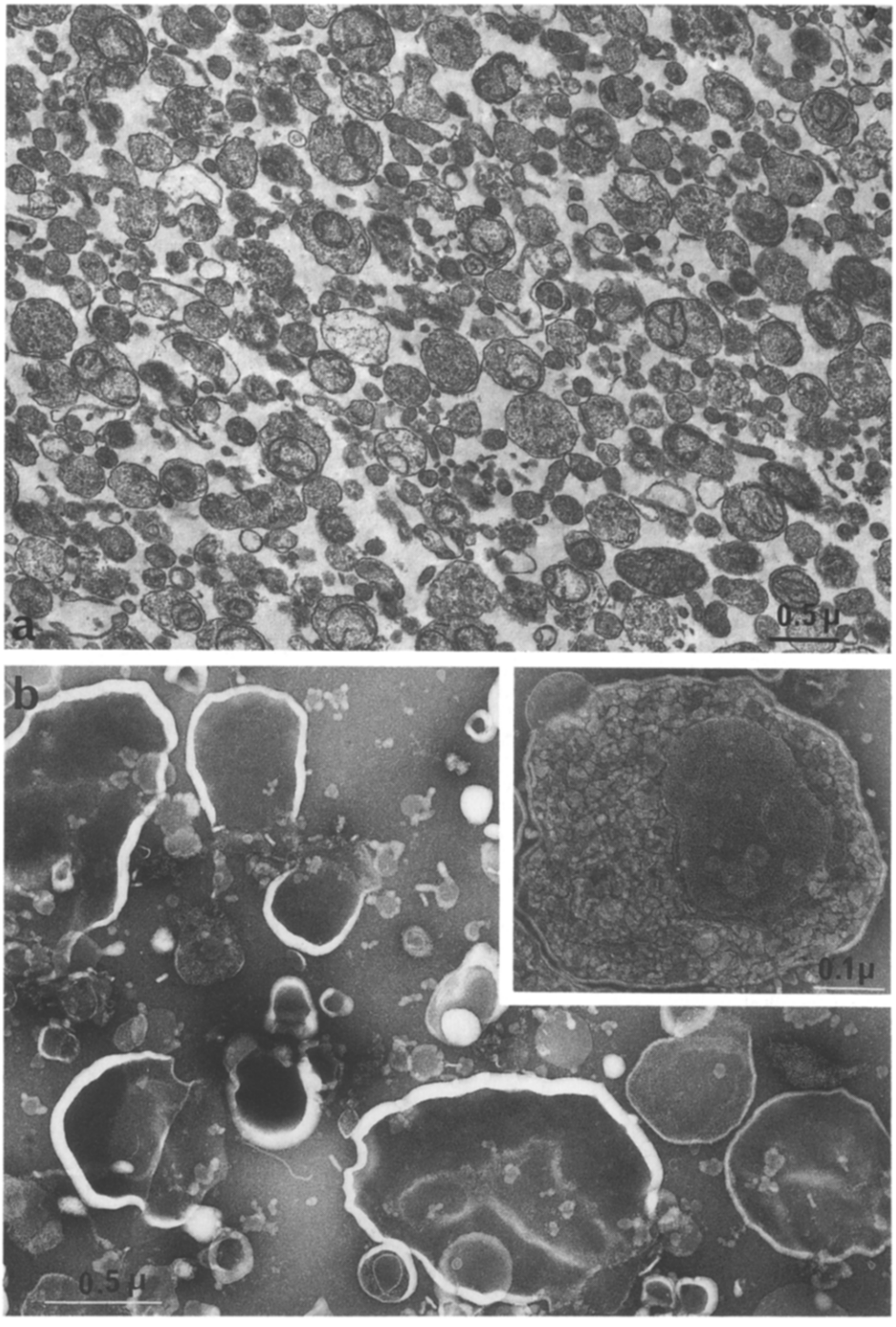

Fig.1. Rat synaptosomal fraction. (a) Electron micrograph of a large field of synaptosomal fraction B. (b) Electron micrograph of synaptosomal membrane ghosts negatively stained. The inset in (b) shows a negatively stained synaptosome before the water shock necessary to isolate the presynaptic membrane.

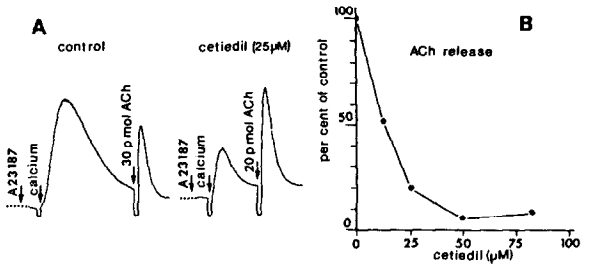

Fig.2. Acetylcholine release from proteoliposomes made with rat presynaptic membrane proteins. (Left trace) ACh release from proteoliposomes made with a mediatophore extract prepared from purified presynaptic membranes and reconstituted into artificial liposomal membranes. When release subsides an ACh standard is injected for calibration. (Middle trace) Inhibition of ACh release from proteoliposomes by cetiedil (25 μM). (B) The curve plots the inhibition of ACh release from these proteoliposomes for increasing cetiedil concentration. In all cases the entrapped ACh in the liposomes was 1 nmol per assay. The incubation medium contained 6 μM EGTA. The release was monitored with the choline oxidase chemiluminescent method, after addition of ionophore A23187 (6.6 μM) and calcium (5 mM).

dense nerve endings, mediatophore activity seems to be associated to synaptosomal membranes, together with the proteolipidic material extracted by the mediatophore extraction procedure.

Table 1

Mediatophore content of different membrane fractions

Membranes from	Mediatophore activity (pmol ACh·s^{-1}·g^{-1})	Protein in mediatophore extract (μg·g^{-1})
Fraction A (myelin)	1.8 ± 0.8 (4)	4.5 ± 1.7 (2)
Fraction B (synaptosomes)	6.3 ± 1.6 (4)	8.3 ± 0.7 (2)
Fraction C (mitochondria)	1.8 ± 0.6 (4)	3.3 ± 0.6 (2)

The fractions were, respectively, the myelin fraction A that also contains axonal membranes, the synaptosomal membrane fraction B and the mitochondrial membranes (fraction C). The three fractions were prepared from 4.5 g of cortex and results normalized for 1 g tissue. Values are means ± SE for the number of experiments in parentheses. The table shows that the proteolipid protein was most abundant in the synaptosomal membrane extract which had the strongest release when reconstituted in the liposomal membrane

3.2. *Mediatophore activity in different brain areas*

Three brain regions were chosen: the caudate plus putamen nuclei, the brain cortex and the cerebellum. We checked that choline acetylase activities in P_2 fractions obtained from these regions

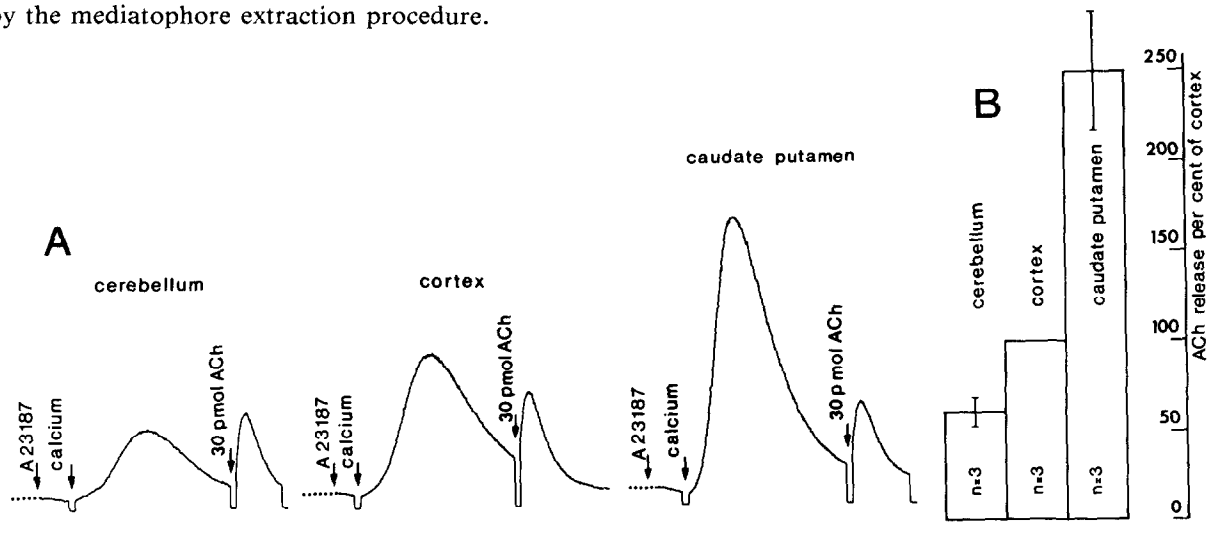

Fig.3. Mediatophore content in different rat brain areas. (A) Traces show a typical experiment in which the mediatophore was extracted from P_2 crude synaptosomal membranes from different brain areas: cerebellum, cortex or caudate-putamen nuclei. Extracts were then incorporated into liposomal membranes as for fig.2 and the proteoliposomes tested for calcium-dependent ACh release. It is clear that the strongest release capability was found for the most cholinergic region (caudate-putamen). (B) Diagrams plot the result of three different experiments normalized by taking the release rate of the cortex proteoliposomes as 100%. The cerebellum was clearly lower and the caudate-putamen region clearly higher.

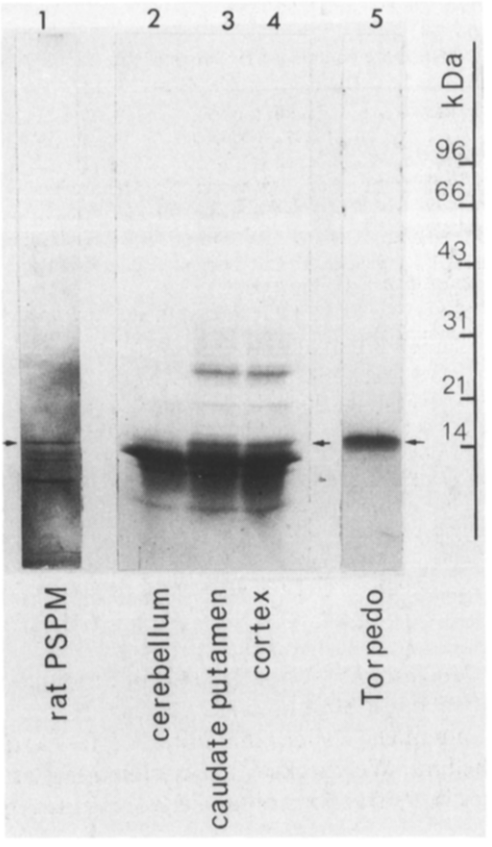

Fig.4. Acrylamide gel electrophoresis of mediatophore extracts of various membrane preparations. Lane 1: 4 bands are stained by Coomassie blue after gel electrophoresis of a mediatophore extract prepared from presynaptic membranes purified from rat cortex synaptosomes. Lanes 2–4: mediatophore extracted from P_2 membranes of different brain regions. The pattern is more complex but a 15 kDa band seems to be low in cerebellum, and more represented in caudate and cortex areas which showed more mediatophore activity. Lane 5: purified *Torpedo* mediatophore showing the characteristic 15 kDa monomer. In all cases fractions were boiled in 5% SDS in the presence of 5% β-mercaptoethanol.

were markedly different: 430, 119 and 6 nmol/h·g tissue, respectively. These P_2 fractions were extracted and tested for mediatophore activity as described in section 2. Mediatophore activity was maximal in caudate-putamen nuclei and lowest in cerebellum (fig.3B). A representative experiment is shown in fig.3A.

The protein patterns of the mediatophore containing extracts obtained from these brain areas were determined after sodium dodecyl sulfate gel electrophoresis and compared to the pattern of extracts prepared from presynaptic plasma membranes purified from cortex synaptosomes (fig.4). Four bands were stained from purified synaptosomal membrane extract in the 10 to 15 kDa region which is to be compared to the 15 kDa *Torpedo* mediatophore monomer. When P_2 fractions are extracted, rather than purified presynaptic membranes, the protein pattern is more complex and a 12 kDa band becomes dominant. Nevertheless the 4 bands of purified presynaptic membrane extract are present and the intensity of the 15 kDa band seems to follow mediatophore activity.

4. DISCUSSION

Two essential functions of the nerve terminal membrane of cholinergic synaptosomes have recently been reconstituted in artificial membranes, the sodium-dependent choline uptake blocked by hemicholinium 3 [16–18] and the calcium dependent ACh release blocked by cetiedil [8,9,19]. The reconstitution of a calcium-dependent ACh release mechanism raised several questions related to the role of synaptic vesicles which are obviously missing in these reconstituted systems. Whatever the future explanation of their role in ACh release will be, it is clear that ACh release from the reconstituted proteoliposomes in many respects resembles the physiological process [8,9] including regulatory properties such as its desensitization [20].

The experimental data demonstrate that *Torpedo* electric organ nerve terminal membranes contain a protein, the mediatophore, which has been purified [1] and which confers to artificial membranes calcium dependent ACh release properties. If mediatophore, described in *Torpedo* nerve terminals, is indeed involved in the mechanism of ACh release, one would expect to find this protein, or a similar one, in other cholinergic synapses.

The present work shows that the purest presynaptic membrane fraction we could prepare from rat brain synaptosomes possessed mediatophore activity, which is blocked by cetiedil as is the case for *Torpedo* preparations [2] and rat brain synaptosomes [15]. Active protein was extracted from rat brain in conditions similar to

those described for *Torpedo* mediatophore. It was more abundant in synaptosomal membranes than in other membranes and is represented more in cholinergic areas. A 15 kDa peptide subunit, close to the *Torpedo* mediatophore monomer, was found in rat brain membrane extracts.

In spite of the qualitative aspect of the functional test used to show the presence of mediatophore in rat brain, a clear-cut result was obtained. It anticipates future works in which one would hope to get an immunological assay of the rat mediatophore and to compare the rat material with the purified *Torpedo* protein.

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