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Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine

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ABSTRACT **A protein, which we call "mediatophore," that mediates calcium-dependent release of acetylcholine from proteoliposomes has been purified from the presynaptic plasma membrane. About 250 μg of this material was obtained from 500 g of *Torpedo marmorata* electric organ. Precipitation of the protein and subsequent removal of associated lipids inactivated the protein, which then became water soluble; this permitted evaluation of its Stokes radius (52 Å) and its sedimentation coefficient (9.8 ± 0.75 S) and, hence, an approximate molecular mass of 210 ± 16 kDa could be determined. PAGE analysis showed that the protein is made of 17-kDa subunits, not linked by disulfide bonds. When this material was observed by electron microscopy after negative staining, the apparently pentameric structures had an average diameter of about 7 nm.**

Two possible explanations, not mutually exclusive, have been advanced to account for the release of cytoplasmic acetylcholine (AcCho). The favored hypothesis holds that a population of synaptic vesicles (those of the active zone) takes up the transmitter from the cytoplasm and releases it by exocytosis. The other possibility attributes AcCho-translocating properties to presynaptic membrane proteins activated by calcium (1–4).

This latter view received some support when it was found that synaptosomal membrane sacs filled with AcCho released transmitter in response to calcium influx (5). In the following years, it became possible to insert several components of the nerve terminal membrane into liposomal membranes. This reconstituted structure had at least three properties in common with the nerve terminal: (*i*) it transported choline by a sodium-dependent carrier (6–8), (*ii*) it responded to a specific neurotoxin (*Glycera convoluta* neurotoxin) that binds to a glycoprotein of the membrane before inducing AcCho release (9), and (*iii*) transmitter release could be elicited by KCl depolarization (10) or by triggering a calcium influx with the calcium ionophore A23187 (9). The observation of numerous intramembrane particles within the liposomal membrane showed incorporation of synaptosomal membrane proteins concomitant with the appearance of several nerve ending functions (9), making more plausible the hypothesis that a presynaptic membrane component mediated the observed calcium-dependent AcCho release.

In the previous experiments total membrane components had been used to make the proteoliposomes. We then tried to purify the membrane components involved in release (11). After cholate solubilization of the presynaptic membrane, most of the component that conferred calcium-dependent AcCho release to liposomes was recovered in a fraction comprised of proteins with molecular masses between 200 and 600 kDa. This component, "mediatophore," was rapidly inactivated in the presence of cholate, limiting its further purification. However, it had also been demonstrated that alkaline treatment of the synaptosomal membrane extracted mediatophore as a protein–lipid domain that retained activity (11). In this work we describe the purification of mediatophore, which became possible for the following reasons: (*i*) large scale purification of the presynaptic plasma membrane could be achieved, (*ii*) alkaline treatment of the membrane extracted mediatophore in a stable form, and (*iii*) mediatophore could be solubilized in organic solvents. Purified mediatophore, a 210-kDa protein in association with lipids, was still able to translocate AcCho from proteoliposomes by a strictly calcium-dependent mechanism.

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METHODS

Large Scale Purification of the Presynaptic Plasma Membrane. We used the procedure published by Morel *et al.* (12). The presynaptic membrane fraction was collected at the 0.6–1.0 M sucrose interface of the gradient, and crude postsynaptic membranes were collected below 1 M sucrose. Fractions were half-diluted with H_2O and centrifuged at 480,000 × *g* for 1 hr. The pellets were kept frozen.

Mediatophore Extraction and Evaluation of Its Activity. The frozen presynaptic membrane pellets derived from 0.5 kg of *Torpedo marmorata* electric organ were thawed and mixed vigorously in 32 ml of 10 mM Tris buffer, pH 11/10 mM NaCl; the tissue lowered the pH to 9.3. After 30 min at 10°C the solution was centrifuged at 48,000 × *g* for 70 min. The supernatant was then collected, dialyzed against H_2O for 5 hr at 4°C, and lyophilized. Similar results were obtained by simply bringing the membrane suspension in H_2O to pH 10 with NaOH and maintaining it constant for 30 min on ice. After centrifugation, the supernatant was lyophilized. The lyophilized powder, 10 mg of protein, was moistened with 400 μl of H_2O and dissolved in 8 ml of chloroform/methanol (1:1). After 30 min at 0°C, the precipitated proteins were eliminated by centrifugation (48,000 × *g* for 50 min), and the supernatant containing mediatophore was evaporated under vacuum.

The activity of the fraction was measured by incorporating it into the liposomal membrane as described (9). Fractions corresponding to 10–20 μg of mediatophore were mixed with 4 mg of lecithin (dipalmitoyl L-α-phosphatidylcholine) in 1 ml of 1-butanol. After evaporation under N_2, the material was suspended in 0.5 ml of 100 mM potassium succinate/10 mM Tris buffer, pH 7.2/10^{-1} mM phospholine (ecothiopate iodide), the phospholine being an esterase inhibitor; this solution served as the interior bathing solution of the proteoliposomes. After the addition of 50 mM AcCho chloride, the suspension was sonicated as previously described (9) to obtain proteoliposomes. These were then filtered twice through 5 ml of Sephadex G-50 (coarse) columns to remove external AcCho. These Sephadex columns were equilibrated with 150 mM Tris buffer, pH 8.6/50 mM NaCl.

The amount of AcCho sequestered in the proteoliposomes was determined by injecting aliquots of the proteoliposome

Abbreviations: AcCho, acetylcholine.

suspension into an iso-osmotic solution containing the enzymes—acetylcholinesterase, choline oxidase, horseradish peroxidase, and luminol (13, 14)—for chemiluminescent AcCho assay. First, any residual external AcCho was immediately hydrolyzed, causing a light emission that rapidly returned to baseline. Detergent addition (Triton X-100) allowed sequestered AcCho to be measured by comparison with known AcCho standards (13, 14). Aliquots (5–10 μl) of the proteoliposome suspension containing 1 nmol of sequestered AcCho were used to follow the calcium-dependent AcCho release. When baseline was reached, the addition of 7 μM of calcium ionophore A23187 was without effect. The addition of 10 mM calcium, 60 sec later, elicited AcCho release, which could be compared with control AcCho standards. The assay mixture must be iso-osmotic with the interior of the proteoliposomes; we used either the same potassium succinate solution with NaCl to compensate for osmolarity differences due to AcCho chloride or simply iso-osmotic sodium chloride/5 mM Tris buffer at pH 8.6.

Delipidification of Mediatophore. Protein in the chloroform/methanol extract (1 volume) was precipitated with five volumes of ether. After 2 hr at −20°C, the precipitate was pelleted (48,000 × *g* for 50 min) and dried under vacuum; this usually sufficed for $NaDodSO_4$ gel electrophoresis. Otherwise, the pellet was surface-washed with ethanol/ether (2:3), resuspended in 100 μl of H_2O, and dissolved in 2 ml of chloroform/methanol (1:1)/0.5% 1 M HCl. Addition of 5 volumes of ether precipitated again the protein that, after 40 min at −20°C, was pelleted as above.

Other Methods. Proteins were determined using the procedure of Lowry *et al.* (15) after dissolving insoluble proteins in $NaDodSO_4$. Polyacrylamide gel electrophoreses were done as described by Laemmli (16). Gels were stained either with Coomassie blue or with silver according to Morrissey (17). *G. convoluta* neurotoxin binding was assayed as a marker for presynaptic membrane (18). Acetylcholinesterase activity was measured according to Ellman *et al.* (19). Nicotinic AcCho receptor was estimated according to Schmidt and Raftery (20) as the binding of α-bungarotoxin (N-[propionyl-^{3}H]propionylated α-bungarotoxin; Amersham) that was inhibited by 1 mM D-tubocurarine.

Negative Staining Method. Water-soluble mediatophore was spread on formvar, carbon-coated grids and stained with 2% sodium phosphotungstate at pH 7.3.

RESULTS

Purification of an AcCho-Releasing Protein: Mediatophore. We had previously observed that alkaline treatment of presynaptic membranes extracted mediatophore as a functional protein–lipid complex; this purification step eliminates most of the lipids and many undesired proteins. The alkaline extraction was done on presynaptic plasma membranes prepared by a large-scale purification procedure that enabled an 8-fold purification of presynaptic membrane proteins. As shown in Fig. 1, from 500 g of electric organ (10 g protein), we isolated a fraction of presynaptic plasma membranes (PSPM) containing ≈75 mg of protein. The alkaline extract (AE) contained 10 mg of protein and about 30% of mediatophore (Table 1). The observation that organic solvents—80% acetone, chloroform/methanol, or 1-butanol—could be used to solubilize mediatophore was valuable, as we had previously found that cholate solubilization led to a progressive loss of activity, thus limiting purification. Chloroform/methanol (1:1) was adopted (Fig. 1) at this stage, because it precipitated most protein in the alkaline extract, which could then be removed by centrifugation. After evaporation of the organic solvent, a pure fraction of fully active mediatophore (≈250 μg of protein) was obtained.

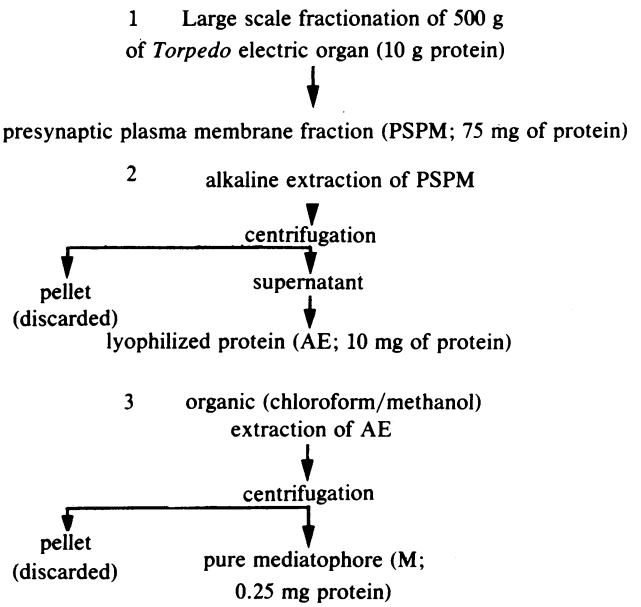

FIG. 1. Scheme of mediatophore purification procedure. See *Methods* section for details of centrifugation procedures.

Proteoliposomes were made from purified mediatophore as described earlier. Fig. 2*A* shows a continuous record of AcCho release from proteoliposomes containing 1 nmol of sequestered AcCho; when the release decayed, it was compared with AcCho standards. Fig. 2*B* shows that the magnitude of calcium-dependent AcCho release is proportional to the amount of mediatophore used in proteoliposome preparation. Table 1 compares the specific activity of mediatophore in presynaptic plasma membranes in the alkaline supernatant extract and in the organic solvent extract. The specific activity, or calcium-dependent AcCho efflux per mg of protein, and the total protein content of each fraction per

Table 1. Mediatophore enrichment and content during the three steps of purification

	PSPM	AE	M
Mediatophore specific activity, pmol AcCho/sec per mg of protein	17 ± 5 (12)	39 ± 5 (4)	350 ± 94 (13)
Protein content, μg/g of tissue	151 ± 8 (19)	20 ± 1 (18)	0.53 ± 0.05 (5)
Mediatophore content, pmol AcCho/sec per g of tissue	2.57	0.78	0.19
Recovery, %	100	30	7

PSPM, presynaptic plasma membrane fraction; AE, alkaline extract; M, organic solvent extract. An amount of each of the different fractions containing ≈20 μg of mediatophore was mixed with 4 mg of lecithin to obtain proteoliposomes. The calcium-dependent AcCho efflux was measured (Fig. 2) and normalized for 1 mg of protein to yield mediatophore specific activity. This value multiplied by the protein content per g of electric organ gives the total mediatophore content per g of tissue and permits calculation of recovery after each step. Values are mean ± SEM of the number (in parentheses) of experiments.

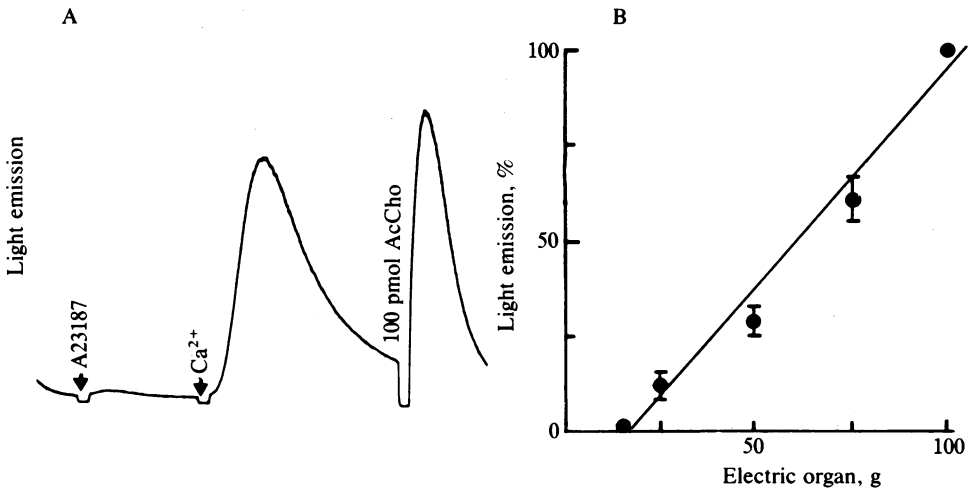

FIG. 2. Calcium-dependent AcCho release from proteoliposomes made with mediatophore. (*A*) An amount of proteoliposomes corresponding to 1 nmol of sequestered AcCho was added to the assay reaction mixture. The ionophore A23187 (7 μM) was then added. Release of AcCho was elicited by calcium (10 mM) and recorded by monitoring light emission. When the curve decays, AcCho standard is added. (*B*) Proteoliposomes were prepared with various amounts of purified mediatophore derived from 20 to 100 g of electric organ. In all cases, liposomes corresponding to 1 nmol of sequestered AcCho were used. The calcium-dependent AcCho release was estimated from the mean slope of the rising phase of light emission. Data (mean ± SEM) were obtained in four different experiments and expressed in percent of AcCho release obtained with the mediatophore derived from 100 g of electric organ.

g of fractionated tissue were determined. Their product gives total mediatophore content in each fraction derived from 1 g of electric organ. The purified mediatophore has a specific activity 20 times greater than that of the presynaptic plasma membrane fraction. Since we isolated 0.5 μg of mediatophore from 150 μg of presynaptic plasma membrane protein, a 7% yield was attained. We may then consider that this protein represents as much as 5% of the presynaptic membrane proteins.

Absence of Mediatophore in Postsynaptic Membranes. The subcellular fractionation methods allowed the separation of presynaptic from postsynaptic membranes, both membrane preparations being characterized by specific markers. Membrane-bound acetylcholinesterase activity and the binding of *G. convoluta* neurotoxin were associated with presynaptic plasma membranes that were clearly separated from a heavier membrane fraction containing most of the nicotinic AcCho receptors and hence the membranes of the postsynaptic cells (12). The two membrane fractions were repurified on separate sucrose gradients, identical with the previous one. When the mediatophore extraction procedure was applied to both membrane preparations, mediatophore closely followed the distribution of presynaptic membrane markers (Table 2). Therefore, this protein is specifically localized in the presynaptic plasma membrane. The comparison was also useful because it provided a good methodological control.

Identification of Mediatophore. After solubilization by $NaDodSO_4$ in the absence of reducing agents, electrophoresis in 7.5% to 20% acrylamide gradient gels (Fig. 3) revealed the existence of numerous protein bands in the presynaptic plasma membranes fraction, only one of which was found in the mediatophore fraction (M). This band was often deformed by the presence of lipids. To obtain a better separation and a more accurate evaluation of its molecular mass, lipids were removed as described. The electrophoretic pattern (M_1) was greatly improved, and a single band at 17 kDa was obtained. This band was unmodified whether or not mercaptoethanol was present, showing that no disulfide bonds link the subunits. No protein bands were detectable in the lipid-containing ether supernatant (E), even after silver staining, demonstrating that ether had precipitated all proteins in fraction M. In a few experiments, faint contaminants at 20 and 30 kDa (in reducing conditions) were visible after overstaining of the gels with silver (data not shown). In all cases, no material remained in the stacking gel despite the fact that the entire sample was deposited without centrifugation.

Molecular Mass of Mediatophore. Both the ether-precipitated protein and the lipid-containing supernatant were inactive, and we were unable to obtain any significant renaturation of the protein. But, as with proteolipids, the lipid removal rendered mediatophore water soluble. This was advantageous, because it allowed evaluation of the molecular mass of mediatophore and a direct observation of the protein by electron microscopy. Filtration of the delipidified mediatophore in 10 mM Tris buffer, pH 7.4, through an Ultrogel ACA 22 column (diameter, 1 cm; height, 28 cm; flow rate, 10

Table 2. Specific localization of mediatophore in presynaptic membranes

	Presynaptic	Postsynaptic
Mediatophore, pmol AcCho/min per mg of protein	31.6 ± 8.2	2.6 ± 1.6
Hydrophobic acetylcholin esterase, mmol/hr per mg of protein	5.8 ± 0.8	0.3 ± 0.1
Glycera convoluta venom binding, gland eq./mg protein	25.1 ± 2.5	2.1 ± 1.2
AcCho receptor, pmol/mg of protein	22 ± 6	415 ± 61

Mediatophore was purified from presynaptic and postsynaptic plasma membranes that had been separated by centrifugation and repurified on density gradients and that contained equivalent amounts of protein. Mediatophore activity was measured after reconstitution of the extracts into proteoliposomes as in Fig. 2. To compare the two membrane fractions, mediatophore activity was here expressed per mg of initial membrane protein, as was also done for the other membrane markers. Gland eq. represents the venom contained in a single gland of *Glycera convoluta*. Results (mean ± SEM from three experiments) were statistically different ($P < 0.01$) in all cases.

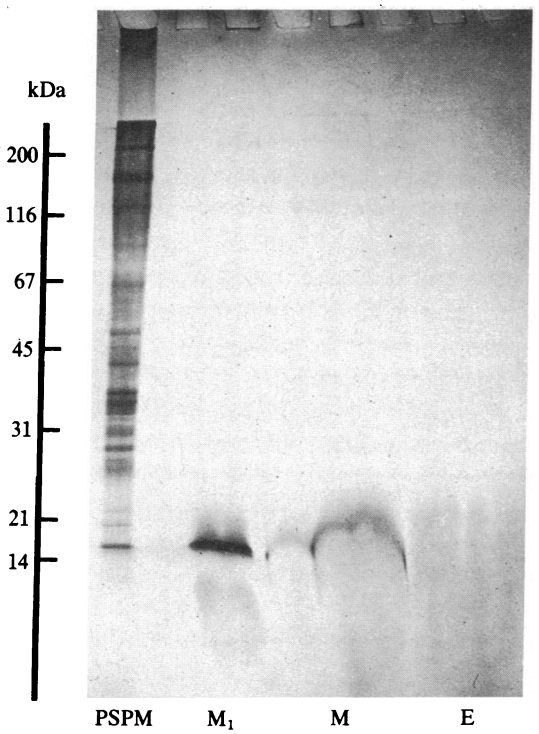

FIG. 3. Identification of mediatophore. Comparison of NaDodSO$_4$ gel electrophoresis patterns of the presynaptic plasma membrane fraction (PSPM, 15 μg of protein), the purified mediatophore fraction M, and the lipid-free mediatophore fraction M_1 ($\approx$25 μg of protein), which was obtained by ether precipitation. E corresponds to the ether supernatant. M, M_1, and E were each derived from 100 g of electric organ. All samples were solubilized in 100 μl of buffer containing 2.5% NaDodSO$_4$ without reducing agents. Electrophoresis was done at 25 mA for 5 hr in a 7.5% to 20% acrylamide gradient gel. Coomassie blue staining.

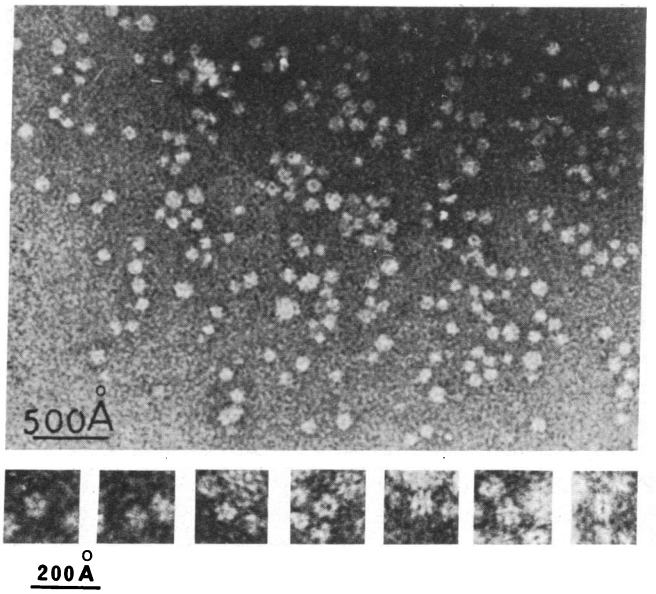

FIG. 4. Ultrastructure of mediatophore. Negative staining of water-soluble, lipid-free mediatophore in 2% neutral sodium phosphotungstate. *Inserts*, high magnifications of face and profile views of the molecule taken from three experiments.

ml/hr) produced a small peak in the void volume followed by a high peak characteristic of a single protein that eluted exactly like the molecular mass marker catalase of 230 kDa. The Stokes radius would be 52 Å. The small peak of void volume aggregates, and the protein peak both gave, after NaDodSO$_4$ gel electrophoresis, a single 17-kDa band. To evaluate the molecular mass of the mediatophore we also needed its sedimentation coefficient. Unfortunately, in 5% to 20% sucrose gradients the aggregated portion of the protein increased, and 60–70% of the material was found in the pellet; yet, a clear peak of nonaggregated mediatophore was recovered at 9.8 $\pm$ 0.75 S (n = 5). We checked that both the aggregate and the peak consisted of 17-kDa subunits. Knowing the Stokes radius, the sedimentation coefficient, and using a partial specific volume of 0.72, we calculated an approximate molecular mass of 210 $\pm$ 16 kDa (21). Despite the errors possible in this determination, we were clearly dealing with a relatively large molecule made of 17-kDa subunits. Direct evidence for a relatively large molecule was obtained by electron microscopy after negative staining. A homogeneous field of protein molecules was found and, on uniformly thin areas, face and profile views of the molecule could be seen (Fig. 4). The shape suggested is that of a slightly elongated pentamer with a hollow center; the mean diameter was 7–8 nm.

DISCUSSION

That neurotransmitter release is triggered by a calcium influx into nerve terminals is generally accepted, but the release mechanism itself is still controversial. A review of all experimental results concerning this question (see refs. 1–4, 22–24 for reviews) is not our purpose here, but we shall mention the line of evidence that led to the present work. After electrical stimulation of nerve endings *in situ* of *Torpedo* electric organ, AcCho release was associated with a preferential mobilization of the cytoplasmic AcCho compartment as compared with vesicular AcCho (25). *Torpedo* synaptosomes, stimulated by various chemical treatments, released an amount of AcCho that exactly matched the depletion of cytoplasmic AcCho (14). In both cases AcCho associated with synaptic vesicles appeared to be unaffected. More recently, synaptosomal membrane sacs, refilled with AcCho, released the neurotransmitter in response to calcium influx (5). It was subsequently possible to prepare proteoliposomes from purified presynaptic membranes and synthetic lecithin. When filled with AcCho, they also were able to release it in a calcium-dependent manner (9, 11). This report details the purification of the presynaptic membrane protein, mediatophore, responsible for this calcium-dependent translocation of AcCho from proteoliposomes.

Are we dealing here with quantal AcCho release or with another phenomenon known as transmitter leakage (26)? The latter case seems unlikely, because the AcCho release here described is strictly calcium dependent, which would not be true for transmitter leakage (27). Moreover, AcCho is released from proteoliposomes against a pH gradient—7.2 inside the liposomes versus 8.6 outside—(see *Methods*); this was shown to inhibit AcCho leakage at neuromuscular junctions (28).

One might surmise that we are purifying the AcCho carrier that concentrates AcCho inside the synaptic vesicles. There is no evidence, however, that this carrier is triggered by calcium. Furthermore, presynaptic plasma membranes were purified under conditions (low speed centrifugations and equilibrium density between 0.65–1.0 M sucrose) that are known to exclude synaptic vesicles, and electron microscopic examination of presynaptic membrane fractions showed very few membrane profiles of synaptic vesicle size. Mediatophore is, therefore, undoubtedly a presynaptic membrane protein. However, it does remain possible that this protein is also present in the membrane of the synaptic vesicle, because the endo–exocytotic cycle of synaptic vesicles could result in

the existence of common proteins. An antimediatophore antibody would help answer this question, but mediatophore appears only poorly antigenic.

Mediatophore was purified by a three-step procedure: (*i*) purification of large amounts of presynaptic plasma membranes (12), (*ii*) alkaline treatment of these membranes, and (*iii*) solubilization in organic solvents. Several integral membrane proteins are selectively extracted in alkaline conditions (29, 30); 30% of mediatophore activity was recovered in our alkaline supernatant. Mediatophore was detached in association with lipids as demonstrated by its sedimentation properties in sucrose gradients with or without cholate (11) and its solubility in organic solvents. In the third step the chloroform/methanol (1:1) mixture extracted only mediatophore, whereas all other proteins were precipitated, thus purifying mediatophore in functional form. This fraction contained lipids that could be biochemically detected and seen by electron microscopy.

After lipid removal and ether precipitation, mediatophore lost its activity but became water soluble as has been found to be the case for proteolipids (31–34); this permitted us to determine the Stokes radius, the sedimentation coefficient, and to calculate a molecular mass of 210 ± 16 kDa. The method introduces large possible errors, because a difference of 0.75 S for sedimentation coefficient gives a difference of ≈20 kDa for molecular mass. The situation is simplified by the fact that a single type of subunit at 17 kDa (apparent molecular mass) associates to form the molecule, these subunits not being linked by disulfide bonds. In addition, the morphological image of the structure is most probably pentameric. Hence, should the five arms be identical, they would each be composed of two chains at 17 kDa and give a molecular mass close to the determined value.

By a simple and rapid procedure, we have obtained significant amounts of a protein representing about 5% of the presynaptic plasma membrane protein. The fraction appears reasonably pure, as a single protein peak was obtained after gel filtration, and a single protein band was recovered after $NaDodSO_4$ gel electrophoresis. An identical band was seen after solubilization of the active material. This membrane protein, mediatophore, was discovered as a consequence to the discussion on the mechanism of AcCho release. Indeed, as shown in a previous report (11), this protein meets a number of criteria generally attributed to the AcCho release mechanism; it was therefore of interest to purify, and describe this "cholinergic" substance.

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