Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 277–281, January 1984
Neurobiology

Reconstitution of a functional synaptosomal membrane possessing the protein constituents involved in acetylcholine translocation

(presynaptic proteoliposomes/acetylcholine release)

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Communicated by Harry Grundfest, September 9, 1983

ABSTRACT **Reconstitution of a functional presynaptic membrane possessing calcium-dependent acetylcholine release properties has been achieved. The proteoliposomal membrane obtained gains its acetylcholine-releasing capabilities from presynaptic membrane proteins. At the peak of acetylcholine release, intramembrane particles became more numerous in one of the proteoliposomal membrane faces. This phenomenon resembles the intramembrane particle rearrangements found in stimulated synaptosomes. No visible structures capable of releasing acetylcholine as a result of the calcium influx were found inside the proteoliposomes. This supports the view that the release of free cytosolic acetylcholine from stimulated nerve terminals can be directly attributed to presynaptic membrane proteins. These proteins were extracted in a functional form from the synaptosomal membrane.**

The intramembrane particles found in the presynaptic membrane undergo important changes in the course of synaptic activity (1–8). They were analyzed during acetylcholine (AcCho) release from *Torpedo* electric organ synaptosomes stimulated with a variety of agents. The only common ultrastructural change found was the appearance of a category of large (8- to 18-nm) particles while smaller particles (5–11 nm) disappeared (6–8). The large particles can be pinched-off with the E or P faces of the membrane according to the conditions used. The hypothesis that Ca^{2+} causes the assembly of large particles in the membrane and that these particles ensure Ca^{2+}-dependent translocation of AcCho has been put forward by Israël *et al.* (6–8).

At present, the view is generally accepted that there is, in the nerve terminal, a genuine cytosolic free AcCho compartment (20–50%) of total AcCho (9, 10) and that the enzyme cholineacetylase, which synthesizes it, is also located in the cytosol (11). Free AcCho decreases and is renewed in the course of stimulation of electric organ slices (9, 12–14). The decrease of free AcCho has also been observed with stimulated synaptosomes, where it was directly measured with a chemiluminescent AcCho assay (15). Cytosolic AcCho also seems to be involved at *Aplysia* synapses (16) or at neuromuscular junctions (17). As for the vesicular AcCho pool (bound AcCho), it is mobilized during intense stimulation in *Torpedo* electric organ (9) and at neuromuscular synapses (17). The possibility of direct release of free AcCho from the cytosolic compartment through a presynaptic membrane element (the operator) (13) is supported by recent experiments in which synaptosomes were depleted of their contents and filled with AcCho under controlled ionic conditions. These sacs were able to release AcCho in proportion to the Ca^{2+} influx and the internal AcCho concentration (18). It therefore became possible to try to recover several essential presynaptic membrane mechanisms such as choline uptake, or AcCho release, in proteoliposomes derived from the presynaptic membrane (19–21). Until recently, proteoliposomes filled with AcCho or choline had been found to exhibit only small AcCho permeability changes on addition of Ca^{2+}/KCl. A small preference for $AcCho^+$ was noticed (21) but choline was also released, suggesting a low specificity of the system. In the present paper, we report that a functional presynaptic membrane can be reconstituted from a lyophilized presynaptic membrane powder mixed with lipid in an organic solvent. The reconstituted proteoliposomes have a Ca^{2+}-dependent AcCho release mechanism that seems to depend on specific presynaptic membrane proteins. Intramembrane particles that are formed in the proteoliposomal membrane while it releases AcCho might be directly involved in the Ca^{2+}-dependent translocation of the transmitter.

MATERIALS AND METHODS

Preparation of Presynaptic Membrane Proteoliposomes. Synaptosomes were isolated from *Torpedo marmorata* electric organ (22, 23). After osmotic shock, their membrane was centrifuged at low speed (12,000 × *g* for 30 min) and then resuspended in 300 μl of H_2O and lyophilized as described (21). The lyophilized membrane powder derived from 15–20 g of tissue (1–2 mg of presynaptic protein) was mixed with 3.5–4 mg of lecithin (dipalmitoyl L-α-phosphatidylcholine) and dissolved in 1 ml of 1-butanol. The organic solvent was evaporated under a stream of N_2. Then, the material was suspended in 0.5 ml of 100 mM potassium succinate/10 mM Tris buffer, pH 7.2/10 μM phospholine (ecothiopate iodide). (Potassium succinate was prepared by neutralization of the acid with KOH.) After 15 min, necessary to block AcCho esterase action, 50 mM AcCho chloride was added to the suspension and it was then sonicated at room temperature (15 s with the 1-cm probe of a Bioblock France sonicator set at maximum power, scale 4). The proteoliposomes were cleaned of external AcCho by two gel filtrations done in succession on columns of 5 ml of Sephadex G-50 (coarse). The Sephadex beads were in a solution of 150 mM Tris buffer, pH 8.4/50 mM NaCl. In this solution, the proteoliposomes are stable for many hours and retain amounts of AcCho in the order of 50 pmol per μl of suspension.

AcCho Release from Proteoliposomes. The chemiluminescence procedure for measuring AcCho was used (15, 24–26). In this method, AcCho esterase hydrolyzes AcCho, the choline generated is oxidized by choline oxidase, and the H_2O_2 produced generates a light emission in the presence of luminol plus peroxidase. We first prepare a mixture of 100 μl of choline oxidase (Wako, Osaka, Japan) (250 units/ml), 50 μl of horseradish peroxidase (Sigma type II) (2 mg/ml), and 100 μl of luminol (Merck) (1 mM in 0.2 M Tris buffer, pH 8.6). Each assay tube receives 15 μl of the mixture and 3 μl of AcCho esterase (Boehringer Mannheim) (1,000 units/ml) (cleaned on Sephadex G-50) in 250 μl of 100 mM potassium

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Abbreviation: AcCho, acetylcholine.

succinate in 10 mM Tris buffer (pH 8.4). This assay mixture is constantly stirred with a small magnet. The release of AcCho is measured as follows: (*i*) the Ca^{2+} ionophore A23187 is added (final concentration, 1–16 μM; the stock solution, 20 or 50 mM, is prepared in dimethyl sulfoxide, which does not interfere with the assay) and (*ii*) 5–20 μl of proteoliposomes are added (this corresponds to 1 nmol of entrapped AcCho). When all traces of choline in the external solution are oxidized, the light emission returns to baseline. The release of AcCho is then triggered by addition of 5–15 mM Ca^{2+}. The spontaneous leakage of AcCho from the proteoliposomes is negligible in comparison with the Ca^{2+}-dependent release. Controls are done by omitting the ionophore. In other experiments, the release of AcCho was triggered by using the neurotoxin extracted from the annelid polychaete *Glycera convoluta* (27).

Morphological Studies. Freeze-fracture experiments were carried out according to the "sandwich technique" of Gulik-Krzywicki and Costello (28). A thin layer (10 μm) of particles in suspension is set between two copper plates and rapidly frozen in Freon (cooled by liquid nitrogen) (29). The intramembrane particles were counted as described (6–8) in the flat parts of the synaptosomes or proteoliposomes. In general, the flat surface had a diameter of about 25% the diameter of the proteoliposomes. Because it was necessary to count many proteoliposomes, they had to be concentrated. After the sonication step, the proteoliposomes that eluted from the first Sephadex G-50 column in 0.5 ml were diluted 1:10 with succinate/Tris medium, pH 8.4, containing the Ca^{2+} ionophore A23187 (8 μM) and 100 μM EDTA. They were then centrifuged at 12,000 × *g* for 20 min and the pellet was resuspended in 50 μl of the same solution. The release of AcCho was triggered in 10 μl of proteoliposome suspension by adding 10 mM $CaCl_2$ before freezing. In a parallel tube, we determined that the peak of AcCho release was reached in 15 s. The control was done by adding NaCl, instead of $CaCl_2$, to the ionophore-containing sample or by replacing the ionophore with buffer in the Ca^{2+}-containing samples.

Protein Separations. The lyophilized membrane powder was suspended in 0.5 ml of 1% sodium cholate in Tris buffer (pH 8). After 40 min, the suspension was separated by gel filtration on Ultrogel ACA 22 (exclusion limit, 3,000,000 daltons). The column (17 × 1 cm) was equilibrated with cholate/Tris buffer and eluted with the same solution. The flow rate was 0.25 ml/min. Three fractions were collected: fraction 1 corresponds to the excluded material (>600,000 daltons), the intermediate fraction 2 corresponds to material of 200,000–600,000 daltons, fraction 3 contains elements of 68,000–200,000 daltons. The three fractions were dialyzed for 30 hr against a total volume of 40 liters of distilled water. The content of the dialysis bags was then lyophilized, and the powders obtained were incorporated into liposomes as described above. The AcCho-releasing properties of these proteoliposomes was tested, and their protein composition was analyzed. They were dissolved in $NaDodSO_4$/2-mercaptoethanol at 100°C and subjected to polyacrylamide gel electrophoresis as described by Laemmli (30), and then the gels were silver stained (31).

RESULTS

Morphology of the Reconstituted Presynaptic Membrane. In the reconstitution procedure described above, a mixture of lyophilized presynaptic membrane powder and additional lecithin is dissolved in 1-butanol, the organic solvent is evaporated, and the material is sonicated in a solution containing AcCho. The proteoliposomes formed are rather large (0.5 μm). The reconstituted proteoliposomes are shown in Fig. 1 *a–c*; their convex and concave faces contain intramembrane particles similar to the ones found in the P and E faces of the synaptosomes shown for comparison in Fig. 1 *d* and *e*.

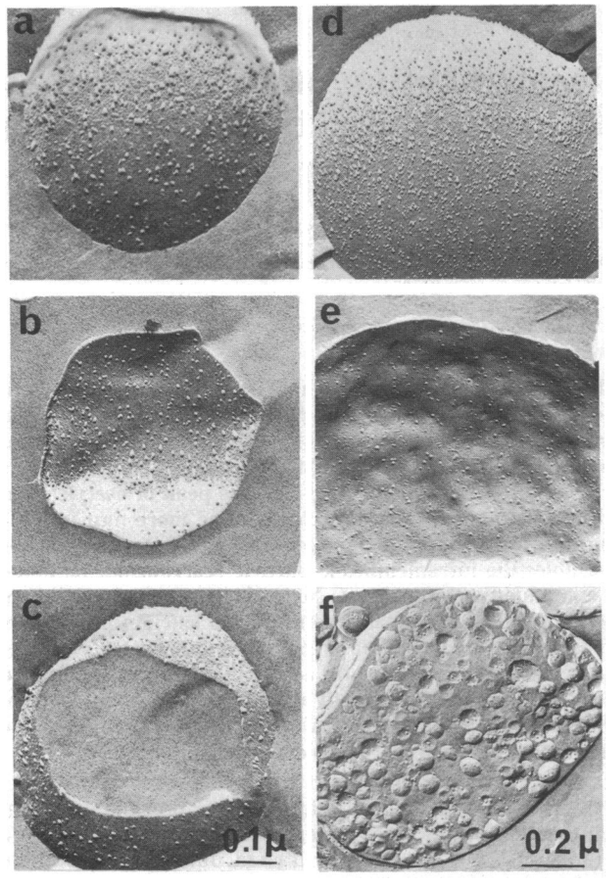

FIG. 1. Morphology of the reconstituted proteoliposomes: comparison with synaptosomes. (*a*) Convex face of the proteoliposomal membrane. (*b*) Concave face of the proteoliposome. (*c*) Plane of the fracture passing through the proteoliposome and showing no visible structure inside the proteoliposome. (*d* and *e*) P and E faces of the synaptosomes, respectively. (*f*) Interior of the synaptosome containing synaptic vesicles.

The ratios of the number of particles in the two faces are quite different in the reconstituted and synaptosomal membranes; the values are close to 0.8 for proteoliposomes and 3.2 for synaptosomes (Table 1). Another difference is that proteoliposomes do not contain any visible structures (Fig. 1*c*) while synaptosomes contain numerous synaptic vesicles (Fig. 1*f*).

AcCho Release from Proteoliposomes. In more than 10 experiments, we have shown that proteoliposomes filled with AcCho under the given conditions are able to release AcCho when a Ca^{2+} influx is generated. The ionophore A23187 was first incorporated into the system. The subsequent addition

Table 1. Distribution of intramembrane particles in reconstituted and native synaptosomal membrane

Proteoliposomes		Synaptosomes	
Convex face	571 ± 23 (68)	P face	1304 ± 72 (19)
Concave face	714 ± 62 (29)	E face	403 ± 57 (13)
Ratio	0.79	Ratio	3.2
	$P < 0.01$		$P < 0.001$

The density of intramembrane particles is higher in the P face than in the E face of the synaptosomal membrane (ratio = 3). In the reconstituted membrane, the ratio of particle density between the convex and concave faces is 0.8. Data are mean particle densities per μm^2 ± SEM. Numbers in parentheses are numbers of synaptosomes or proteoliposomes counted. Statistical significance was determined by Student's *t* test.

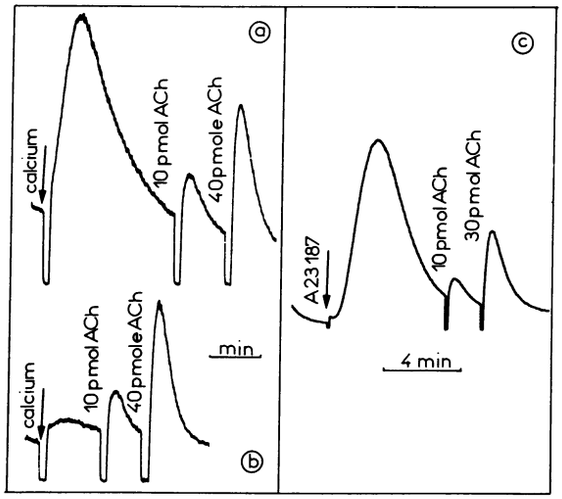

FIG. 2. AcCho release from presynaptic proteoliposomes: comparison with synaptosomes as measured with the chemiluminescence method. (*a*) Proteoliposomes were prepared with a lyophilized presynaptic membrane powder. Each sample contained 1 nmol of AcCho entrapped in the proteoliposomes. After addition of the ionophore A23187 (6.7 μM), the release of AcCho is triggered by the addition of Ca^{2+} (17 mM). When the luminescence decays, AcCho standards are added. (*b*) Control without the ionophore. Addition of Ca^{2+} fails to induce release of AcCho. (*c*) Release of AcCho from synaptosomes. The physiological medium contained Ca^{2+} (10 mM); therefore, the release of AcCho was triggered by addition of A23187 (3.8 μM). The synaptosomes contained 1 nmol of occluded AcCho.

of Ca^{2+} triggered the efflux of AcCho measured with the chemiluminescence reaction (Fig. 2*a*). In the absence of ionophore, no AcCho release occurred on Ca^{2+} addition (Fig. 2*b*). In Fig. 2*c*, we show for comparison the release of AcCho from synaptosomes stimulated by A23187 in the presence of Ca^{2+}. In both cases, the amount of entrapped AcCho was about 1 nmol. A neurotoxin extracted from the venom glands of the annelid *G. convoluta* (27) triggered an important increase of the miniature end-plate frequency at neuromuscular junctions or electric organ synapses (27) and an important AcCho release from synaptosomes. When the toxin was applied to proteoliposomes, it induced the release of AcCho (Fig. 3*a*). When the buffer without toxin was added, the record remained flat (Fig. 3*b*); for comparison, the release of AcCho from synaptosomes stimulated with the toxin is shown in Fig. 3*c*. In both cases, the AcCho content was similar (1 nmol). We have also checked that the AcCho content of the proteoliposomes decreased after AcCho release; this was done by solubilizing them in the reaction mixture by addition of a detergent (Triton X-100) either before or after triggering the release of AcCho. In general, 20–50% of the proteoliposomal AcCho content was released in a few minutes. The release is slower after neurotoxin action than with the calcium ionophore.

Intramembrane Particle Rearrangements Associated with the Release of AcCho from Proteoliposomes. The intramembrane particle density of proteoliposomes was studied at the peak of AcCho release in the presence of A23187/Ca^{2+}. This required counting a large number of proteoliposomes, which were therefore concentrated before use. AcCho release was triggered in 10-μl aliquots. This was followed by quick freezing of the samples. As shown in Table 2, the number of intramembrane particles increased in the convex face of the stimulated proteoliposomes at the peak of AcCho release. The controls done either without Ca^{2+} or without ionophore were comparable with each other. Stimulation did not change the particle density in the concave face (data not given). Table 2 also shows that an intramembrane particle density increase is found in the E face of stimulated synaptosomes. It is known from previous work with synaptosomes that the occurrence of large particles is associated with an opposite change in the number of small particles (6–8).

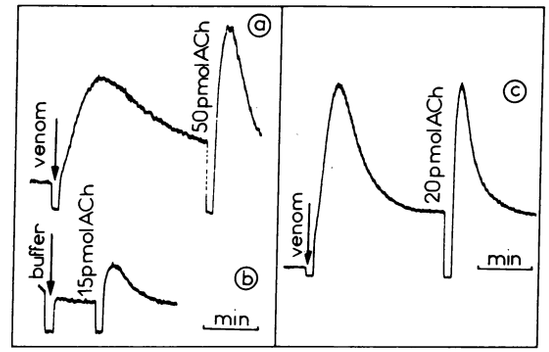

FIG. 3. AcCho release from presynaptic proteoliposomes and synaptosomes stimulated with *G. convoluta* neurotoxin. (*a*) Chemiluminescence due to the release of AcCho from the presynaptic proteoliposomes treated with *G. convoluta* neurotoxin (one gland per ml). When the luminescence decays, an AcCho standard is injected. (*b*) Control in which buffer alone was injected. (*c*) Release of AcCho from synaptosomes stimulated with *G. convoluta* neurotoxin (one gland per ml). The Ca^{2+} concentration was 15 mM in *a* and *b* and 5 mM in *c*. In all cases, the amount of occluded AcCho was 1 nmol.

Role of Presynaptic Membrane Proteins in the Release of AcCho. In two experiments, we treated the lyophilized presynaptic membrane powder with Pronase before reconstitution of the membrane. It was found that the Pronase-treated liposomes had lost most of their ability to release AcCho (Fig. 4 *a* and *b*). The protein content of the reconstituted structure was reduced to 30% of the control value. In three additional experiments, we compared the AcCho release capabilities of proteoliposomes prepared with presynaptic membranes or with other plasma membranes. These were obtained from the nuclear fraction of a *Torpedo* liver homogenized in physiological solution. Erythrocytes are abundant in the nuclear fraction, which also contains hepatocyte plasma membranes. After washing the pellet in 0.8 M sucrose, erythrocytes were sedimented at 27,000 × *g* for 30 min and disrupted in H_2O. The plasma membrane fraction was floated in 0.8 M sucrose and sedimented after diluting the sucrose

Table 2. Effect of influx of Ca^{2+} on density of intramembrane particles

	Proteoliposomes (convex face)	Synaptosomes (E face)
Control		
Ca^{2+} alone	555 ± 31 (39)	403 ± 57 (13)
A23187/EGTA	594 ± 33 (29)	
Stimulated (A23187/Ca^{2+})	812 ± 47 (27)	665 ± 79 (11)
P	<0.001	<0.02

Intramembrane particles counted in stimulated proteoliposomes (A23187/Ca^{2+}) become more numerous in the convex face than in controls done without A23187 or without Ca^{2+}. The density of intramembrane particles remained unchanged in the concave face (data not given). The release was triggered by 11 mM Ca^{2+} in the presence of 8 μM A23187. The sample was frozen after 15 s. For comparison, the increased particle density in the E face of stimulated synaptosomes after 280 s of A23187 (14 μM) treatment in the presence of 10 mM $CaCl_2$ is shown. In this case, the intramembrane particles that appear as a result of stimulation are pinched-off with the E face. There is a parallel decrease of small intramembrane particles in the P face (data not shown). Data are mean particle densities per μm^2 ± SEM. Numbers in parentheses are numbers of proteoliposomes or synaptosomes counted. Statistical significance was determined by Student's *t* test.

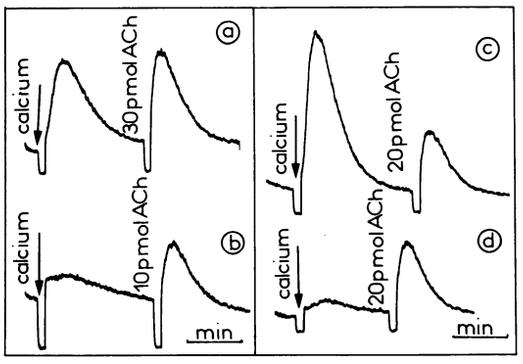

FIG. 4. Role of protein in the release of AcCho. (*a*) Release of AcCho from the proteoliposomes was induced as in Fig. 2, consequent to the influx of Ca^{2+}, and should be compared with the situation in which the liposomes were formed after treating the lyophilized membrane suspension with Pronase (1 mg/ml for 15 min) (*b*). The AcCho release capability is greatly reduced by Pronase action. (*c*) Ca^{2+}-dependent AcCho release from the presynaptic proteoliposomes should be compared with the situation in which the proteoliposomes were formed with a nonsynaptosomal membrane (*d*). The presynaptic membrane proteoliposomes released AcCho much more efficiently. In all cases, the entrapped AcCho was 1 nmol and an AcCho standard was injected after each release experiment. (*a* and *b*) The ionophore A23187 and Ca^{2+} concentrations were 4 μM and 16 mM, respectively. (*c* and *d*) These concentrations were 1.5 μM and 10 mM, respectively.

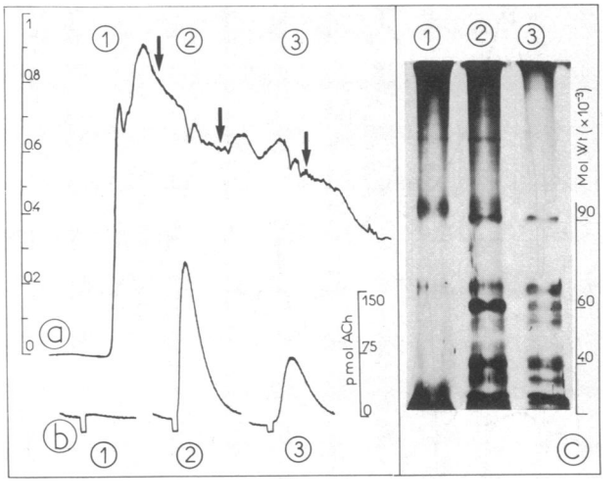

FIG. 5. Fractionation of a cholate extract of lyophilized presynaptic membrane. (*a*) Protein profile of the Ultrogel ACA 22 column loaded with the cholate extract of lyophilized presynaptic membranes. The absorption was monitored at 280 nm. Arrows give the boundaries of the fractions collected: 1, excluded volume (>600,000 daltons); 2, 200,000–600,000 daltons; 3, 68,000–200,000 daltons. (*b*) Ca^{2+}-dependent AcCho release obtained with the proteoliposomes prepared with fractions 1, 2, and 3. The proteoliposomes made with fraction 2 proteins had the greatest AcCho release capacity; fraction 3 was less active. (*c*) Proteoliposomes of fractions 1, 2, and 3 were dissolved in $NaDodSO_4$/2-mercaptoethanol at 100°C and subjected to polyacrylamide gel electrophoresis. A typical subunit pattern is found in the active fractions (92,000, 60,000, 42,000, and 36,000 daltons).

to 0.4 M. The membrane pellet was resuspended in H_2O and lyophilized. Proteoliposomes were prepared from this material in parallel to the presynaptic ones. The amounts of AcCho entrapped and the proteins associated with the two fractions were similar, but only the presynaptic membrane proteoliposomes were able to release AcCho in the presence of A23187 and Ca^{2+} efficiently (Fig. 4 *c* and *d*).

Separation of Presynaptic Membrane Proteins Involved in the Release of AcCho. The protein structures involved in the Ca^{2+}-dependent AcCho translocation through the presynaptic membrane were extracted by treating the lyophilized powder for 40 min with a mild detergent (1% sodium cholate in 10 mM Tris buffer, pH 8). The suspension was subjected to gel filtration on Ultrogel ACA 22. The column was equilibrated and eluted with the same detergent solution. Four experiments gave similar results. The elution profile (Fig. 5*a*) of the column was monitored by the absorption at 280 nm. The turbid material recovered in fraction 1 corresponds to material of >600,000 daltons, fraction 2 corresponds to material of 200,000–600,000 daltons, and fraction 3, to material of 68,000–200,000 daltons. These apparent molecular masses were evaluated by passing known protein standards through the column. The detergent was removed by dialysis, then the material was lyophilized, and proteoliposomes were tested for their ability to release AcCho. In spite of a similar AcCho content, it was found that most of the Ca^{2+}-dependent AcCho release activity was recovered in fraction 2; some activity was also present in fraction 3. The AcCho release curves for the three fractions are shown in Fig. 5*b*. In the experiment shown, the slopes of the release curves (expressed in pmol of AcCho released per sec per mg of protein) were, respectively, for the three fractions 442, 12,217, and 4,598. Because fraction 2 was the most active, we compared its protein composition with that of the two others. The proteoliposomes were boiled in 2.5% $NaDodSO_4$/5% 2-mercaptoethanol, and protein subunits were analyzed by polyacrylamide gel electrophoresis. The protein pattern (Fig. 5*c*) was simpler than that of the whole presynaptic membrane studied elsewhere (32). Four major bands were always found in fraction 2 (92,000, 60,000, 42,000, and 36,000 daltons). These bands were found only after boiling in the presence of 2-mercaptoethanol, suggesting that they derive from heavier protein complex, which is in accordance with the gel filtration results. Other presynaptic proteins, for example a 68,000-dalton protein previously described (32) was detected by immunological methods.

DISCUSSION

AcCho release has been intensively studied in the last decade, and it has now become possible to analyze its properties at the molecular level. Starting with the whole tissue, it was shown that the characteristic decay curve of the electrical discharge of a stimulated electric organ slice is associated with a characteristic variation of the free AcCho compartment (9, 12, 13). The correlation was followed down to a few stimuli (14). The release of free AcCho from isolated cholinergic synaptosomes was more recently studied (15). It was found that synaptosomes can be emptied of their contents and refilled with AcCho and ions. These synaptosomal sacs are able to release AcCho as a result of Ca^{2+} influx (18). Because stimulated electric organ, isolated synaptosomes, and synaptosomal sacs are all able to release their free AcCho content on Ca^{2+} entry, it was envisaged to try to reconstitute a functional presynaptic membrane. The presynaptic membrane was lyophilized and the powder was dissolved in 1-butanol/lecithin. Then, the organic solvent was evaporated and proteoliposomes were formed by sonication. They can be filled with AcCho and ions, and the composition of the external solution can be changed by gel filtration or centrifugation. The reconstituted presynaptic membrane proteoliposome show intramembrane particles evenly distributed between the two faces, demonstrating the incorporation of presynaptic proteins, but the nonsymmetrical distribution of particles found in the synaptosomal membrane is lost. Incorporation of a Ca^{2+} ionophore to the proteoliposomes permitted triggering the release of AcCho on Ca^{2+} en-

try. Release of AcCho was also obtained with the *G. convoluta* neurotoxin, which is known to increase the release of transmitter from tissues (27) or synaptosomes (6). A highly significant increase in the number of intramembrane particles was found in the convex face of the proteoliposomal membrane at the peak of AcCho release; no change was observed in the concave face. A similar phenomenon has been described in isolated synaptosomes stimulated with a variety of agents inducing AcCho release (6–8) but, in this case, the intramembrane particles formed were pinched off mainly with the E (concave) face. The mechanical and electrical forces that control the partition of these particles between the membrane faces are far from being understood. Nevertheless, these particles that appear on Ca^{2+} action are believed to be polymers of proteins that might ensure the passage of the transmitter. The fact that proteoliposomes made with Pronase-treated membrane powders are unable to release AcCho, and the observation that other nonsynaptosomal membranes are inactive, suggests that presynaptic membrane proteins (perhaps those that form the intramembrane particles) are directly involved in the release of AcCho.

In several previous works, the term "operator" (13) has been used to describe the elements of the presynaptic membrane that permit the translocation of AcCho. We have tried to extract these elements from the presynaptic membrane by treating the lyophilized material with a mild detergent. When the material was gel filtered, the fraction at 200,000–600,000 daltons carried most of the AcCho release capability. The protein pattern of the fractions eluted from the column was analyzed by $NaDoSO_4$ gel electrophoresis. The AcCho release activity seems to coincide with the presence of protein subunits of 92,000, 60,000, 42,000, and 36,000 daltons; however, other less-abundant proteins may be involved, and it is premature to correlate these proteins with the observed AcCho release.

Probably the reconstituted system is simply a model system that may not reflect the complexity of synaptic mechanisms. Nevertheless, the decrease in free AcCho previously found in intact tissues, synaptosomes, sacs, and now in reconstituted presynaptic membranes may well be the consequence of direct translocation of the transmitter through membrane proteins, modified by the action of Ca^{2+}.

We thank Professor R. Couteaux for his encouragement and helpful discussion, S. O'Regan for her help with the manuscript, and S. Lazereg for technical assistance. This work was supported by Delegation Generale a la Recherche Scientifique et Technique Grant 81E1381 and Institut National de la Sante et de la Recherche Medicale Grants 816019.

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