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ACETYLCHOLINE RELEASE FROM PROTEOLIPOSOMES EQUIPPED WITH SYNAPTOSOMAL MEMBRANE CONSTITUENTS

MAURICE ISRAEL, BERNARD LESBATS, ROBERT MANARANCHE and NICOLAS MOREL

Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire, C.N.R.S., 91190 Gif-sur-Yvette (France)

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A lyophilized presynaptic membrane powder prepared from *Torpedo* electric organ synaptosomes was incorporated into liposomes. These proteoliposomes had a large internal volume. The P and E faces of their membrane showed particles which were comparable to the presynaptic membrane ones. The synaptosomal ecto-esterase activity was also incorporated. A large amount of acetylcholine could be entrapped in the proteoliposome which became permeable to acetylcholine in the presence of calcium. Acetylcholine was released in preference to choline. The calcium-induced acetylcholine release depended on the incorporation of a presynaptic membrane constituent. Proteoliposomes prepared from postsynaptic membrane powders gave a much slower acetylcholine efflux. The protein pattern of presynaptic and postsynaptic membrane proteoliposomes were compared.

Introduction

Most authors working with cholinergic synapses accept presently the view that there is a genuine cytosolic acetylcholine compartment synthesized by cholineacetylase, an enzyme which is localized in the cytosol of cholinergic nerve terminals [1]. The amount of cytosolic acetylcholine ranges between 22 and 50% of the total. The lowest estimates were obtained with an isotopic dilution method [2] while highest figures were found by subtracting the amount of bound acetylcholine, protected from cholinesterases within the vesicles, from the total acetylcholine present (see Refs. 3 and 4). More recently the cytosolic and vesicular pools could be directly evaluated and physically separated from each other. This was performed with the aid of the chemiluminescent procedure developed by Israël and Lesbats [5–8]. In a reaction mixture containing cholinesterase, choline oxidase, peroxidase and luminol, the presence of acetylcholine gives rise to a light emission. If

cholinergic synaptosomes isolated from *Torpedo* electric organ [9,10] are frozen-thawed in such a reaction mixture there is an initial light emission giving the size of the cytosolic compartment (40%). Then the light measured by opening the synaptic vesicles with a detergent gives the size of the bound acetylcholine pool [7]. It was also possible to confirm with the chemiluminescent method all previous observations showing that the cytosolic pool (i.e. free acetylcholine) could be depleted by stimulation [7]. This result was also obtained on intact tissues immediately frozen and extracted after a few electrical impulses or a single one in the presence of 4-aminopyridine, a drug which enhances considerably the release of transmitter [11,12]. The release of cytoplasmic acetylcholine can certainly be performed by a special population of vesicles 'operative vesicles' which would be submitted to a rapid recycling process at the active zone [4]. The phenomenon would be related to a process called 'futile recycling' in the model proposed by Suszkiw [13] and Weiler et al. [2]. Such a

model explains at least the great stability and slow turn over of the great part of the vesicular pool, a stability which was quite unexpected when it was described [3]. The other possibility would be that the release of cytoplasmic acetylcholine is not performed through a special population of vesicles but results from a typical property of the presynaptic membrane. Such a mechanism should be considered since it was indeed found that synaptosomal sacs refilled with acetylcholine released acetylcholine in proportion to their internal acetylcholine concentration and in proportion to the calcium influx triggered by a calcium ionophore [14]. It was also found that important ultrastructural changes take place within the presynaptic membrane when synaptosomes are stimulated. There is a population of small intramembrane particles which disappears while larger particles are formed as a result of stimulation [15]. This phenomenon is the only common ultrastructural change found in a variety of conditions leading to the release of acetylcholine [16,17].

Since synaptosomes or emptied and refilled synaptosomes exhibit a calcium-dependent release of a great part of their acetylcholine content, it appeared possible to go one step ahead and try to reconstitute a proteoliposome which would have incorporated presynaptic membrane elements and find out if it would show a calcium-dependent acetylcholine permeability. It is not reasonable to hope to get a reconstituted system with all the properties of intact synaptosomes or refilled synaptosomal sacs (i.e. channels, membrane potential, metabolism, etc.); and our present aim was just to test if the permeability for acetylcholine of the reconstituted system was calcium dependent, and if some substance of the presynaptic membrane was essential to obtain it.

We describe in the present work a procedure for preparing large proteoliposomes derived from a lyophilized presynaptic membrane powder. They possess a membrane with a P and E face similar to those of the presynaptic membrane. These proteoliposomes were filled with acetylcholine and shown to possess a calcium dependent permeability for the transmitter. The specificity and selectivity of such a permeability supports the view that the reconstituted presynaptic membrane contains the substance which normally ensures a calcium-dependent release of the transmitter.

Methods

Preparation of presynaptic membrane proteoliposomes

In a typical experiment the synaptosomes from about 60 g of *Torpedo marmorata* electric organ were isolated in two successive runs as previously described [9,10]. In the final purification step they were half diluted in the physiological medium and centrifuged at $10\,800 \times g$ for 20 min. The pellets were osmotically shocked in a hypoosmotic solution obtained by diluting 10-fold the physiological solution with glass distilled water. The pellets were resuspended in a final volume of the hypoosmotic solution of 66 ml (about 1.1 ml per g original tissue). The shocked material was centrifuged at low speed $11\,000 \times g$ for 30 min. Three synaptosomal membrane pellets were obtained (each derived from about 20 g tissue). Each pellet was resuspended in 300 μ l glass distilled water, frozen in liquid nitrogen and lyophilized. The lyophilized presynaptic membrane powder derived from 15 to 20 g original tissue (1 to 2 mg protein) was mixed with 3 to 4 mg L-dipalmitoylphosphatidylcholine (lecithin) (Sigma) and resuspended in 0.5 ml 1-butanol. The opalescent solution was evaporated to dryness under a flow of nitrogen. Then the material was resuspended in 500 μ l of a solution consisting of 100 mM potassium succinate, 50 mM K_2SO_4 , 10 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.1) and 10^{-5} M phospholine (ecothiopate iodide). After 15 min, 25 μ l of acetylcholine chloride 1 M were added to give a final concentration of 50 mM. The solution was then sonicated for 15 s in a vial of 2.5 cm diameter with the 1 cm probe of a sonicator (type 20-200 s Bioblock France). The sonicator was set at maximum power scale 4. The sonicated material (0.25 ml) was then submitted to gel filtration on two successive 5 ml Sephadex G-50 coarse columns equilibrated in a solution of 275 mM NaCl, 10 mM Tris-HCl buffer (pH 8.6). The material was recovered in a volume of 0.7 ml. The gel filtration removed the external acetylcholine and phospholine. Any residual trace of external acetylcholine will further be eliminated in the chemiluminescent acetylcholine assay mixture, where choline is converted to betaine by choline oxidase.

When proteoliposomes from other membranes

were prepared, the postsynaptic membranes were either collected at the pellet of the gradient (Fraction E) [9,10] or directly spun down from a homogenate of electric organ. In order to remove most of attached nerve terminals the postsynaptic membranes were passed several times through the 200 mesh metallic grid (like in the synaptosome preparation procedure), but in a 0.5 M sucrose saline solution. This solution is isodense to the pinched-off synaptosomes and does not allow their sedimentation. The pellet was then shocked and lyophilized as described for the synaptosomal membranes. The protein to lipid ratio was kept as close as possible to that of the presynaptic membrane preparation. The amount of acetylcholine occluded in postsynaptic proteoliposomes was similar to that of presynaptic ones.

Acetylcholine release from proteoliposomes (chemiluminescent determination)

The acetylcholine determination was performed in 250 μ l of a mixture consisting of NaCl (5 ml of 275 mM NaCl in 10 mM Tris-HCl buffer (pH 8.6)); choline oxidase (EC 1.1.3.17, obtained from Wako, Japan, 100 μ l of a stock solution at 250 units/ml H_2O); horseradish peroxidase (EC 1.11.1.7, type II Sigma, 50 μ l of a stock solution at 2 mg/ml H_2O); luminol (5-amino-2,3-dihydrophthalazine-1,4-dione obtained from Merck, 100 μ l of a 1 mM stock solution) prepared as previously described [5–8,14]. Each tube receives acetylcholinesterase (EC 3.1.1.7) from *Electrophorus electricus* (Boehringer) passed through Sephadex (see Israël and Lesbats [6–8]). The stock solutions of enzymes can be kept at $-40^\circ C$. Upon addition of about 20 μ l of the proteoliposomes in the reaction mixture, the residual non occluded acetylcholine is hydrolyzed, choline is oxidized and the light emission returns to baseline. The calcium dependent acetylcholine permeability of the proteoliposomes was then tested by increasing the osmolarity of the external solution with KCl (100 mM final concentration) in the presence or absence of $CaCl_2$. The addition of $CaCl_2$ (7 mM or 17 mM final concentrations) gives an initial light emission followed by a more important light emission when the osmotic jump is performed with KCl. If the KCl is added in the absence of calcium only a very small acetylcholine release is recorded.

The light signals are calibrated with acetylcholine standards.

Morphological studies

Freeze-fracture studies were performed as previously described [15]. A thin layer of suspension is set between two copper plates and frozen in liquid freon. It is then fractured, shadowed and replicated according to the 'sandwich technique' of Gulik-Krzywicki and Costello [18] and examined in a Siemens Elmiskop 102 electron microscope.

Protein analysis

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [19]. The stacking gel and the separating gel (3 and 7.5% acrylamide, respectively) each contained 0.1% SDS. Samples (50–60 μ g protein) were heated at $100^\circ C$ for 3 min in a sample buffer containing 5% SDS and 10% 2-mercaptoethanol. Electrophoresis was carried out at 40 mA (constant current) for about 4 h. Gels were stained and fixed in 0.0125% Coomassie Brilliant Blue, 20% methanol, 7% acetic acid.

Results

Properties of proteoliposomes obtained from lyophilized presynaptic membranes

The first property we expected from these proteoliposomes was that they would entrap an important amount of acetylcholine. This was indeed the case, since a volume of 1 μ l of the fraction collected after the final gel filtration step liberated upon addition of Triton X-100 an amount of acetylcholine of 56 ± 4.6 pmol of acetylcholine ($n = 10$). Since the proteoliposomes were formed in a solution of 50 mM acetylcholine (i.e. 50 pmol/nl) it is concluded that the proteoliposomal volume in 1 μ l fraction is $56/50 = 1.12$ nl (0.1% of the fraction volume). For comparison, 1 μ l of the initial synaptosomal fraction represents a synaptosomal volume of 1.5 nl [20]. One ml of the proteoliposomes is associated to 300 μ g sedimentable protein. It is concluded that the reconstituted proteoliposomes prepared with lyophilized presynaptic membranes have an internal volume and a protein content close to the native synaptosomes.

This result was in accordance with the very close resemblance, of synaptosomal and proteoliposomal membranes. Fig. 1A shows the proteolipo-

somes as visualized by freeze-fracture methods. The fracture shows either a P or an E face of the reconstituted membrane. Notice, on the proteo-

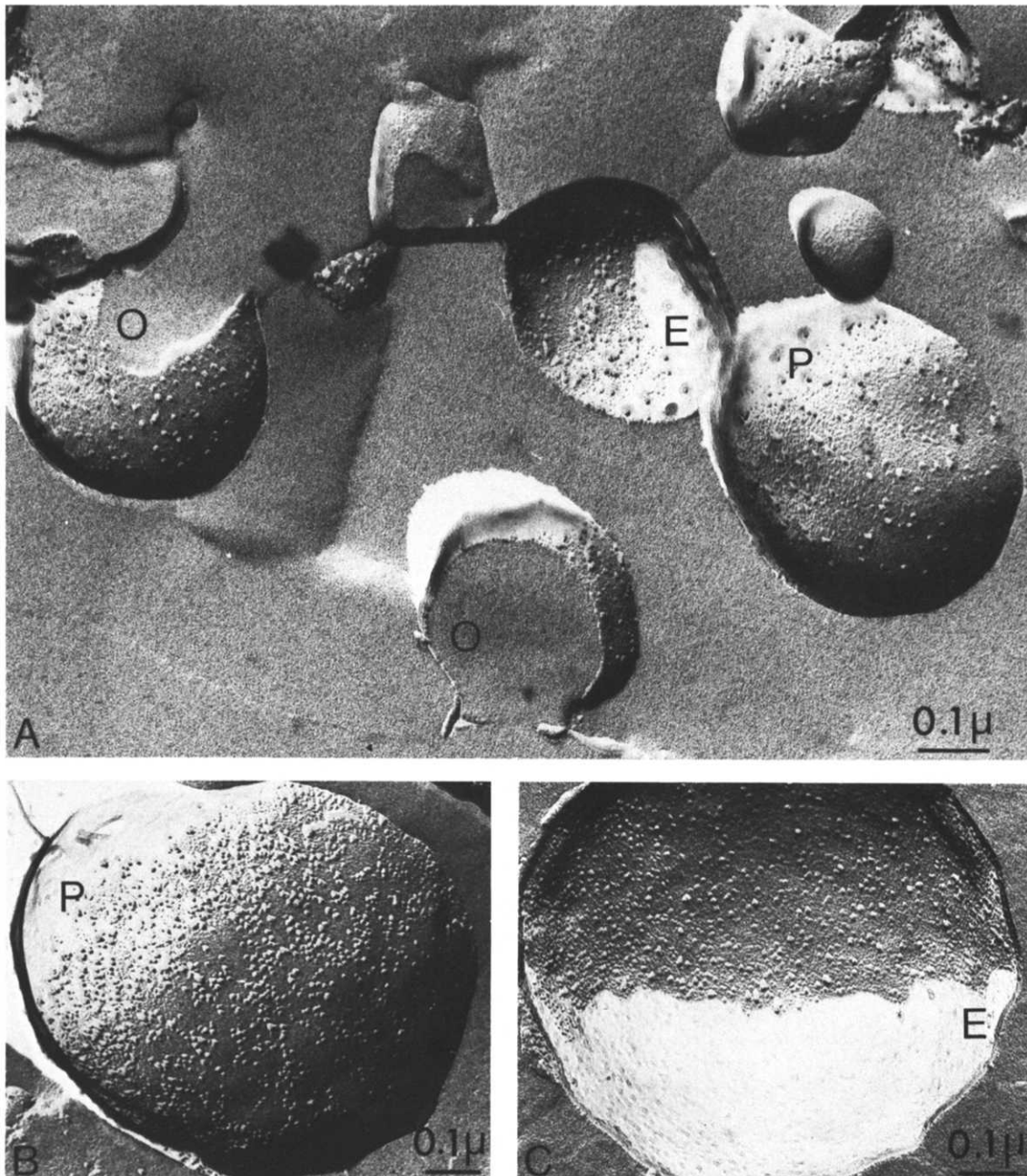


Fig. 1. Freeze-fractured presynaptic membrane proteoliposomes. (A) The P and E faces of proteoliposomes show numerous intramembrane particles. When the fracture opens the proteoliposomes (O) no structure is seen inside. Note that the membrane shows a single leaflet. Magnification $\times 100000$. (B and C) The P and E faces of synaptosomes are shown to be compared to the proteoliposomes, they are richer in intramembrane particles. Magnification $\times 80000$.

liposomes opened by the fracture that the interior of the sac is empty and that the membrane is made of a single leaflet. The method described produces very large proteoliposomes (diameter about $0.5\ \mu\text{m}$). The presence of intramembrane particles in the proteoliposomal membrane indicates that synaptosomal membrane constituents have been incorporated in the reconstituted membrane. Figs. 1B and 1C show for comparison the P and E faces of a synaptosome. Notice that they resemble very much the reconstituted proteoliposomes. The hydrophobic form of acetylcholinesterase [21–23] can be considered as a marker enzyme of the presynaptic membrane [24,25] its active site is extracellularly oriented [26]. It was found that the proteoliposomes have an acetylcholinesterase activity externally oriented (Fig. 2) since they were capable of hydrolysing the acetylcholine added to the medium and since the slope of the recorded curve

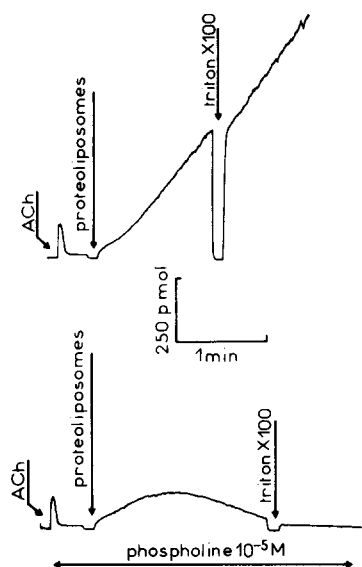


Fig. 2. Sidedness of the incorporated ecto enzyme acetylcholinesterase. (Top) When acetylcholine ($400\ \mu\text{M}$) is added to the reaction mixture, the acetylcholinesterase activity brought by the proteoliposomes leads to a linear increase of the light emission. The disruption of the liposomes with Triton X-100 does not change the slope, showing that no internal enzyme was masked. (Bottom) Same experiment but proteoliposomes were added just after the esterase inhibitor phospholine (ecothiopate iodide). A hump is recorded showing the rapid inactivation of the esterase sites; the disruption with Triton X-100 does not show any enzyme which would have escaped the inhibitor.

was not increased by adding Triton X-100. Moreover when phospholine was added to the medium, it immediately inhibited the esterase and no trace of activity could be elicited when the detergent was added. It is concluded that acetylcholinesterase was incorporated to the reconstituted proteoliposome membrane with its active site externally oriented like on intact synaptosomes.

Calcium-dependent acetylcholine permeability of the reconstituted presynaptic membrane proteoliposomes

It is presently not reasonable to try to reconstitute with a presynaptic membrane powder a structure which would show all the properties of synaptosomes or of a refilled synaptosomal sacs (i.e. membrane potential: metabolism, etc.). We therefore developed a method to simply test the calcium-dependent permeability for acetylcholine of the reconstituted presynaptic membrane proteoliposomes. The Gibbs-Donnan equilibrium reached when the proteoliposomes are in their external medium can be displaced by increasing the external NaCl or KCl concentration. Since the proteoliposomes contain impermeant anions together with permeant cations, the downhill Cl^- entry and the cations which follow, is balanced by the efflux of cations together with Cl^- , until a new equilibrium is reached. The result showed that the predicted efflux of cations does not contain the acetylcholine cation when calcium is absent, but when calcium is added before the osmotic jump, the membrane becomes permeable to the acetylcholine cation which will appear in the efflux. Fig. 3A, top row, shows that the addition of calcium ($17\ \text{mM}$) to proteoliposomes triggers a first moderate acetylcholine release; then the KCl osmotic jump triggers the release of about $20\ \text{pmol}$ acetylcholine (i.e. 1 to 3% of the content). The middle row shows that reducing calcium ($7\ \text{mM}$) diminishes the release of acetylcholine. In the lower row two KCl additions in the absence of calcium do not trigger a significant acetylcholine release. Fig. 3B shows the same phenomenon with proteoliposomes prepared with different amounts of presynaptic lyophilized membrane powder. The release triggered by the osmotic jump in the presence of calcium in the top row is greater than in the middle row an experiment in which the synaptosomal powder was reduced from 3.7 to $2.4\ \text{mg}$

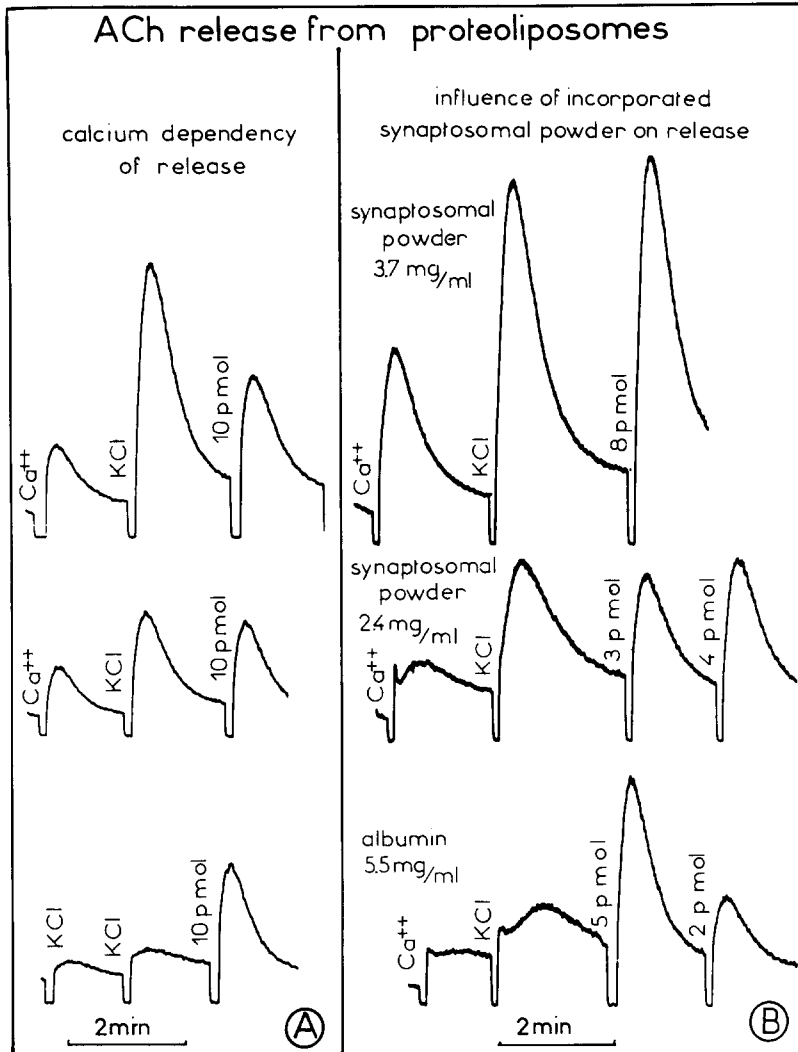


Fig. 3. Acetylcholine release from proteoliposomes. (A) Calcium dependency of acetylcholine release. The proteoliposomes (20 μl) are added to the 'chemiluminescence reaction mixture' (250 μl). When the light emission due to the oxidation of residual amounts of acetylcholine or choline has declined (about 1 min) the release experiment can be performed. Top row: The addition of CaCl_2 (17 mM final concentration) triggers a first acetylcholine release measured as a transient light emission. The subsequent addition of KCl (100 mM final concentration) triggers a new light emission representing the release of about 20 pmol acetylcholine, in comparison to the 10 pmol acetylcholine standard. Middle row: The same test but with a lower CaCl_2 concentration (7 mM) the release triggered by the calcium addition followed by the KCl addition (100 mM) is substantially reduced. Lower row: Two successive KCl additions change the osmolarity as above but no significant acetylcholine release is triggered while the injected acetylcholine standard responds as above. (B) Influence of incorporated synaptosomal powder. In this experiment the calcium-dependent acetylcholine efflux was measured as in (A) by adding CaCl_2 and then KCl. But the phosphatidylcholine was mixed with different amounts of synaptosomal membrane lyophilized powder. Top row: The addition of calcium and KCl induces release from the proteoliposomes made with an amount of lyophilized powder resulting from 3.7 mg presynaptic protein/ml (the addition of KCl releases about 8 pmol). Middle row: The same test as above performed on proteoliposome made with a lower amount of powder resulting from 2.4 mg presynaptic protein/ml. The addition of KCl released about 4 pmol in comparison to the standards. This shows that the calcium-dependent efflux is reduced when the synaptosomal powder is decreased. Lower row: No synaptosomal material was used, it was replaced by 5.5 mg albumin. Neither the calcium addition nor the KCl released significant acetylcholine amounts (compare with the 5 and 2 pmol standards). In all cases the entrapped acetylcholine was the same.

of presynaptic protein per ml. In the last row the synaptosomal powder was replaced by albumin (5.5 mg/ml), and the release was negligible. Moreover, when the presynaptic membrane powder was not added, the pure phosphatidylcholine liposomes did not show any calcium-dependent acetylcholine release, only a slow osmotic leakage was noticed. In another experiment we reconstituted proteoliposomes directly from the presynaptic membrane powder without adding the phosphatidylcholine. These structures entrapped acetylcholine but were osmotically unstable, the calcium dependency of acetylcholine release appeared difficult to establish above the high osmotic leakage of acetylcholine. In all cases the amount of occluded acetylcholine was the same. In conclusion, a calcium-dependent acetylcholine permeability of reconstituted presynaptic membrane proteoliposomes is found, and it depends on the amount of synaptosomal membrane proteins incorporated to the proteoliposomes.

Specificity of acetylcholine release from proteoliposomes

With the chemiluminescent procedure, it is easy to measure the choline and acetylcholine contents of proteoliposomes after disrupting them with Triton X-100. In a mixture containing phospholine, only choline (Ch) is read while in the presence of esterase the acetylcholine and choline contents are measured. Table I describes two experiments in which the acetylcholine (ACh) and choline (Ch) contents are different. The Ca^{2+} -dependent release of both substances was measured. It can be seen in the first experiment, that the ACh/Ch ratio was $31/20 = 1.5$ for contents and that the calcium dependent release gave an ACh/Ch ratio of 2.3. Notice that as expected, the release increases when the volume of fraction tested was increased. In the second experiment we reduced the molarity of acetylcholine to half its value and the acetylcholine content decreased from 31 to 13 pmol/ μl liposomes. The acetylcholine release measured on an aliquot of 10 μl fraction decreased from 7.2 pmol to 3.0 pmol showing that the release of acetylcholine is in proportion to the content. As for the ACh/Ch ratio it was 1.8 for the contents and 3.7 for the released amounts, showing again that acetylcholine is released in preference to choline.

TABLE I

ACETYLCHOLINE AND CHOLINE RELEASE FROM PROTEOLIPOSOMES

The table gives the acetylcholine (ACh) and choline (Ch) contents of the proteoliposomes and the Ca^{2+} -dependent release of both substances measured for 5, 10 or 30 μl of liposomal fraction. The ACh/Ch ratio is indicated in brackets for content and release in each determination. The table also shows that when the acetylcholine content is reduced from 31 to 13 pmol/ μl liposome, the release of acetylcholine decreases from 7.2 to 3 pmol. In all cases there is a preferential release of acetylcholine in comparison to choline.

	Liposomal content (pmol/ μl)	Calcium-dependent release; volume of fraction tested		
		5 μl	10 μl	30 μl
ACh	31 \pm 1.2	1.5	7.2 \pm 1.2	11.5
Ch	20.4 \pm 0.4 (1.5)–(1.55)	0.65 (2.3)	3.2 \pm 1.37 (2.3)–(1.8)	4.12 (2.8)
ACh	13		3	
Ch	7 (1.8)		0.8 (3.7)	
Mean ACh/ Ch	1.62 \pm 0.009		2.76 \pm 0.33 0.05 > α > 0.02 (n = 8)	

The mean ACh/Ch ratio for contents (1.62) was significantly different from the mean ACh/Ch ratio for the calcium dependent release (2.76). When proteoliposomes were only filled with choline, the calcium-dependent choline release was relatively low in comparison with the choline content of proteoliposomes. The diagram (Fig. 4) (on the right) gives the choline release normalized for a choline content of 30 pmol/ μl proteoliposomes. The release was measured for increasing amounts of fraction. For comparison we show (Fig. 4) (on the left) the acetylcholine and choline release normalized for equal acetylcholine and choline contents of 30 pmol/ μl . It can be seen that even when choline is not in competition with acetylcholine, which is the case when the proteoliposome contains only choline, it is less efficiently released than acetylcholine.

Comparison of ACh and choline release

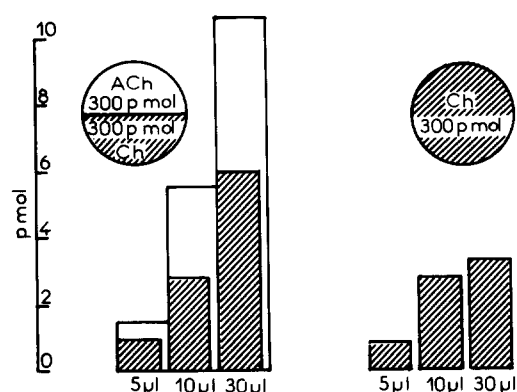


Fig. 4. Comparison of acetylcholine and choline release from proteoliposomes. Proteoliposomes containing acetylcholine plus choline are compared to proteoliposomes containing only choline. The efflux was normalized for a mixed content of 300 pmol of each substances, or for a content of 300 pmol choline (in 10 μ l liposomes). The calcium-dependent efflux of acetylcholine \square or choline \blacksquare was measured in 5, 10 or 30 μ l liposomal fractions. It can be seen that acetylcholine is released in preference to choline even when choline is not in competition with acetylcholine.

Comparison of presynaptic membrane proteoliposomes with those derived from other membranes

Are we dealing with a specific substance ensuring the calcium dependent acetylcholine permeability of the presynaptic membrane and present in the lyophilized powder, or are all membranes provided with substances capable to ensure this release of acetylcholine when activated by calcium?

The answer was obtained by comparing proteoliposomes made either with a lyophilized presynaptic membrane powder or with a lyophilized postsynaptic membrane powder. The postsynaptic membranes in fraction E (see Methods) correspond either to the dorsal non innervated faces of the electroplaques and to the ventral faces which have lost much of their nerve terminals during the fractionation procedure [10]. They were passed once more through the 200 μ m grid and pelleted again in 0.5 M sucrose-saline solution in order to avoid the sedimentation of pinched-off nerve terminals. Postsynaptic membranes were lyophilized and submitted to the same reconstitution method as synaptosomal membranes (see Methods). It was found that the slope of the calcium-

dependent acetylcholine efflux triggered by the addition of KCl was the most suitable parameter for comparing the release from proteoliposomes made with different membranes. This slope obtained by dividing the maximum amplitude by the time to peak was measured in the presence or absence of calcium for presynaptic proteoliposomes (S) and for postsynaptic proteoliposomes (E). Since the two sorts of proteoliposomes contain the same acetylcholine concentration, the slope will be directly related to the number of 'channels' introduced by the lyophilized membrane powder. The slope in pmol/s obtained for one mg of presynaptic or postsynaptic proteins permits the comparison of the two sorts of proteoliposomes (Table II). It can be seen that the ratio of mean efflux is 3.6-times greater for presynaptic proteoliposomes. If this ratio is calculated in each experiment the mean ratio is 5.8-times greater for the presynaptic proteoliposomes. This shows that the proteoliposomes derived from the synaptosomal membrane contain much more of the substance involved in the calcium-dependent acetylcholine permeability.

Analysis of the protein composition of proteoliposomes

The polypeptide composition of proteoliposomes prepared either from synaptosomal (S)

TABLE II

COMPARISON OF PROTEOLIPOSOMES PREPARED FROM SYNAPTOSOMAL OR OTHER MEMBRANES

The slope of acetylcholine (ACh) efflux was obtained by dividing the maximum amplitude by the time to peak. This was performed after a KCl addition in the presence or absence of calcium. The result in pmol was calculated per s and per mg protein for each fraction. It was checked that an equivalent acetylcholine amount was entrapped in the proteoliposomes. Results are means \pm S.E. of *n* experiments. The means were significantly different with $\alpha = 0.001$. Mean S/mean E = 3.65. Mean S/E = 5.8 ± 1.6 .

	S (Synaptosomal membranes)	E (other membranes)
Calcium-dependent ACh efflux (pmol/s/mg protein)	347 ± 59.7 (<i>n</i> = 9)	95 ± 27 (<i>n</i> = 10)

or postsynaptic (E) lyophilized membranes was analysed by one dimensional SDS-polyacrylamide gel electrophoresis. Proteoliposomes were half diluted in water and spun down in a Beckmann airfuge ($160\,000 \times g_{\max}$ for 40 min). The washed pellets were directly resuspended in the sample buffer (see Methods). The Fig. 5 compares their protein pattern with that of whole synaptosomes (C₁). Three protein bands were always present in proteoliposomes (prepared from synaptosomal membranes) as major bands (90 000, 60 000 and 42 000 mol.wt.). These bands were not found in proteoliposomes E (prepared from postsynaptic membranes). They are also present as major bands in whole synaptosomes (C₁) together with several other bands which are likely to correspond to soluble proteins since they are eliminated after osmotic disruption of the synaptosomes and centrifugation. It has been checked that all proteins of the lyophilized S powder were incorporated to the liposomes with about the same ef-

ficiency. We have recently checked that the 67 kDa protein, which is specific for presynaptic membranes [25], was immunologically detected in the proteoliposome membrane. It is of interest that Michaelson and Avissar [27] have reported that, among several other proteins of *Torpedo* synaptosomes, the 90 and 60 kDa protein band can be phosphorylated. However stimulation did not increase their phosphorylation as it is the case of a 100 kDa protein [27].

Discussion

The reconstitution of a completely functional presynaptic membrane is still a remote project, which would require the insertion in the artificial structure of the various channels, uptake mechanisms, and the incorporation of a source of energy with adequate substrates. Such a project is not unconceivable. The fact that synaptosomes emptied from their acetylcholine content and refilled with transmitter were functional [14] encouraged us to go one step ahead and find out if proteoliposomes filled with acetylcholine would gain calcium-dependent release properties if provided with elements present in the presynaptic membrane. The incorporation of choline pump activity to liposomes appeared to us as a breakthrough [28,29] and we hoped to obtain similar results with the calcium-dependent acetylcholine release mechanism. A major difficulty was to keep acetylcholine within the proteoliposomes. The technique which was successful for the incorporation of the nicotinic acetylcholine receptor (see, for example, Refs. 30 and 31) uses a detergent and even traces of it would be sufficient to deplete the acetylcholine content. In addition the small spheres (10–100 nm diameter) obtained with the most current methods entrap extremely small amounts of acetylcholine, and do not seem convenient for our purpose. The technique described here leads like other methods using organic solvents [32,33] to the reconstitution of a very large objects (0.5 to 1 μm diameter) with a membrane very similar indeed to the synaptosomal membrane itself (Fig. 1). In addition, the presynaptic acetylcholinesterase with its active site extracellularly oriented was incorporated to the proteoliposomal membrane. The synaptosomes are slightly larger (2 to 3 μm) than

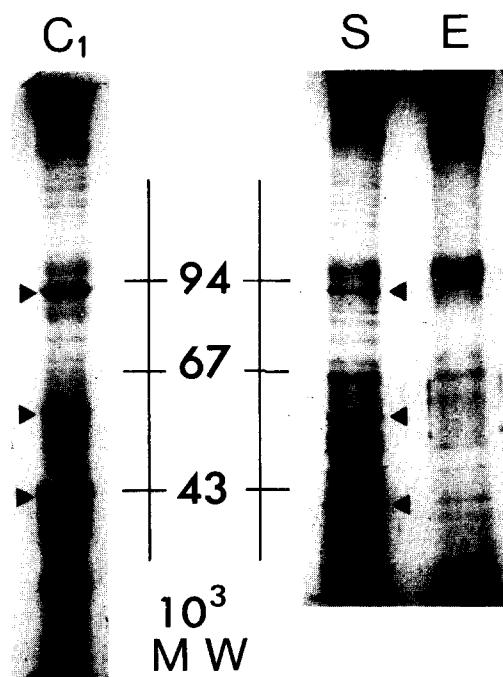


Fig. 5. Protein composition of proteoliposomes. Protein patterns of proteoliposomes prepared either from synaptosomal (S) or postsynaptic (E) lyophilized membranes. They are compared with that of whole synaptosomes (C₁). Each sample contains 50–60 μg protein.

these reconstituted proteoliposome, which did not show any internal organelle as it was judged on diametrical freeze-fractures, or after conventional electron microscopy. These proteoliposomes resemble more a synaptosomal sac [14] than do usual liposomes. This is in full agreement with the observation that they entrap a very high acetylcholine content in their internal volume (0.1% of the total volume). Another advantage of the method used is that butan-1-ol does not impair, like many other organic solvents, the structural properties of proteins [34].

In spite of the close resemblance of the reconstituted membrane of proteoliposomes with the synaptosomal membrane, there is however a major difference. We previously showed that synaptosomes or synaptosomal sacs could be depleted upon calcium entry from a great part of their acetylcholine, while the presynaptic proteoliposomes released not more than 3% of their content. Evidently we are far from having all the properties of a synaptosome (potential, channels, energy supply, etc.). We are simply showing here that the membrane of the reconstituted structure becomes permeable for acetylcholine in the presence of calcium. But the released acetylcholine cannot be greater than expected from the osmotic change; therefore the acetylcholine content is not greatly affected. The depletion test would certainly have been a very convenient way to rule out possible trivial artifacts. We therefore had to be more cautious with the presynaptic proteoliposomes, and the following controls were done to check that the observed release was not an artifact. (1) When the acetylcholine content of the presynaptic proteoliposomes was reduced, the calcium-dependent acetylcholine release declined in proportion. When proteoliposome were formed in the absence of acetylcholine, no release was observed. (2) When the synaptosomal powder incorporated to the proteoliposomes increased, the calcium-dependent acetylcholine release was higher. When no synaptosomal powder was added, or when it was replaced by albumin, the calcium dependent release was negligible. (3) Acetylcholine was released in preference to choline when the proteoliposomes contained acetylcholine and choline, the internal ratio was lower than the acetylcholine-to-choline released ratio. (4) The release observed was fully

calcium dependent and magnesium was much less effective. (5) If other lyophilized membranes were used instead of synaptosomal membranes, the reconstituted system was much less efficient. (6) The proteoliposomes have a large capacity, their membrane is very similar to the synaptosomal membrane (notice the presence of numerous intramembrane particles in the P and E faces) in addition, acetylcholinesterase was incorporated in the right position. Consequently we may consider that we are probably not dealing with an artefact, but with a genuine acetylcholine permeability change of the reconstituted system. Certainly the osmotic jump technique described to test the acetylcholine permeability of the system is presently useful. We do hope, however, to find methods to provide the proteoliposome with a potential and energy, which might well be important to sustain the flow of transmitter. Some of the observed intramembrane particles, believed to be proteins, are perhaps the structures ensuring the flow of acetylcholine. The comparison of the protein pattern of the reconstituted synaptosomal and postsynaptic membranes shows that some 90, 60 and 42 kDa proteins are incorporated, we have also detected the 67 kDa band which has been shown to be enriched in highly purified presynaptic membranes [26]. But it has to be emphasized that some of these bands (90, 60 and 42 kDa) are absent in the proteoliposomes prepared from the postsynaptic membranes which on the other hand contain two heavy bands about 95 and 190 kDa. If one assumes that proteins are the structures responsible for the calcium-dependent acetylcholine permeability change, the 90, 60 or 42 kDa bands could be concerned with acetylcholine release. While this work was in progress, proteoliposomes were prepared from rat cortical synaptosomes by Meyer and Cooper, a calcium-dependent acetylcholine release was also found (Cooper, personal communication).

In conclusion a reconstituted presynaptic membrane resembling the original synaptosomal membrane has been obtained. The structure is a large and well sealed proteoliposome almost identical to synaptosomal sacs. The structure retains large acetylcholine amounts and releases up to 3% of its content in a calcium-dependent manner. No internal organelles were observed in the reconstituted

system. A substance present in the synaptosomal membranes and poorly represented in postsynaptic membranes is able to ensure the calcium-dependent acetylcholine permeability change when inserted into the proteoliposome.

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