Isolation of a Presynaptic Plasma Membrane Fraction from *Torpedo* Cholinergic Synaptosomes: Evidence for a Specific Protein

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ABSTRACT Synaptosomal plasma membranes were isolated from *Torpedo* cholinergic synaptosomes which had been purified as previously described or repurified by equilibrium centrifugation. The synaptosomal plasma membrane could be distinguished from postsynaptic membranes by the absence of postsynaptic specific markers (nicotinic AChR) and by its low intramembrane particle complement after freeze fracture. In addition, the presynaptic membrane fraction contained acetylcholinesterase. Gel electrophoresis permitted the identification of a major protein component of the presynaptic membrane fraction which had a molecular weight of 67,000. This protein was not found in postsynaptic membrane or synaptic vesicle fractions. Thus it appeared to be specific to the nerve terminal plasma membrane.

Since the works of Whittaker (49), De Robertis et al. (8), and Gray and Whittaker (16), it has been known that, during homogenization of brain tissue, nerve endings pinch off and reseal: this leads to the formation of particles limited by a continuous plasma membrane, the synaptosomes, which can be purified on density gradients. Synaptosomes retain the main structural and functional characteristics of nerve endings and have been extensively used to study presynaptic metabolism and mechanisms of transmitter release (for a review see reference 26). The synaptosomal plasma membrane (SPM) can be considered to be a specialized part of the neuronal plasma membrane because it can be differentiated morphologically by structures such as the "active zones" (7, 9, 18, 39). Chemical analysis of the composition and organization of the SPM, after its isolation from synaptosomal preparations, has been reported by several workers (see reference 44 for a review). The purification of the SPM, however, raises two major difficulties. First, the biological heterogeneity of brain tissue results in a heterogeneity of synaptosomal fractions. Second, there is no known specific biochemical or morphological marker for the SPM.

The electric organ of *Torpedo marmorata* provides a very favorable tissue for fractionation studies due to the abundance of its pure cholinergic innervation (12, 13). Synaptosomes can be isolated from this tissue and the preparation is of good purity as estimated from morphological and biochemical criteria (23, 34, 36). Because these synaptosomes are all cholinergic, the SPM resulting from subfractionation will also be homogeneous. Moreover, these synaptosomes are devoid of postsynaptic membrane attachments and very often lack intraterminal mitochondria (34, 36), two frequent sources of contamination when isolating SPM. Another advantage is that the plasma membrane of these synaptosomes has retained the following major properties of the presynaptic membrane: (*a*) Transport mechanisms for choline and acetate, the precursors of acetylcholine synthesis (36) and for adenosine (32). (*b*) Very low permeability to ions such as Na^+ and Cl^- as shown by osmotic swelling experiments on synaptosomes (35). (*c*) Increased calcium entry after KCl depolarization (31) and a calcium-dependent release of acetylcholine (20, 35, 37) and of ATP (24, 38). In addition we have observed ultrastructural changes in these synaptosomes when they were depolarized in the presence of calcium. This was accomplished by ultrarapid freezing of the synaptosomes followed by freeze fracture. The changes consisted of the appearance of endo-exocytotic pits (37) and of modifications of the density and distribution of intramembrane particles (22).

Stadler and Tashiro (46) were able to disrupt synaptosomes isolated by the method we have described and to isolate several membrane fractions; one of these fractions was rich in plasma membrane markers and was considered to be the SPM. Such an identification suffered from the lack of a specific marker. However, there were several membrane fractions in the aforementioned work and we wanted to know whether the SPM could be recovered in a single fraction, the others containing

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contaminants, or whether it was distributed among several fractions with different domains of the SPM being separated. In the present work, we have separated two different fractions of the SPM and distinguished them from contaminating membranes.

MATERIALS AND METHODS

Isolation of Synaptosomal Plasma Membranes

Torpedo synaptosomes were prepared as previously described (23, 36). Usually three successive runs were made, 25–30 g of electric organ being fractionated in each run, and the synaptosomal fractions (fraction C) from 18 Beckman SW 27 gradients were pooled (Beckman Instruments, Inc., Richmond, CA). They were diluted (1:1) in *Torpedo* physiological medium, and synaptosomes were allowed to equilibrate for 15 min at 4°C. The *Torpedo* physiological medium consists of 280 mM NaCl, 3 mM KCl, 1.8 mM $MgCl_2$, 3.4 mM $CaCl_2$, 1.2 mM Na phosphate buffer (pH 6.8), 5.5 mM glucose, 300 mM urea, and 100 mM sucrose. After equilibration with O_2, $NaHCO_3$ (4–5 mM) was added to adjust the solution to pH 7.0–7.2. Synaptosomes were then pelleted by centrifugation at 10,000 *g* for 20 min. The pellet C_1 was resuspended in a volume (usually 5 ml) of "lysis buffer" consisting of 0.1 mM EDTA, 5 mM Tris, pH 8.0 (46) by several passages through a hypodermic needle and frozen overnight (−70°C). After thawing at 4°C, lysis of synaptosomes was completed by addition of "lysis buffer" (up to 40 ml final volume). A pellet (C_2) was obtained by centrifugation (360,000g_{max} for 30 min). C_2 was resuspended in the "lysis buffer" (~0.6 mg protein/ml) by several passages through a hypodermic needle. C_2 was layered onto a discontinuous sucrose gradient (1.5 ml per gradient). This gradient was composed, from bottom to top, of 2.5 ml of 1.2 M sucrose, 2.5 ml of 1.0 M sucrose, 2.5 ml of 0.8 M sucrose and 3 ml of 0.6 M sucrose in the "lysis buffer" (0.1 mM EDTA, 5 mM Tris, pH 8.0). Gradients were centrifuged for 240 min at 40,000 rpm in a SW41 Beckman rotor (Beckman Instruments, Inc.). The supernatant (F_1) and the bands at the interface (F2, F3, F4, F5) were collected from the top to the bottom of the gradient. The pellet (F6) was resuspended in the remaining portion of the 1.2 M sucrose layer.

Refractionation of SPM

Fractions F3, F4, and F5 were pooled, diluted in 5 mM Tris pH 7.1 (40 ml final volume) and centrifuged (360,000g_{max} for 30 min). The pellet P_3 was resuspended in a small volume of 5 mM Tris pH 7.1 (4 mg protein/ml), and 0.12 ml of P_3 was layered onto a "continuous" sucrose gradient obtained after diffusion (3 h at room temperature) of the following layers: 4 ml of 1.4 M sucrose, 4 ml of 1.0 M sucrose and 4 ml of 0.6 M sucrose in 0.1 mM EDTA, and 5 mM Tris pH 8.0. After 19-h centrifugation in a Beckman SW41 rotor at 40,000 rpm, 0.5-ml fractions were collected starting from the top of the gradient.

Equilibrium Centrifugation of Intact Synaptosomes

In some experiments, synaptosomes were diluted and concentrated by centrifugation (10,000 *g* for 20 min) as described above. The pellet was resuspended in 0.3 M sucrose in *Torpedo* physiological medium (0.6 mg of protein/ml) and 1.5 ml were layered onto each of six continuous sucrose gradients. These were obtained after 3-h diffusion of the following 2-ml layers: from top to bottom, 0.3, 0.5, 0.7, 0.9, and 1.2 M sucrose in *Torpedo* physiological medium (without sucrose and urea). Gradients were centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 12–14 h (Beckman Instruments, Inc.). Most of the material was recovered as a major band (fraction C'). It contained the majority of synaptosomes (see Results) which were then subfractionated as described above.

Enzymatic and Biochemical Estimations

Protein was determined by amidoschwarz staining (42) using bovine serum albumin (BSA) as a standard. Sucrose was estimated using an Abbe-type refractometer. Lactate dehydrogenase (LDH) was measured as described by Johnson and Whittaker (25). Choline acetyltransferase (CAT) was estimated by the acetylation of choline from radioactive acetyl coenzyme A (acetylCoA; Amersham Corp., Arlington Heights, IL) by the method of Fonnum (15). Acetylcholinesterase (AChE) was determined according to Ellman et al. (11). Nicotinic acetylcholine receptor (AChR) was measured (after solubilization) by the binding of tritiated α-bungarotoxin (*N*-[^{3}H]propionyl-propionylated; TRK 603 Amersham Corp., England) as described by Schmidt and Raftery (43). We have only taken into account the binding which was sensitive to 15 min of preincubation in the presence of 10^{-3} M *d*-tubocurarine (Serva, Feinbiochemica, Heidelberg RFA). 5'-Nucleotidase (EC 3.1.3.5) was measured by the appearance of phosphate after AMP (1.5 mM) hydrolysis in a medium containing 100 mM Tris, 100 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$ (pH 7.2). Phosphate was determined according either to Fiske and Subbarow (14) or to Ames and Dubin (2). It was checked that αβ-methylene ADP (Sigma Chemical Co., St. Louis, MO), a specific inhibitor of 5' nucleotidase (4), inhibited most of the activity. (Na^+K^+) activated-ouabain sensitive ATPase (EC 3.6.1.4) was measured as the difference in the amount of phosphate produced by the hydrolysis of ATP (1.5 mM) in the presence and absence of ouabain (10^{-3}M). The medium contained 100 mM Tris, 100 mM NaCl, 5 mM KCl, and 3 mM $MgCl_2$ (pH 7.4). Mg^{++} dependent ATPase activity was defined as the amount of phosphate released by ATP hydrolysis in the same medium containing 1 mM ouabain either with or without 5 mM $MgCl_2$.

All enzymatic activities were measured at 18°C. ACh was measured in trichloracetic acid extracts either by the frog rectus muscle bioassay (30) or by the method recently described by Israël and Lesbats (19, 20) which was also used to determine the gramicidin-induced ACh release.

Morphological Methods

For conventional electron microscopy, synaptosome pellets were fixed in 3% glutaraldehyde in 0.5 M cacodylate buffer as described previously (36). Freeze-fracture experiments were performed using the "sandwich freezing" procedure described by Gulik-Krzywicki and Costello (17) as adapted by Morel et al. (37).

SDS PAGE

Samples (40–50 μg protein) were mixed with an equal volume of dissociation buffer (10% glycerol, 10% β-mercaptoethanol, 5% SDS, 5 mM Tris, pH 6.8) and incubated in boiling water for 3–5 min before being applied to the gel. Pyronin G (BDH Chemicals, Poole, England) was used as the tracking dye. SDS PAGE was performed in a slab gel apparatus (28): an 8.75% acrylamide running gel and a 3% acrylamide stacking gel were used. Electrophoresis was carried out either for ~4 h at a constant current of 50 mA or overnight at 6 mA. Gels were stained and fixed in 0.0125% Coomassie Brilliant Blue, 40% methanol, 5% acetic acid for 1–2 h. They were destained for 2 d in 5% methanol, 7% acetic acid. Molecular weights were estimated by comparison with protein standards: myosin from rabbit muscle (220,000), β-galactosidase (130,000), phosphorylase *a* (94,000), BSA (68,000), catalase (60,000), aldolase (38,000), chymotrypsin (25,000), actin from calf muscle (43,000) which were kindly provided by Dr. Guilbert (Institut Pasteur), and tubulin prepared from pig brain (55,000) by Dr. D. Pantaloni (Centre National de la Recherche Scientifique, Gif sur Yvette).

RESULTS

Isolation of Synaptosomal Plasma Membrane

Synaptosomes, fraction C, were diluted and centrifuged at 10,000 *g* for 20 min. The resulting synaptosomal pellet is very pure as shown by morphological controls (23, 36). This moderate centrifugation, the last step of the purification procedure, permits the removal of soluble proteins and of small membrane fragments. This is shown by biochemical analysis which reveals an increase in the specific activities of presynaptic markers (CAT was 1.7 ± 0.2 [$n = 7$] times higher), whereas postsynaptic parameters were reduced (AChR was 0.89 ± 0.04 [$n = 3$] times lower). Lysis of the synaptosomes was performed essentially as described by Stadler and Tashiro (46). It combined an osmotic shock at alkaline pH and a freeze-thawing step. Table I demonstrates that 90% LDH activity is recovered in a soluble form (supernatant S_2) with 40% of the synaptosomal protein, whereas membrane markers are sedimented. The C_2 pellet is then layered onto a discontinuous sucrose density gradient. After centrifugation (40,000 rpm for 240 min in a Beckman Instruments SW41 rotor) bands were visible at each interface.

Biochemical Characterization of Membrane Fractions

Table II shows the distribution of some enzyme markers. The remaining LDH activity appeared to be restricted to the supernatant fractions (F1 and F2). AChE activity was found in fractions F3 and F4 (0.6–0.8 and 0.8–1.0 M sucrose interfaces) as was also the case for 5'-nucleotidase activity. Mg-dependent

TABLE I

Lysis of Synaptosomes: Biochemical Markers

	Protein	LDH	AChR	AChE	5′-Nucleotidase	NaK-ATPase
	μg/g	*Δ E/min·g*	*pmol/g*	*μmol/h·g*	*nmol/h·g*	*nmol/h·g*
C_1	160 ± 16	2.7 ± 0.3	23 ± 7	584 ± 72	536 ± 142	475 ± 104
	(7)	(4)	(6)	(7)	(2)	(4)
S_2	61 ± 7	2.3 ± 0.5	0.2 ± 0.1	6 ± 1	96 ± 11	24 ± 14
	(6)	(3)	(5)	(5)	(2)	(4)
C_2	96 ± 21	0.34 ± 0.04	20 ± 7	442 ± 62	359 ± 83	573 ± 112
	(9)	(3)	(7)	(7)	(2)	(4)
R	0.98	0.98	0.88	0.77	0.85	1.26

Results are mean ± SEM of the number of experiments indicated in brackets. They are expressed per gram of electric organ fractionated. *R*: recovery after lysis and centrifugation.

TABLE II

Synaptosomes (Fraction C): Distribution of Biochemical Markers in Subfractions

	Protein	LDH	AChR	AChE	Mg ATPase	5′-Nucleotidase	NaK-ATPase
	μg/g E.O.	*Δ E/min·mg*	*pmol/mg*	*mmol/h·mg*	*μmol/h·mg*	*μmol/h·mg*	*μmol/h·mg*
C_2	96 ± 21	3.7 ± 0.6	248 ± 57	4.5 ± 0.4	2.8 ± 0.1	2.2 ± 0.7	6.7 ± 0.9
F_1	1.1 ± 0.5	0.3 ± 0.3	0	0.03 ± 0.01	0	0.5 ± 0.4	0
F_2	13 ± 2	13.7 ± 2.8	7 ± 2	1.4 ± 0.7	4.9 ± 1.1	0.9 ± 0.4	3.5 ± 0.4
F_3	16 ± 3	3.7 ± 1.8	35 ± 4	9.8 ± 1.6	4.2 ± 0.4	11.3 ± 6.2	12.3 ± 3.5
F_4	15 ± 3	2.2 ± 0.3	175 ± 26	11.3 ± 2.5	1.8 ± 0.5	8.8 ± 3.7	6.1 ± 1.5
F_5	34 ± 9	0.9 ± 0.2	528 ± 118	1.5 ± 0.2	1.0 ± 0.7	4.6 ± 3.8	4.1 ± 0.9
F_6	8 ± 2	0.8 ± 0.4	285 ± 59	3.4 ± 0.4	0.1 ± 0.1	1.5 ± 0.5	2.7 ± 1.2
n	8	3	6	7	3	5	4
R	0, 91	0, 90	0, 90	0, 99	0, 61	0, 91	0, 84

Results are mean ± SEM of the number of experiments indicated (*n*). They are expressed per milligram of protein. *R*: recovery on the gradients.

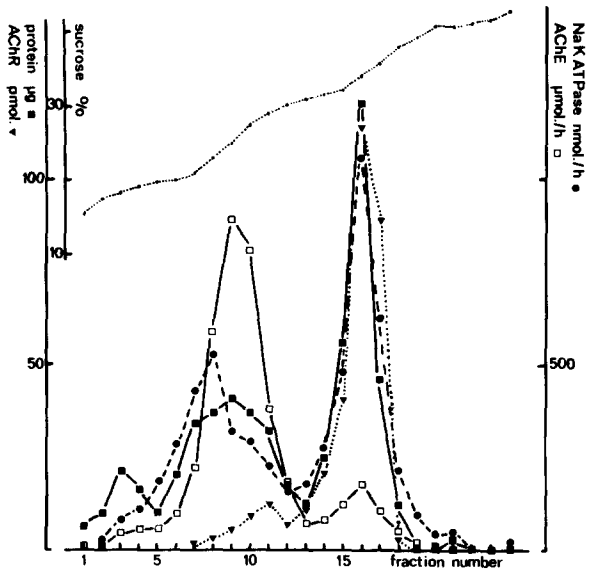

FIGURE 1 Refractionation of plasma membrane fractions. Membrane fractions F3, F4, and F5 were pooled and refractionated on a continuous sucrose gradient. Results are presented in absolute amounts or activities per 0.5-ml gradient fractions. Two protein peaks were obtained: one had an equilibrium density between 0.65 and 0.90 M sucrose and contained most of the AChE activity (with a maximum sp act of 23 mmol/h·mg protein); the other, equilibrating around 1.1 M sucrose, contained the AChR sites (maximum sp act of ~1,000 pmol/mg protein).

ATPase, a widespread enzyme which is also associated with *Torpedo* synaptic vesicles (3, 33), is maximal in F2 (above 0.6 M sucrose where monodispersed synaptic vesicles are expected) and in F3. The ACh receptor sites were measured to detect contamination by the postsynaptic membrane. They are mainly recovered in F5 (1.0–1.2 M sucrose interface which corresponds to the density of isolated postsynaptic membranes); some are also found in F4. The Na^+K^+-dependent, ouabain-inhibited ATPase is generally considered to be a marker of any plasma membrane. It is extremely abundant in membranes of the dorsal, noninnervated face of the electroplaques. This enzyme was recovered in F3, F4, and F5. From the results presented in Table II, three fractions (F3, F4, and F5) appeared to contain plasma membranes. There were clear-cut differences between F3 and F4, which have high specific activities for AChE and 5′-nucleotidase, and F5 which contains the peak of AChR sites. To define with better accuracy the density of these fractions of plasma membranes, F3, F4, and F5 were pooled, concentrated, and layered onto a continuous sucrose gradient ranging from 0.5 to 1.4 M sucrose. After equilibrium centrifugation, two bands were apparent: the upper band was wide and hazy while the lower one was narrow and dense. Fig. 1 presents the distribution of protein and of membrane markers. Two peaks of protein were well separated, the lighter banding between 0.65–0.90 M sucrose and the heavier banding very sharply around 1.1 M sucrose. AChE was associated with the light peak and AChR with the heavy one, whereas Na^+K^+-dependent ATPase activity was recovered with both. From this experiment it is apparent that the AChR-containing peak corresponds to F5 and contains the large majority of postsynaptic membrane fragments contaminating the synaptosomal fraction. The specific activity of α-bungarotoxin binding sites was ~1,000 pmol/mg protein which is to compare with the specific activity of 4,000 pmol/mg protein reported by Sobel et al. (45) for purified postsynaptic membrane microsacs. F5 is clearly separated, on the basis of membrane density, from the AChE-containing peak which corresponds to fractions F_3 and F_4. The

light and broad protein peak shown in Fig. 1 appears to be heterogeneous because the peaks of Na^+, K^+-dependent ATPase and of AChE were not coincident and because a shoulder appeared on the ATPase peak. This suggests that there are two membrane populations; one, which is enriched in NaK-ATPase activity, has a lower density and could correspond to F3, and the other, which contains more AChE activity, and few AChR sites, could correspond to F4.

Morphological Characterization

We have therefore examined by electron microscopy the three plasma membrane fractions. Fig. 2 shows large fields of F3, F4, and F5 fixed in glutaraldehyde and observed after conventional treatment. Vesicular profiles of various sizes can be seen in the three fractions. The most important observation is that myelin, an occasional contaminant of synaptosomal fractions, was not detected in either of these fractions. Synaptic vesicles (Diam ~850 Å) were also absent. F5 appears heterogeneous, containing large fragments probably derived from the noninnervated face of electroplaques. After rapid freezing of synaptosomal suspensions and freeze fracture, it was previously shown that the appearance of SPM was very different from that of postsynaptic membranes, its intramembrane particle density being three to four times lower (37). Fig. 3 and Table III demonstrate, using this technique, that the intramembrane particle density increases for the heaviest gradient fractions. It is clear from Table III that membranes from F3 and F4 have a mean particle density equal to that of intact nerve terminals and synaptosomes, a density which is markedly lower than ventral and dorsal electroplaque membranes. Fragments of these membranes are found in F5. Myelin sheets, which are extremely poor in intramembrane particles (440 ± 59; $n = 4$) and recognizable by their lamellar structure, were absent from these fractions. As for Schwann cell membranes, they were so poorly represented in freeze-fractured electric organ that we were not able to get an estimate of their intramembrane particle density.

Repurification of Synaptosomes on a Continuous Sucrose Gradient

Synaptosomes were concentrated by low-speed centrifugation as described in Materials and Methods, resuspended in 0.3 M sucrose in physiological medium, and layered onto a continuous sucrose gradient ranging from 0.3 M to 1.2 M sucrose. Fig. 4 shows the distribution of some enzyme markers in the gradient after equilibrium centrifugation. Protein and the cytoplasmic markers (choline acetyltransferase and LDH) were recovered in a single peak at 0.45 M sucrose (fraction C′) and in the pellet. C′ contained 35–40% of the gradient protein and LDH. AChE activity was recovered in the 0.45 M sucrose peak but was slightly shifted towards the bottom of the gradient. A background of AChR was found below 0.45 M sucrose.

The peak fractions for cytoplasmic markers (4, 5, 6, Fig. 4) were pooled (fraction C′) and their physiological activity was tested. They contained 14 ± 2 nmol ACh/g of electric organ

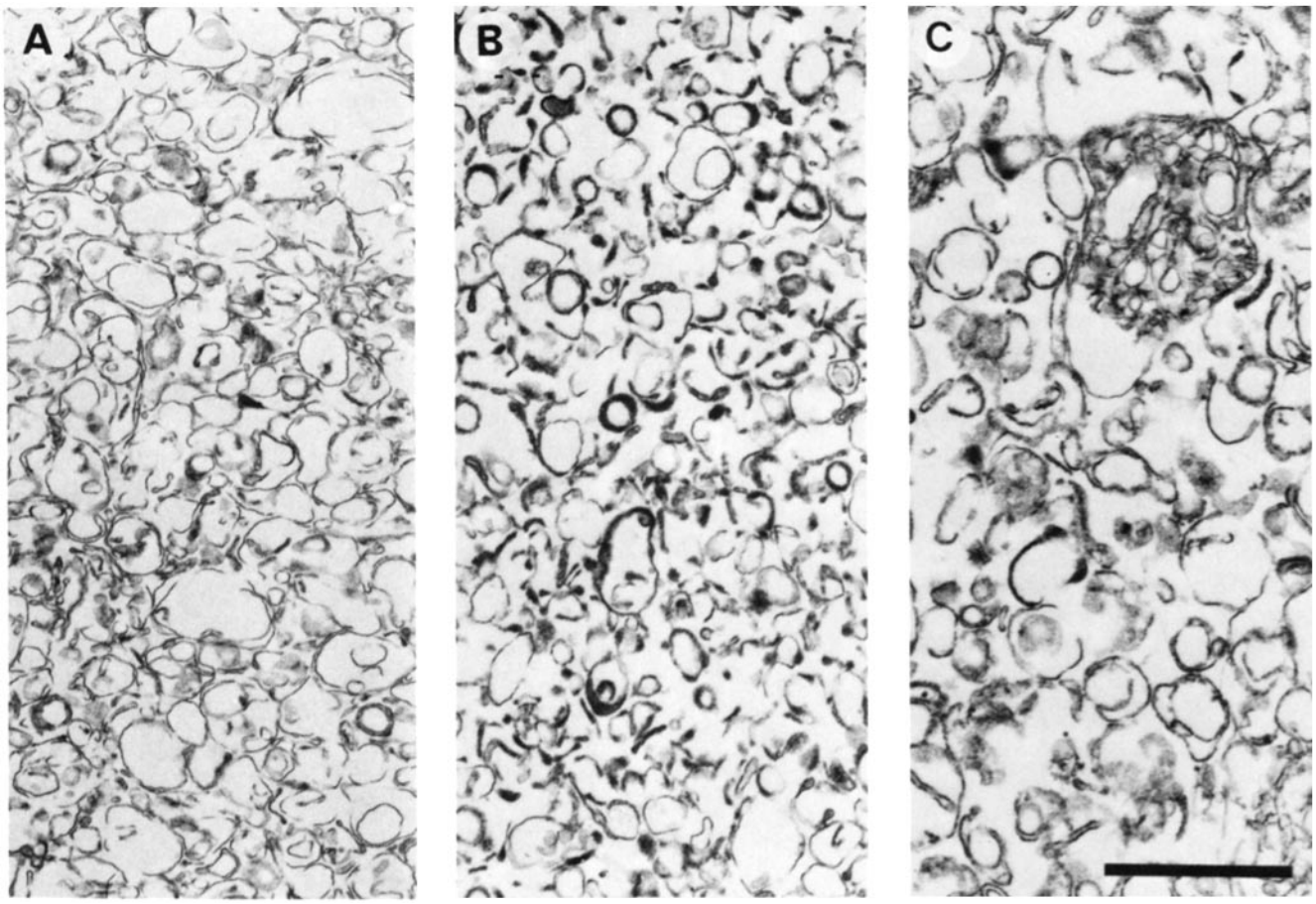

FIGURE 2 Electron micrograph of plasma membranes. Fractions F3 (*A*) and F4 (*B*) are the synaptosomal plasma membrane fractions: notice the absence of myelin fragments or synaptic vesicles. Fraction 5 (*C*) contained postsynaptic membrane contaminants removed from the SPM fractions. Bar, 1 μm. × 25,000.

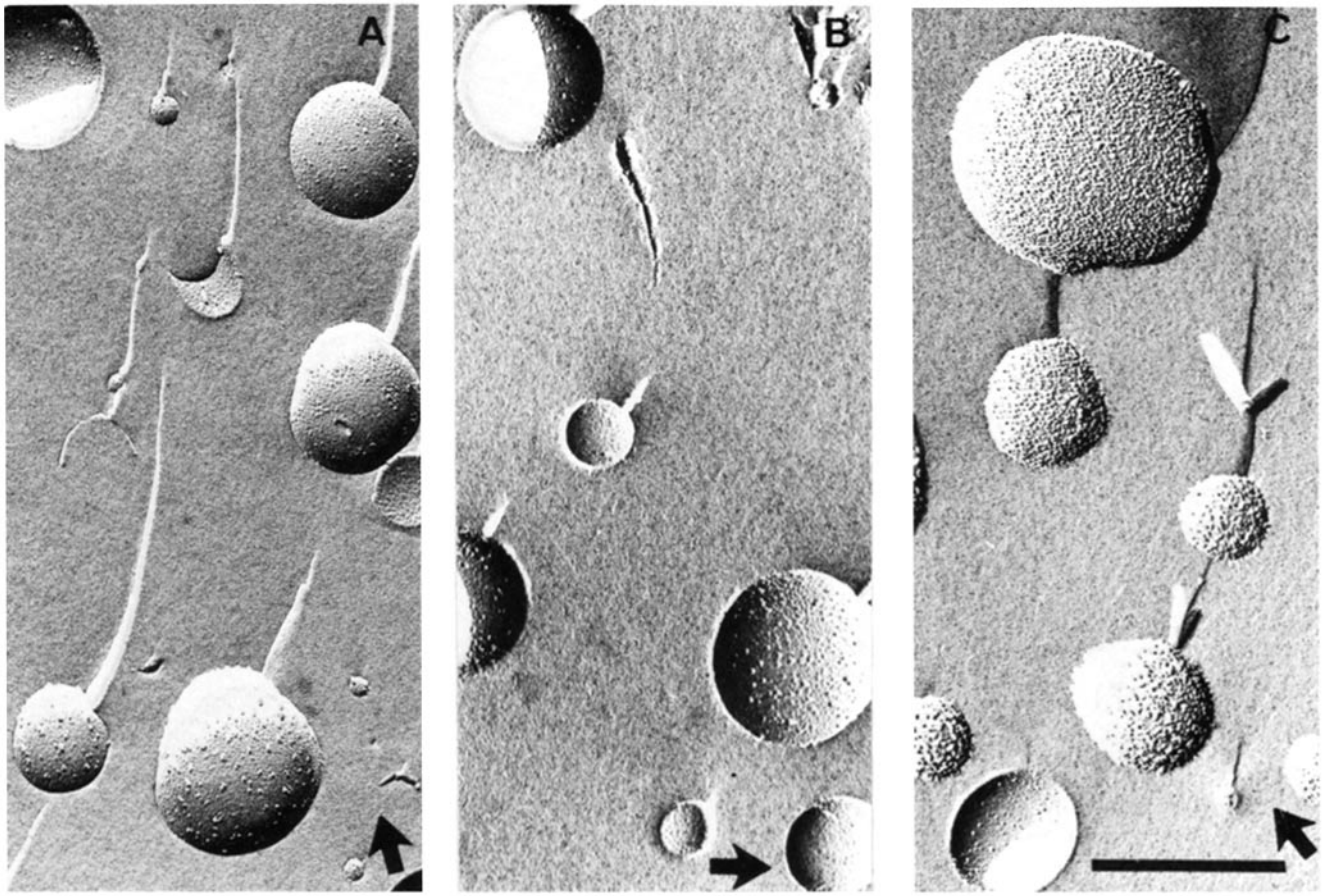

FIGURE 3 Freeze-fractured plasma membranes. Membrane in F3 (*A*) and F4 (*B*) had a low intramembrane particle density when compared to postsynaptic membranes in F5 (*C*). Bar, 0.5 μm. × 50,000.

TABLE III

Comparison of Intramembrane Particle Densities of Membrane Fractions and of Membranes In Situ

	Convex faces	Concave faces	Total
F3	1186 ± 229 [11]	972 ± 120 [19]	2,158
F4	1642 ± 860 [5]	1240 ± 588 [6]	2,882
F5	5153 ± 318 [14]	2036 ± 301 [10]	7,189
Synaptosomal plasma membrane*			2,040
Nerve endings *in situ**			1,780
Postsynaptic plasma membrane‡			
Postsynaptic innervated face‡			6,000–8,000
Postsynaptic dorsal face§			5,000
Myelin sheets			440 ± 59 [4]

F3 and F4 had an intramembrane particle density similar to that of presynaptic plasma membrane. That of F5 was in the range of that of postsynaptic plasma membranes. As for myelin, recognizable by its lamellar structure, it had very low intramembrane particle density and was absent from the fractions. Results are expressed in particles per square micrometer and mean ± SEM of the numbers in brackets.

* See reference 37.

‡ See references 1, 5, and 41.

§ See reference 1.

fractionated (mean ± range of two different experiments) with specific activities of 331 ± 32 nmol ACh/mg protein. This represents 33 ± 7% of the ACh content of control synaptosomes, kept at 4°C during all the centrifugation procedure. As a functional control, it was tested that these synaptosomes were able to release their transmitter after depolarization with gramicidin. Electron microscopy did not reveal any morphological modification. Their purity appears to have been increased on the basis of relative specific activities as compared to the parent fraction C (see Discussion) but at the expense of yield (only 30–40% of the synaptosomes were recovered after the continuous gradient centrifugation).

Fraction C′ was fractionated as described for C (see Materials and Methods). Table IV presents the distribution of protein, AChE, and AChR in the discontinuous sucrose gradient. Repurification of synaptosomes virtually eliminates F5 which now represents 10% of total proteins as compared to 40% for routine synaptosomes (Table II). Under the same conditions, F3 and F4 now represent 33 and 22% of total proteins as compared to 18 and 17% (Table II).

Analysis of the Peptide Composition of SPM Fraction

The polypeptide composition of membranes in F3 and F4 was analyzed by one-dimensional SDS PAGE. It appears to be rather complex as numerous bands were found. Fig. 5 compares the protein pattern of SPM with that of whole

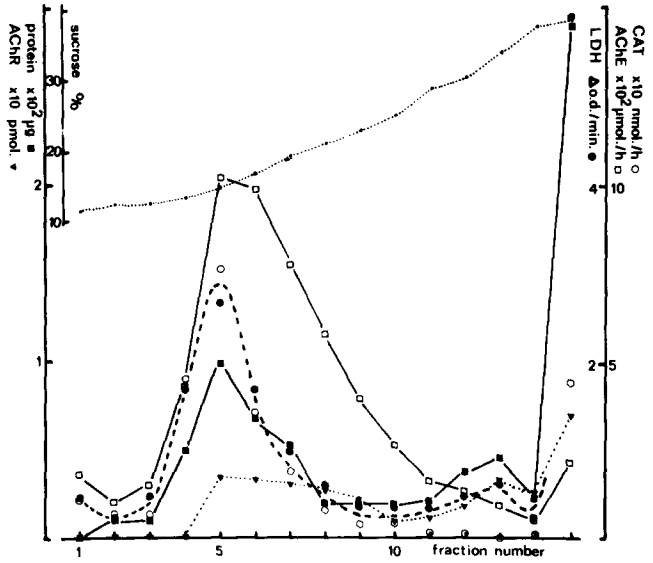

FIGURE 4 Repurification of synaptosomes on a continuous sucrose gradient. A broad synaptosomal peak (fraction C') was recovered in fractions 4, 5, and 6 as shown by the LDH and CAT activities; it corresponded also to a peak of AChE activity while the AChR sites remained at a low level. Results are activities per 0.8-ml fraction.

TABLE IV

Biochemical Markers in Subfractions of Synaptosomes Repurified on Continuous Sucrose Gradient

Fraction	Protein	AChE	AChR
	µg/g	*mmol/h · mg*	*pmol/mg*
$F_1 + F_2$	3.0 ± 1.1	2 ± 1	31 ± 21
F_3	3.4 ± 1.2	25 ± 15	64 ± 37
F_4	2.3 ± 0.0	30 ± 15	292 ± 82
F_5	1.0 ± 0.1	5 ± 1	383 ± 216
F_6	0.6 ± 0.1	1 ± 0	123 ± 33

Fractions F1 and F2 were pooled. Results are mean ± range of two different experiments.

synaptosomes, crude synaptic vesicles, and postsynaptic membranes recovered in fraction F5. A number of high-molecular-weight proteins were present. The patterns of F3 and F4 were rather similar but F4 presents some bands also found in F5 which were absent in F3 (for instance, in the 95,000 and 40,000 mol wt regions). These bands were markedly reduced when SPM were prepared from recentrifuged synaptosomes. Some bands were found in F3 and not in F4 even when prepared from repurified synaptosomes (~135,000, 37,000, and 32,000 mol wt). A major band (43,000 mol wt) comigrating in one-dimensional electrophoresis with actin was present in F3 and F4 (as well as in synaptic vesicles and in F5); this is in accordance with the results of Stadler and Tashiro (46). The most striking result was the presence in F3 and F4 of a major band (67,000 mol wt) which was not observed in F5 nor in synaptic vesicles. This band was highly enriched in SPM when compared to synaptosomes and could be specific for the SPM.

DISCUSSION

In our earlier reports (23, 36) we found that morphologically pure synaptosomes could be obtained by low-speed centrifugation of the gradient fraction C. Biochemical calculations based on specific activities of presynaptic markers (ACh and CAT) such as those reported by Kelly et al. (27) showed that

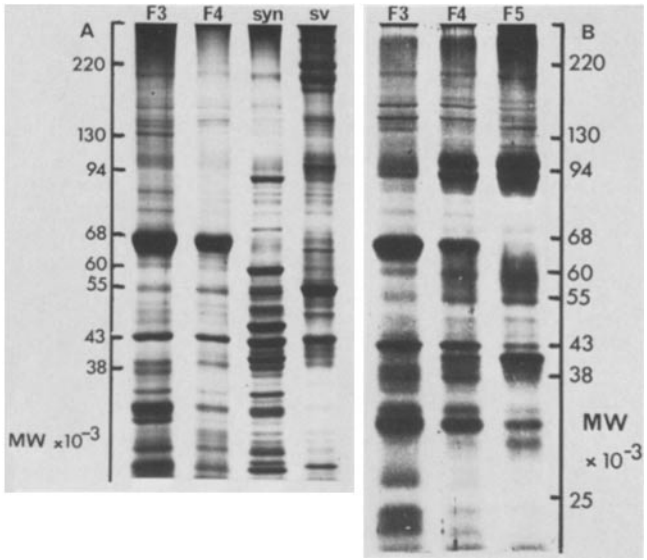

FIGURE 5 Protein pattern of SPM. SPM were prepared either (*A*) from recentrifuged synaptosomes (fraction C') or (*B*) from standard synaptosomes (fraction C). The protein pattern of SPM (*F3, F4*) is compared with that of synaptosomes (*syn*), crude synaptic vesicles (*SV*) isolated as described by Israël et al. (21), and of contaminating postsynaptic membranes (*F5*). Each sample contained ~40–50 µg protein.

only 20–40% of fraction C proteins could be attributed to synaptosomes; however, low-speed centrifugation of fraction C yielded purer synaptosomes; according to these biochemical criteria, it gave a 1.7-fold enrichment in presynaptic markers. This was due to the removal of soluble proteins and of postsynaptic membrane fragments. Thus we could conclude that presynaptic proteins represent 35–70% of the fraction C_1 proteins. In electron micrographs of this fraction, some small membrane fragments could be found between synaptosomes. They were likely to be of postsynaptic origin, and to be very rich in proteins, explaining the apparent discrepancy between morphological and biochemical data. Here, we have tried to get rid of these contaminants by recentrifugation on continuous sucrose gradients. This modification permitted us to reach an ACh specific activity of ~350 nmol/mg protein.

After synaptosomal disruption, equilibrium centrifugation permitted us to isolate three fractions of plasma membranes as shown by the distribution of plasma membrane markers (NaK-dependent ATPase and 5'-nucleotidase). One of them (F5), which contained most of the AChR sites, was markedly decreased when very pure synaptosomes were subfractionated and was therefore not of presynaptic origin. An important criterion for the recognition of the presynaptic plasma membrane came from freeze-fracture experiments. Membranes of fraction F3 and F4 contain a low intramembrane particle density, like the nerve terminal membrane in situ (37). This is in marked contrast to the plasma membrane of the postsynaptic electroplaque cells. Another criterion that was used by Stadler and Tashiro (46) is the presence of AChE in nerve terminal membranes (29, 40). Fractions F3 and F4 had a high AChE activity which remained associated with these membranes during the fractionation procedure. This is in contrast to postsynaptic AChE activity which was separated from the AChR-containing membranes during their isolation (6, 10). Fractions F3 and F4 appeared to be closely related as shown by refractionation on continuous sucrose gradient (Fig. 1). A single

protein peak and single AChE peak were found. But F3 and F4 were not identical as suggested by the nonsymmetrical peak of NaK-dependent ATPase and by the distribution of Mg-dependent ATPase which was more abundant in F3. Differences were also noticed in the SDS PAGE patterns (Fig. 5). These differences might be artificial, due, for instance, to postsynaptic membrane attachments in F4. The presence of AChR in F4 would favor this possibility in spite of the lack of morphological observations of such membrane attachments in intact synaptosomes. Alternatively, these differences might result from a heterogeneity of the SPM, domains with different intramembrane particle densities, and protein composition being separated during fractionation. This would be compatible with the fact that some protein bands present in F3 are absent in F4 (as well as in synaptic vesicles). The lack of a known specific marker for the presynaptic membrane makes it difficult to evaluate the purity of SPM in F3 and F4. The most likely contaminants of SPM are: (*a*) postsynaptic membranes, (*b*) myelin fragments, and (*c*) intrasynaptosomal constituents such as synaptic vesicles. (*Torpedo* electric organ synaptosomes are extremely poor in mitochondria [36]). Contamination by myelin fragments or synaptic vesicles ought to have been detected in morphological controls of F3 and F4 (Fig. 2); it seemed negligible. Postsynaptic membranes have a high equilibrium density and thus could be separated from the SPM by centrifugation (Fig. 1). Nicotinic AChR (measured as α-bungarotoxin binding sites blocked by *d*-tubocurarine) were virtually absent from F3. Their specific activity in F4 (~200 pmol/mg protein) was very low compared to that of purified postsynaptic membranes (4,000 pmol/mg protein; reference 45). Even if all the activity we saw was postsynaptic in origin and not due to presynaptic receptors, contamination by postsynaptic membrane proteins would have accounted for <10% of F4 proteins. Therefore, SPM fractions appear reasonably pure.

The analysis of the polypeptide profile of SPM by one-dimensional SDS PAGE revealed a complex pattern. A major band (~67,000 mol wt) was always found in fractions F3 and F4. This band was highly enriched in SPM when compared to fraction C synaptosomes. It was absent in postsynaptic membranes (fraction F5) and in synaptic vesicles (see Fig. 5 and results of Stadler and Tashiro [46] and Wagner and Kelly [47]). It appears therefore to be specific for SPM. Such a band has not been noticed in previous work except perhaps as a minor, externally oriented polypeptide in the work of Wang and Mahler (48) on rat brain SPM. Another major band comigrating with actin (in one-dimensional electrophoresis) was found in fractions F3 and F4 as well as in synaptic vesicles and in F5; this is in good accordance with the results of Stadler and Tashiro (46).

The aim of the present work was to use membrane markers to follow postsynaptic contamination and to subfractionate the purest synaptosomal fraction we could get to identify a fraction of presynaptic membrane. We were able to characterize a protein band which is the major protein constituent of this membrane. It is not found in postsynaptic membranes and synaptic vesicles and appears therefore specific for the SPM. The present work offers the possibility of an immunochemical characterization of the plasma membrane of cholinergic nerve terminals.

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