Large-scale Purification of Presynaptic Plasma Membranes from *Torpedo marmorata* Electric Organ

N. MOREL,* J. MARSAL,‡ R. MANARANCHE,* S. LAZEREG,* J. C. MAZIE,§ and M. ISRAEL*

*Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire et Moléculaire, 91190 Gif-sur-Yvette, France; ‡Département de Histologia i Biologia Cellular, Facultad de Medicina, Barcelona 36, Spain; §Hydridolab, Institut Pasteur, 75015 Paris, France.

ABSTRACT The presynaptic plasma membrane (PSPM) of cholinergic nerve terminals was purified from *Torpedo* electric organ using a large-scale procedure. Up to 500 g of frozen electric organ were fractioned in a single run, leading to the isolation of >100 mg of PSPM proteins. The purity of the fraction is similar to that of the synaptosomal plasma membrane obtained after subfractionation of *Torpedo* synaptosomes as judged by its membrane-bound acetylcholinesterase activity, the number of *Glycera convoluta* neurotoxin binding sites, and the binding of two monoclonal antibodies directed against PSPM. The specificity of these antibodies for the PSPM is demonstrated by immunofluorescence microscopy.

Synaptic transmission occurs in organized areas where specialized domains of the nerve ending plasma membrane, the so-called "active zones" (6), are facing specialized domains of the postsynaptic cell membrane. A detailed knowledge of the protein composition of the presynaptic plasma membrane (PSPM)[1] is essential to understand the events leading to transmitter release. *Torpedo marmorata* electric organs possess a very abundant and homogeneous cholinergic innervation (11). The nerve electroplaque junction has proven to be a useful model for biochemical studies on peripheral cholinergic transmission (for reviews see references 12, 16, 33, and 36). In addition to synaptic vesicles (13, 35), the presynaptic organelles where acetylcholine (ACh) is stored, nerve terminals (synaptosomes) have been isolated and purified from *Torpedo* electric organ (15, 23). PSPM fractions were obtained after subfractionation of these highly purified synaptosomes (24, 32). This two-step procedure permitted us to overcome a major difficulty, i.e., the lack of specific markers for the PSPM, but only small quantities of PSPM proteins could be obtained.

The aim of the present work was to develop a large-scale procedure permitting the purification of PSPM directly from *Torpedo* electric organ. This was possible since several proteins were recently shown to be specific for, or at least highly concentrated in, the PSPM. First, a high acetylcholinesterase (AChE) activity was found to be associated with this plasma membrane (24, 32). This AChE activity corresponds to the hydrophobic dimeric form of the enzyme (18, 22). Second, a presynaptic neurotoxin extracted from the venom glands of the polychaete annelid *Glycera convoluta* (*GCV*), which triggers a massive quantal ACh release in neuromuscular junctions and *Torpedo* electric organ (19), was found to bind specifically to a protein of the PSPM (26). Finally, two monoclonal antibodies to PSPM antigens were obtained: one (C1-8) is directed against a 67-kD ectocellularly exposed protein (21); the second (H6-1) is characterized in the present report.

Using these protein markers, we were able to isolate large amounts of PSPM by fractionating in a single run up to 500 g of frozen electric organ. The purity of the PSPM fraction was similar to that of the fraction purified from isolated synaptosomes. This large-scale procedure will enable us to attempt the purification of presynaptic membrane proteins involved in synaptic activity.

MATERIALS AND METHODS

Preparation of the PSPM Fraction: *Torpedo marmorata* were obtained alive from the Marine Station of Arcachon (France). Electric organs were dissected out and cut into 0.5-g pieces after removal of the skin and large nerve trunks. Pieces were washed in 300 mM NaCl, 1 mM EDTA, 10 mM Tris buffer, pH 8.0 (~25 g tissue per 100 ml solution) for 2 h in a cold room. They were then blotted dry and frozen in 50-g batches at −70°C. The frozen tissue

[1] *Abbreviations used in this paper:* ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; ELISA, enzyme-linked immunosorbent assay; *GCV, Glycera convoluta* venom; PSPM, presynaptic plasma membrane.

can be stored for several weeks.

PSPM were prepared from 500–550 g frozen electric organ. The tissue was allowed to thaw in 2 liters of a hypoosmotic solution (1 mM EDTA in 10 mM Tris buffer, pH 8.0) at room temperature. All following steps were performed in a cold room (about 8°C). Tissue pieces were transferred into 1.5 liters of 1 mM EDTA, 10 mM Tris, pH 8.0, and incubated for about 30 min. This solution, which contained a lot of soluble proteins, was discarded and fresh solution was added to the tissue pieces to reach a final volume of 1.5 liters. The tissue was then homogenized during 1 min using a large blender (MX 79, purchased from Dynamic, 06140 Vence, France) plunged into the suspension. The homogenization was completed in a Teflon glass homogenizer (5 up and down strokes in a 60-ml Potter). The homogenate was then filtered under vacumm through 50-μm nylon gauzes. Much material was retained on the filters, clogging the gauze which had to be replaced several times. A prefiltration through a 100-μm nylon gauze renders this step much easier with a similar result. The resulting filtrate was centrifuged (25,000 g_{max} for 60 min) in six 250-ml polycarbonate bottles in a Sorvall GSA rotor. The resulting pellets (P1) were resuspended in 0.1 mM EDTA, 10 mM Tris buffer, pH 8.0 (510 ml, final volume). The suspension was then gently deposited (170 ml × 3) in 3 GSA polycarbonate bottles above a 75-ml layer of 1.0 M sucrose in 0.1 mM EDTA, 10 mM Tris, pH 8.0, and centrifuged (25,000 g_{max} for 60 min). The supernatants (S2) were decanted carefully in order to minimize resuspension of the viscous pellets. After dilution in 0.1 mM EDTA, 10 mM Tris, pH 8.0, (1.5 liters, final volume), S2 was centrifuged (25,000 g_{max} for 180 min) in six GSA polycarbonate bottles. Pellets (P3) were resuspended in 0.1 mM EDTA, 10 mM Tris, pH 8.0 (60 ml, final volume). The suspension (10 ml × 6) was layered onto six sucrose density gradients composed of two 13-ml layers of 0.6 M and 1.0 M sucrose in 0.1 mM EDTA, 10 mM Tris, pH 8, and centrifuged overnight (25,000 rpm for about 16 h) in a Beckman SW27 rotor. PSPM were recovered at the 0.6–1.0 M sucrose interface (fraction 2). Material in the band above 0.6 M sucrose (fraction 1) and in the pellet (fraction 3) were also collected.

Biochemical Methods: Protein was determined by amidoschwarz staining (29). AChE activity (EC 3.1.1.7) was measured at room temperature according to Ellman et al. (9). Nicotinic acetylcholine receptor (AChR) was estimated by the binding of tritiated α-bungarotoxin (N-^{3}H propionyl-propionylated; TRK 603, Amersham Corp., England) as described by Schmidt and Raftery (30); we only considered the binding which was inhibited after a 15-min preincubation in the presence of 10^{-3} M d-tubocurarine (Serva, Feinbiochemica, Heidelberg, Germany).

Succinodehydrogenase activity (EC 1.3.99.1) was determined at room temperature according to Arrigoni and Singer (2). (Na^+ K^+)-activated ouabain-sensitive ATPase (EC 3.6.1.4) was measured (at room temperature) as described by Diebler and Lazereg (7). The medium contained 120 mM NaCl, 30 mM KCl, and 0.3 mM ATP. The amount of inorganic phosphate, liberated in the presence or absence of 8×10^{-5} M ouabaïne, was estimated using malachite green (27).

Estimation of GCV Neurotoxin Binding Sites: These experiments were performed as described previously (26). The polychaete annelids, *Glycera convoluta*, were collected on the seashore near the Station de Biologie Marine at Roscoff (France). The venom gland complexes were immediately dissected out and kept frozen (−70°C). Fifty complexes (corresponding to 200 venom glands) were homogenized in 3 ml of 10 mM sodium phosphate buffer, pH 7.4. After centrifugation (120,000 g_{max} for 30 min), the supernatant was filtered through a Sephadex G 75 column and the neurotoxin was recovered in the void volume (2.5 ml, final volume). Aliquots of GCV (0.1 ml, deriving from 6.7 venom glands) were incubated with known amounts of membrane fractions for 90 min at 0°C (1.5 mg protein for homogenates, 0.25 mg protein for P3 and fractions 2 and 3 in 0.2 ml *Torpedo* physiological medium, see reference 26). After centrifugation (27,000 g_{max} for 30 min) the neurotoxin activity remaining in supernatants was assayed using *Torpedo* synaptosomes and compared to that of GCV treated in parallel but without membranes.

Torpedo synaptosomes were isolated from *Torpedo* electric organ (15, 23). ACh release, triggered by the *GCV* neurotoxin, was then continuously monitored using the chemiluminescent method described by Israel and Lesbats (14), the rate of ACh release being proportional to the neurotoxin concentration (26).

Preparation of Monoclonal Antibodies: Monoclonal antibodies to synaptosomal plasma membranes were prepared as described in detail in reference 21. Briefly, mice were immunized against *Torpedo* synaptosomal plasma membranes (prepared as described in reference 24). Cell fusions were performed (as described in reference 17) with a nonsecreting myelomia clone SP 20 and spleen cells. Hybrids were selected in hypoxanthine/aminopterin/thymidine medium. We selected cell clones secreting antibodies to *Torpedo* synaptosomal plasma membranes (fractions 3 and 4 in reference 24) and not to plasma membranes of the electroplaques (fraction 5 in reference 24) by an enzyme-linked immunosorbent assay (ELISA) (see below). These clones were expanded in vivo in syngenic mice (primed with Pristane) to obtain ascitic fluids which were stored frozen at −20°C.

Solid-Phase Assay of Antibody Binding: The binding of monoclonal antibodies to Torpedo plasma membranes was measured using the ELISA (10). Microtiter plates (with 96 flat-bottomed 300-μl wells, Nunc, Denmark) were coated with PSPM (10 μg protein/ml in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.02% NaN_3) as described previously (25). Plates were rinsed with Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.4, containing 0.1% Tween 20). Monoclonal antibodies, at various dilutions in TBS containing 1% bovine serum albumin (BSA), were incubated (200 μl/well) for 2 h at room temperature. After extensive washing in TBS, peroxidase-labeled antibodies to mouse Ig (purchased from Institut Pasteur, Paris, France) diluted 200 times in TBS + 1% BSA (200 μl/well for 2 h at room temperature) were used to detect bound monoclonal antibodies. After washing of the microtiter plates, bound peroxidase activity was measured using orthophenylene diamine (Sigma Chemical Co.) as the substrate.

To compare the antigen concentration in different membrane fractions, a competition binding assay was used. A fixed monoclonal antibody dilution which gives a half-maximal binding to PSPM upon an ELISA was determined (a 25,000-fold dilution for the H6-1 ascitic fluid). Increasing amounts of the different membrane fractions were preincubated (30 min at room temperature) with the diluted ascitic fluid (in 250 μl, final volume, of TBS + 1% BSA). Each preincubated sample was then transferred to a well of a microtiter plate coated with PSPM and the monoclonal antibody binding to PSPM fixed on the plate measured by ELISA as above. The higher the amount of antigen in the preincubation sample, the lower the amount of free antibody able to bind to PSPM in the ELISA. The amount of antigen which gives, in this competition ELISA, a 50% inhibition of antibody binding was taken as one arbitrary unit.

Immunoblot Experiment: SDS PAGE was performed in a 5–15% acrylamide gradient gel overlayered with a 3% acrylamide stacking gel. Samples (~40 μg protein) were boiled (3 min in a lysis buffer (containing 2.5% SDS and 5% β-mercaptoethanol, final concentration). Electrophoresis was carried out at 40 mA constant current. Protein bands were transferred by diffusion onto two nitrocellulose filters according to Bowen et al. (4) as described previously (21). One filter was used to detect monoclonal antibody (C1--8 ascitic fluid) binding (19) and the second blot for protein staining by the amidoschwarz method (29). Molecular weight protein markers were obtained from Bio-Rad Laboratories, Richmond, CA.

Electron Microscopy: The PSPM fraction was pelleted (160,000 g_{max} for 10 min in a Beckman airfuge) and the pellet was fixed in 3% glutaraldehyde in 0.5 M cacodylate buffer, postfixed in 2% OSO_4, dehydrated, and embedded in Spurr's resin as described in reference 23.

Immunofluorescence: Tissue pieces were fixed in 3% formaldehyde in 0.5 M Tris, pH 7.5, for 1 h at 4°C. We used either prisms of electric organ or the rostrolateral muscle which runs, internally to the electric organ, from a marked cartilagenous apophyse to the anterior part of the rostra. Electroplaques or muscles fibers were then dissected and carefully placed on microscope slides coated with gelatin. H6-1 monoclonal antibody (1:100 to 1:500 dilution of ascitic fluid in 1% BSA, 0.1% Triton X-100 in 0.5 M Tris buffer, pH 7.5, was allowed to bind overnight at 4°C. H6-1 binding was visualized by indirect immunofluorescence using fluoresceine-labeled anti-mouse IgG antibody (purchased from Institut Pasteur Production).

RESULTS

Isolation of a PSPM Fraction

It has been shown previously that the synaptosomal plasma membrane could be clearly separated from plasma membranes of the postsynaptic cell—i.e., the electroplaque—on the basis of their different equilibrium densities (24). Postsynaptic membrane fragments with a high nicotinic AChR content and fragments of the dorsal non-innervated face of the electroplaques with a high Na^+ K^+-ATPase activity have similar equilibrium densities heavier than 1.0 M sucrose (1, 8). We took advantage of this observation to develop a large-scale procedure that allowed PSPM to be purified from 500 g of electric organ in a single run. Several features of the procedure have to be stressed. (*a*) All the fractionation was done in calcium-free medium containing EDTA since this was shown to minimize proteolysis in *Torpedo* electric organ (28). (*b*) The disruption of large amounts of electric organ was achieved by a combination of three procedures: freezing

and thawing, hypoosmotic shock, and homogenization using a large blender. (*c*) The large volumes involved required centrifugation in the Sorvall GSA rotor which has a high (1.5-liter) capacity but allows only moderate accelerations (25,000 g_{max}). Nevertheless it was possible to separate PSPM from 80% of the particulate protein before the final untracentrifugation step: filtration through nylon gauzes removed a lot of white fibrous material and a centrifugation over a 1-M sucrose layer permitted one to discard material associated with high density particles which sedimented. (*d*) Membranes still in suspension could be pelleted after a prolonged centrifugation of the supernatant S2, diluted to decrease its sucrose concentration below 0.1 M. The amount of material was now compatible with equilibrium centrifugation in sucrose density gradients. The PSPM-containing fraction was recovered at the 0.6–1.0 M sucrose interface.

Characterization of the PSPM Fraction

We have identified the PSPM–containing fraction by titrating several proteins which have been shown to be specific for, or at least highly concentrated in, the presynaptic plasma membrane (Table I).

AChE activity was maximum in fraction 2, with a high specific activity (Table I). None of this AChE activity was extracted in the presence of 1 M $MgCl_2$ (94 ± 1%, mean ± SEM from three experiments) but it was solubilized by 0.1% Triton X-100 (95 ± 3%, three experiments). Therefore, AChE activity in fraction 2 behaves as has been reported for presynaptic AChE activity (18, 22).

A presynaptic neurotoxin of *GCV* (19) binds specifically to an ectocellularly exposed protein of the plasma membrane of *Torpedo* synaptosomes (26). The number of binding sites in the membrane fractions can be indirectly estimated (26) (see also Materials and Methods) in spite of the fact that the neurotoxin is not available in a pure and active form. It was related to the amount of neurotoxin (extracted from *x* venom glands) bound to the membranes (prepared from *y* g electric organ) and expressed as gland equivalents/g electric organ. The number of *GCV* neurotoxin binding sites was five times higher in fraction 2 as compared to fraction 3 and was not detectable in fraction 1. Membranes in fraction 2 were able to bind the neurotoxin extracted from 13.3 ± 1.7 venom glands/mg protein.

Most of the nicotinic AChR was recovered in fraction 3 and the fraction 2 content was low with a very low specific activity.

Finally, we have estimated the number of antigenic sites binding a monoclonal antibody H6-1 directed to synaptosomal plasma membrane fractions (prepared as in reference 24). The specificity of H6-1 binding will be illustrated by indirect immunofluorescence microscopy in the second part of this work. The antigen H6-1 was mainly recovered in fraction 2 with a specific activity five times higher than that of fraction 3.

In addition, the activity in fraction 2 of the Na^+ K^+-dependent ATPase, a nonspecific membrane marker, was measured: 624 ± 43 nmol/h per g electric organ (mean ± SEM from three experiments) which corresponds to 2% of the homogenate activity (35,000 nmol/h per g electric organ, mean from two experiments). Virtually no succinodehydrogenase, an enzyme associated with internal mitochondrial membranes, was recovered in fraction 2 (0.06 ± 0.06 O.D. unit/h. per g electric organ, mean ± SEM from three experiments), whereas the homogenate activity was 12.4 ± 0.4 O.D. units/h. per g electric organ (mean ± SEM from two experiments).

It was checked, after SDS PAGE of the membrane proteins and immunoblotting, that the 67,000-D protein recognized by the monoclonal antibody C1-8 (21) was enriched in the PSPM fraction as compared to the other membrane fractions (Fig. 1). After SDS gel electrophoresis, monoclonal antibody H6-1 was no longer able to bind to PSPM proteins. The

TABLE I. *Protein Markers in Membrane Fractions*

			Fractions			Number of experiments
	Homogenate	Pellets (P3)	1	2	3	
Protein						
mg/g electric organ	12.2 ± 1.7	1.0 ± 0.1	0.05 ± 0.01	0.21 ± 0.03	0.87 ± 0.20	8
AChE						
mmol/h per g electric organ	20.9 ± 3.8	3.8 ± 0.8	0.11 ± 0.02	2.14 ± 0.47	1.25 ± 0.36	
(mmol/h per mg protein)	(1.8 ± 0.3)	(3.7 ± 0.6)	(2.2 ± 0.2)	(10.1 ± 1.1)	(1.5 ± 0.3)	8
Liaison GCTx						
gland equivalents/g electric organ	20.4 ± 1.7	5.0 ± 1.0	—	3.6 ± 0.5	0.7 ± 0.5	
(gland equivalents/mg protein)	(1.5 ± 0.2)	(4.5 ± 1.2)		(13.3 ± 1.7)	(1.1 ± 0.7)	5
Nicotinic AChR						
pmol/g electric organ	720 ± 303	187 ± 33	0.2 ± 0.2	8.7 ± 3.2	242 ± 71	
(pmol/mg protein)	(44 ± 11)	(156 ± 23)	(3 ± 3)	(30 ± 7)	(204 ± 27)	4
Ag H6-1						
A.U./g electric organ	878 ± 409	253 ± 46	9 ± 2	151 ± 20	90 ± 21	
(A.U./mg protein)	(69 ± 34)	(211 ± 14)	(169 ± 44)	(563 ± 80)	(106 ± 34)	5

Results are mean ± SEM of the number of experiments indicated. They are expressed per gram of electric organ fractionated. Specific activities (in brackets) are mean ± SEM of specific activities determined in each experiment. Fraction H corresponds to the initial homogenate, P3 is the fraction layered onto the sucrose gradient, and fraction 2 is the PSPM.
A.U., arbitrary units.

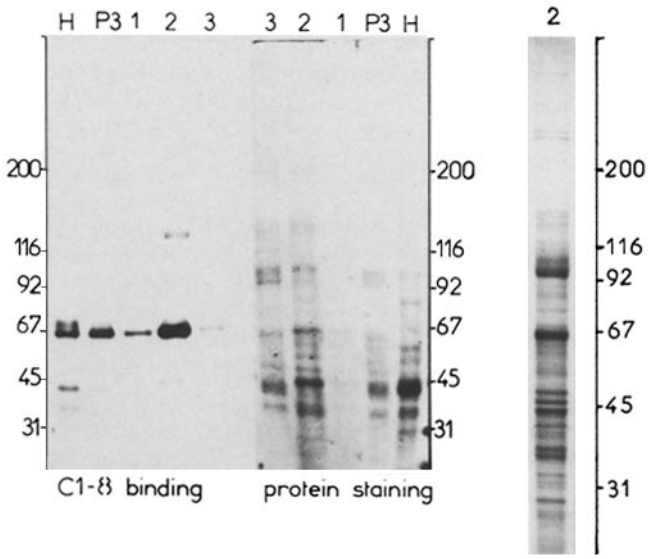

FIGURE 1 Binding of C1-8 to membrane fractions. Membrane proteins (40 μg/fraction) were submitted to SDS PAGE under reducing conditions and protein bands transferred onto nitrocellulose filters. A blot (*left*) was used to detect the binding of the monoclonal antibody, C1-8 (1:25,000 dilution of the ascitic fluid), while the second filter (*middle*) was stained for protein. PSPMs were recovered in fraction 2. For comparison, the protein pattern of the PSPM fraction is shown (*right*) after Coomassie Blue staining of the gel.

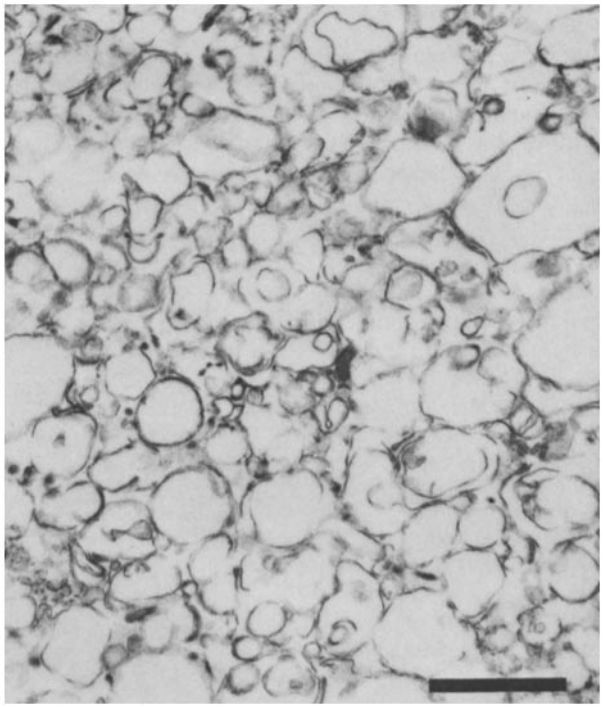

FIGURE 2 Electron micrograph of the PSPM-containing fraction (fraction 2). Bar, 1 μm.

identification of the protein carrying the antigenic site H6-1 was therefore not possible using immunoblot techniques.

The membrane fraction 2 was examined by electron microscopy. Fig. 2 shows a large field of membranes fixed in glutaraldehyde and observed after conventional treatment. It must be stressed that myelin sheets were absent from fraction 2.

Binding of the H6-1 Monoclonal Antibody

The monoclonal antibodies C1-8 and H6-1 were prepared from mice immunized with PSPMs obtained by subfractionation of *Torpedo* synaptosomes (24) and were selected on the basis of their binding to these synaptosomal plasma membranes and not to postsynaptic membranes (prepared as in reference 24). Using immunoblot techniques, C1-8 was shown to bind to PSPMs in *Torpedo* electric organ and not to electroplaque plasma membranes (either from dorsal or ventral faces) or to membranes prepared from electric nerve trunks or electric lobes (21). C1-8 antibody binding to PSPMs was visualized on frozen sections of electric organ by indirect immunofluorescence (Fig. 3*a*). A similar pattern of binding to the electroplaque innervated face was observed for H6-1 antibody (Fig. 3*b*). When electric organ membrane sheets with attached nerve terminals were incubated with H6-1 (Fig. 3*c*), the fluorescence was restricted to the unmyelinated axons and the nerve endings.

As shown by ELISA (see Materials and Methods), H6-1 binds to fraction 2 membranes (Fig. 4*a*). This binding was suppressed after adsorption of H6-1 ascitic fluid with the

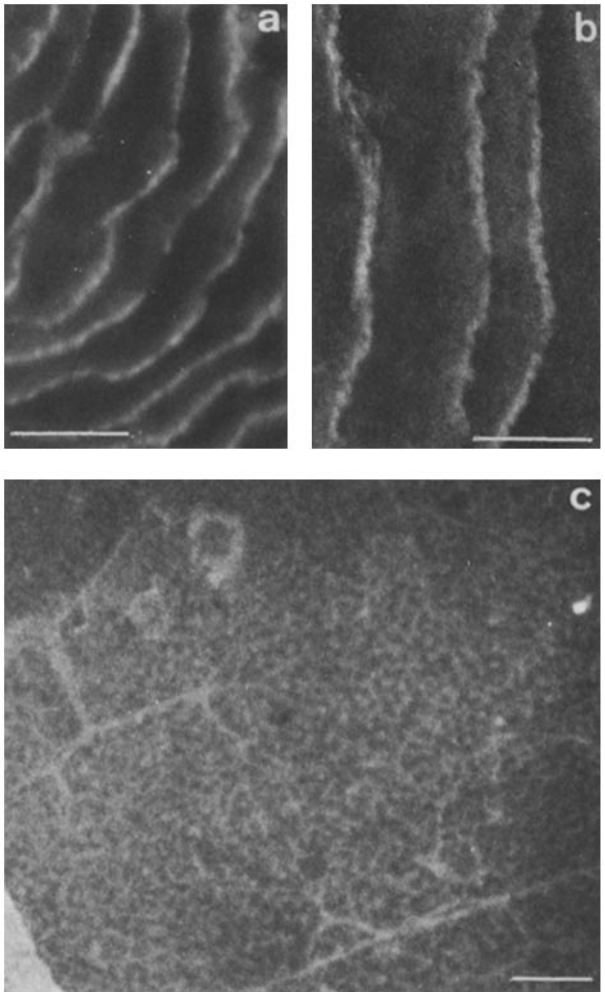

FIGURE 3 Visualization of H6-1 binding in *Torpedo* electric organ. Electric organ frozen sections were incubated with either C1-8 (*a*) or H6-1 (*b*) ascitic fluid. Monoclonal antibody binding was visualized by indirect immunofluorescence. In c, an electric organ membrane sheet was incubated with H6-1, which binds to the nerve terminal network covering the postsynaptic membrane. Bar, 20 μm.

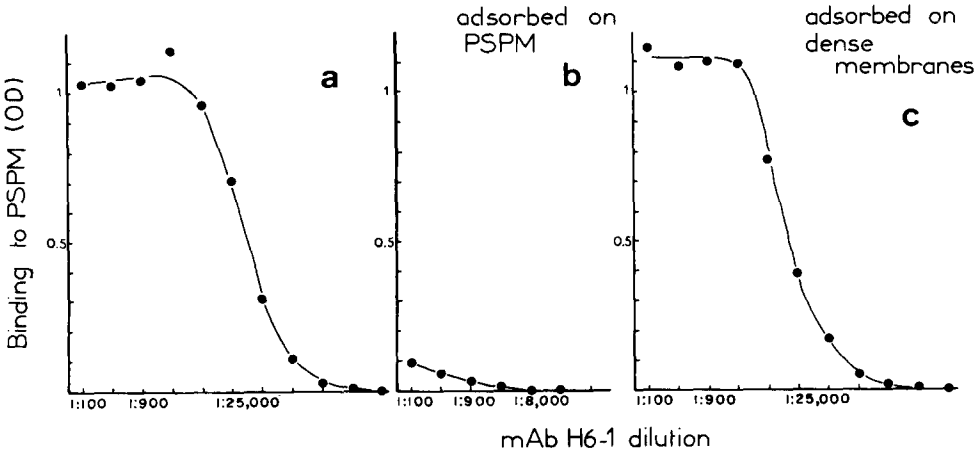

FIGURE 4 H6-1 binding to PSPM. Microtiter plates were coated with PSPM. Monoclonal antibody binding was measured by an ELISA as a function of increasing dilutions of H6-1 ascitic fluid. The binding to PSPM of H6-1 (*a*) is compared with that of the ascitic fluid after adsorption either on fraction 2 membranes (*b*) or fraction 3 membranes (*c*).

membrane fraction 2 (50 μl H6-1 for 9 mg PSPM protein) as demonstrated Fig. 4*b*. In contrast, no modifications were observed (Fig. 4*c*) when H6-1 was adsorbed on fraction 3 membranes which have been resedimented through a 1-M sucrose layer (50 μl H6-1 ascitic fluid for 21 mg of fraction 3 protein). We have studied in parallel, by indirect immunofluorescence, the binding of the monoclonal antibody H6-1 to *Torpedo* neuromuscular junctions (Fig. 5). It can be seen that motor end plates are labeled by H6-1 (Fig. 5*a*) showing that the antigen is not restricted to the electric organ. It was possible to find one end-plate for almost all the muscular fibers examined. After adsorption of H6-1 with PSPM, the fluorescent labeling was completely abolished (Fig. 5*b*). Adsorption of H6-1 with fraction 3 membranes did not modify the motor end-plate staining (Fig. 5*c*). Since fraction 3 contained a significant amount of postsynaptic membrane as shown by nicotinic AChR measurements (Table I), H6-1 binds to a presynaptic membrane antigen. It should be noticed that the fluorescent staining is restricted to nerve terminals.

DISCUSSION

In the present report, we describe a large-scale procedure to purify the PSPM from *Torpedo* electric organ. Procedures used up to now took advantage of the high purity of *Torpedo* synaptosomes (15, 23) to isolate the PSPM after subfractionation of these pure cholinergic synaptosomes (24, 32). Nonspecific plasma membrane markers (5'nucleotidase, Na-K-ATPase) were found associated with the PSPM which also presented a very high membrane-bound AChE activity (24, 32). This result is not in agreement with the report that these enzymatic activities were not associated with presynaptic membranes after lysis of immunoadsorbed *Torpedo* synaptosomes (20). These enzymes might have been inactivated during the lysis-sonication step rather than released as contaminating proteins (no measurements of activities in the supernatant were reported). The AChE activity bound to the PSPM corresponds to the hydrophobic dimeric form of the enzyme (18, 22) which is easily distinguished by its solubilization properties from the collagen-tailed forms (3, 34) which appear to be associated to the basal lamina. About 50% of AChE activity in *Torpedo* electric organ homogenates corresponds to the hydrophobic form (3, 34). Therefore up to 20% of the membrane-bound AChE is recovered in the PSPM fraction (Table I) with a high specific activity (10 mmol/h per mg protein) which is comparable to that of PSPM fractions prepared from lysed *Torpedo* synaptosomes (10.5 mmol/h per mg protein in reference 24 and 7 mmol/h.mg protein in reference 32).

Another protein appears of interest to follow the purification of the PSPM, the presynaptic neurotoxin of *GCV*. This neurotoxin was shown to trigger a massive calcium-dependent ACh release in *Torpedo* electric organ and at frog neuromuscular junctions (19). This effect requires the binding of the neurotoxin to a PSPM-specific protein (26). The number of binding sites for the presynaptic neurotoxin in the PSPM fraction has been estimated to be 13.3 gland equivalents/mg protein, a value comparable to that determined on PSPM prepared from purified synaptosomes (about 20 gland equivalent/mg protein). As much as 18% of *GCV* neurotoxin binding sites, present in the homogenate, were recovered in the PSPM fraction (Table I).

Two monoclonal antibodies to antigens specific for the PSPM have been obtained. One (C1-8) was previously reported, on an immunochemical basis, to bind to a 67,000-D protein which would be specific for the nerve-ending plasma membrane (21). This monoclonal antibody might recognize the same antigen as the monoclonal antibody tor 23 studied by Buckley et al. (5). The antigen recognized by C1-8 is concentrated in the PSPM fraction, as expected (Fig. 1). The second monoclonal antibody (H6-1) is directed to another antigen in the PSPM, the number of antigenic sites for H6-1 being 2 times that for C1-8 (not shown). By competitive ELISA, it was possible to demonstrate that 17% of the antigen in the homogenate was recovered in the PSPM fraction (Table I) with an eightfold enrichment. It is shown by immunofluorescence microscopy that H6-1 binds to a presynaptic antigen that is also present at *Torpedo* neuromuscular junctions (Fig. 5).

The nicotinic AChR was used as a negative marker to estimate the contamination of the PSPM fraction by postsynaptic membrane fragments. About 1% of the homogenate AChR was found in the PSPM fraction with a specific activity of 30 pmol/mg protein. Considering that purified postsynaptic membrane fragments have about 4,000 pmoles AChR/mg protein (31), <1% contamination of PSPM fraction by postsynaptic plasma membrane is expected. Taking into account the specific activity of fraction 3 (210 pmol AChR/mg protein), a 15% contamination of the PSPM fraction by heavy membranes can be estimated. On the other hand, when compared to highly purified PSPM prepared from synaptosomes recentrifuged on continuous sucrose gradients (AChE specific activity = 25 mmol/h.mg protein [see Table IV in

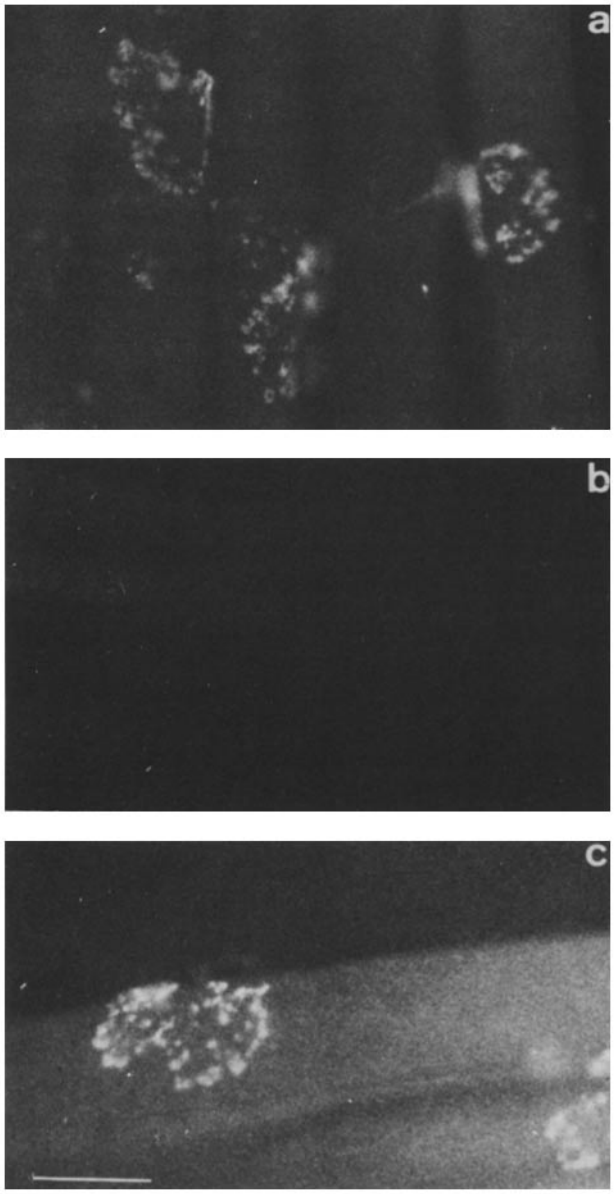

FIGURE 5 H6-1 binding to neuromuscular junctions. *Torpedo* muscular fibers were fixed and incubated with H6-1 ascitic fluid (1:500 dilution). H6-1 binding was visualized by indirect immunofluorescence. The labeling observed with H6-1 (a) is suppressed after adsorption of the ascitic fluid on fraction 2 membranes (*b*) but is not modified after adsorption on fraction 3 membranes (c). Same antibodies as in Fig. 4. Bar, 50 μm.

reference 24), this large scale preparation of PSPM would be 40% pure.

Based on these data, the large-scale procedure described permits the isolation of a PSPM fraction of similar purity as those prepared from *Torpedo* synaptosomes. About 20% of the PSPM of *Torpedo* electric organ homogenates are recovered in the PSPM fraction and, considering the large amount (500 g) of tissue fractionated, >100 mg of presynaptic membrane can be prepared in a single run. This will allow the purification of PSPM proteins of physiological importance for the release of transmitter and synaptic transmission.

Received for publication 19 April 1985, and in revised form 30 May 1985.

REFERENCES

1. Allen, T., and L. T. Potter. Postsynaptic membranes in the electric organ of *Narcine*. III. Isolation and characterization. *Tissue & Cell.* 9:609–622.
2. Arrigoni, O., and T. P. Singer. 1962. Limitations of the phenazine methosulfate assay for succinic and related dehydrogenases. *Nature (Lond.).* 193:1256–1258.
3. Bon, S., and J. Massoulie. 1980. Collagen-tailed and hydrophobic components of acetylcholinesterase in *Torpedo marmorata* electric organ. *Proc. Natl. Acad. Sci. USA.* 77:4464–4468.
4. Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub. 1980. The detection of DNA binding proteins by protein blotting. *Nucleic Acids Research.* 8:1–20.
5. Buckley, K. M., E. S. Schweitzer, G. P. Miljanich, L. Clift-O'Grady, P. D. Kushner, L. F. Reichardt, and R. B. Kelly. 1983. A synaptic vesicle antigen is restricted to the junctional region of the presynaptic plasma membrane. *Proc. Natl. Acad. Sci. USA.* 80:7342–7346.
6. Couteaux, R., and M. Pecot-Dechavassine. 1974. Les zones spécialisées des membranes présynaptiques. *C.R. Acad. Sci., Paris D.* 278:291–293.
7. Diebler, M. F., and S. Lazereg. 1985. Mg-ATPase and Torpedo cholinergic synaptic vesicles. *J. Neurochem.* 44:1633–1641.
8. Duguid, J. R., and M. A. Raftery. 1973. Fractionation and partial characterization of membrane particles from *Torpedo californica* electroplax. *Biochemistry.* 12:3593–3597.
9. Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone. 1961. A new and rapid colorimetric determination of AChE activity. *Biochem. Pharmacol.* 7:88–95.
10. Engvall, E. 1980. Enzyme Immunoassay ELISA and EMIT. *In* Methods *Enzymol.* 70:419–438.
11. Feldberg, W., A. Fessard, and D. Nachmansohn. 1940. The cholinergic nature of the nervous supply to the electric organ of the *Torpedo. J. Physiol. (Lond.).* 97:3P.
12. Israel, M., Y. Dunant, and R. Manaranche. 1979. The present status of the vesicular hypothesis. *Prog. Neurobiol. (Oxf.).* 13:237–275.
13. Israel, M., J. Gautron, and B. Lesbats. 1968. Isolement des vésicules synaptiques de l'organe électrique de la *Torpille* et localisation de l'acétylcholine à leur niveau. *C.R. Acad. Sci. Paris D.* 266:273–275.
14. Israel, M., and B. Lesbats. 1981. Chemiluminescent determination of acetylcholine, continuous detection of its release from *Torpedo* electric organ synapses and synaptosomes. *Neurochem. Int.* 3:81–90.
15. Israel, M., R. Manaranche, P. Mastour-Frachon, and N. Morel. 1976. Isolation of pure cholinergic nerve endings from the electric organ of *Torpedo marmorata. Biochem. J.* 160:113–115.
16. Kelly, R. B., J. W. Deutsch, S. S. Carlson, and J. A. Wagner. 1979. Biochemistry of neurotransmitter release. *Ann. Rev. Neurosci.* 2:399–446.
17. Kohler, G., and C. Milstein. 1975. Continuous culture of fused cells secreting antibodies of predefined specificity. *Nature (Lond.).* 256:495–497.
18. Li, Z. Y., and C. Bon. 1983. Presence of a membrane-bound acetylcholinesterase form in a preparation of nerve endings from *Torpedo marmorata* electric organ. *J. Neurochem.* 40:338–349.
19. Manaranche, R., M. Thieffry, and M. Israel. 1980. Effect of the venom of *Glycera convoluta* on the spontaneous quantal release of transmitter. *J. Cell. Biol.* 85:446–458.
20. Miljanich, G. P., A. R. Brasier, and R. B. Kelly. 1982. Partial purification of presynaptic plasma membrane by immunoadsorption. *J. Cell Biol.* 94:88–96.
21. Morel, N., S. Birman, S. Rouyre, J. C. Mazie, and M. Israel. 1985. Characterization of two ectocellularly oriented 67 K presynaptic proteins. Lack of effect of their removal on acetylcholine release. *Neurochem. Int.* 7:589–600.
22. Morel, N., and P. Dreyfus. 1982. Association of acetylcholinesterase with the external surface of the presynaptic plasma membrane in *Torpedo* electric organ. *Neurochem. Int.* 4:283–288.
23. Morel, N., M. Israel, R. Manaranche, and P. Mastour-Frachon. 1977. Isolation of pure cholinergic nerve endings from *Torpedo* electric organ. Evaluation of their metabolic properties. *J. Cell Biol.* 75:43–55.
24. Morel, N., R. Manaranche, M. Israel, and T. Gulik-Krzywicki. 1982. Purification of a presynaptic plasma membrane fraction from *Torpedo* cholinergic synaptosomes: evidence for a specific protein. *J. Cell Biol.* 93:349–356.
25. Morel, N., R. Manaranche, and M. Israel. 1982. Evidence for a specific protein associated to the plasma membrane of cholinergic synaptosomes. *J. Physiol. (Paris).* 78:433–443.
26. Morel, N., M. Thieffry, and R. Manaranche. 1983. Binding of a *Glycera convoluta* neurotoxin to cholinergic nerve terminal plasma membranes. *J. Cell Biol.* 97:1737–1744.
27. Muszbek, L., T. Szabo, and L. Fesus. 1977. A highly sensitive method for the measurement of ATPase activity. *Anal. Biochem.* 77:286–288.
28. Saitoh, T., R. Oswald, L. P. Wennogle, and J. P. Changeux. 1980. Conditions for the selective labelling of the 66,000 dalton chain of the acetylcholine receptor by the covalent non-competitive blocker 5-azido-^{3}H trimethisoquin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 116:30–36.
29. Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive and specific method for the determination of proteins in dilute solution. *Anal. Biochem.* 56:502–514.
30. Schmidt, J., and M. A. Raftery. 1973. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* 52:349–354.
31. Sobel, A., M. Weber, and J. P. Changeux. 1977. Large scale purification of the acetylcholine-receptor-protein in its membrane bound and detergent extracted forms from *Torpedo marmorata* electric organ. *Eur. J. Biochem.* 80:215–224.
32. Stadler, H., and T. Tashiro. 1979. Isolation of synaptosomal plasma membranes from cholinergic nerve terminals and a comparison of their proteins with those of synaptic vesicles. *Eur. J. Biochem.* 101:171–178.
33. Tauc, L. 1979. Are vesicles necessary for release of acetylcholine at cholinergic synapses? *Biochem. Pharmacol.* 27:3493–3498.
34. Viratelle, O. M., and S. A. Bernhard. 1980. Major component of acetylcholinesterase in *Torpedo* electroplax is not basal lamina associated. *Biochemistry.* 19:4999–5007.
35. Whittaker, V. P., W. B. Essman, and G. H. C. Dowe. 1972. The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family *Torpidinidae. Biochem. J.* 128:833–845.
36. Zimmermann, H. 1979. Commentary: vesicle recycling and transmitter release. *Neuroscience.* 4:1773–1803.