Partial Purification of Presynaptic Plasma Membrane by Immunoadsorption

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ABSTRACT During transmitter release, synaptic vesicle membrane is specifically inserted into the nerve terminal plasma membrane only at specialized sites or "active zones." In an attempt to obtain a membrane fraction enriched in active zones, we have utilized the electric organ of the marine ray. From this organ, a fraction enriched in nerve terminals (synaptosomes) was prepared by conventional means. These synaptosomes were bound to microscopic beads by an antiserum to purified electric organ synaptic vesicles (anti-SV). The success of this immunoadsorption procedure was demonstrated by increased specific activities of bead-bound nerve terminal cytoplasmic markers and decreased specific activities of markers for contaminating membranes. To obtain a presynaptic plasma membrane (PSPM) fraction, we lysed the bead-bound synaptosomes by hypoosmotic shock and sonication, resulting in complete release of cytoplasmic markers. When the synaptosomal fraction was surface-labeled with iodine before immunoadsorption, 10% of this label remained bead-bound after lysis, compared with 2% of the total protein, indicating an approximately fivefold enrichment of bead-bound plasma membrane. Concomitantly, the specific activity of bead-bound anti-SV increased ~30-fold, indicating an enrichment of plasma membrane which contained inserted synaptic vesicle components. This PSPM preparation is not simply synaptic vesicle membrane since two-dimensional electrophoresis revealed that the polypeptides of the surface-iodinated PSPM preparation include both vesicle and numerous nonvesicle components. Secondly, antiserum to the PSPM fraction is markedly different from anti-SV and binds to external, nonvesicle, nerve terminal components.

Membranes appear to move from one compartment within a cell to another in the form of membrane vesicles of various sizes. How the membrane-transporting vesicle recognizes its appropriate target membrane and fuses with it is unknown. An example of such a pathway involves the synaptic vesicle in the neuron. The synaptic vesicle discharges its contents by fusing its membrane with the plasma membrane, but only at specialized sites on the nerve terminal plasma membrane, the so-called active zones (5). The active zone region of nerve terminal plasma membrane should contain, therefore, the molecular machinery involved in membrane-vesicle recognition and fusion. As a first step towards understanding the events involved in the recognition of the plasma membrane by the synaptic vesicle, we are isolating the subcellular elements that are involved. The purification of synaptic vesicles has been reported (2). Here we describe a partial purification of the presynaptic plasma membrane.[1]

The isolation of nerve terminal surface membrane is facilitated if large amounts of nerve terminals are available in a reasonable degree of purity. The synaptosome preparation from the electric organ of marine rays, first described by Israel et al. (13) and subsequently in modified form by others (18; footnote 2) seems to fulfill these requirements. A preparation of putative presynaptic plasma membrane (PSPM) has already been described (26) involving fractionation of lysed synaptosomes on density gradients. However, in this work the evidence for a PSPM-enriched fraction was somewhat equivocal since no unique biochemical or morphological marker is available in electric organ that distinguishes this plasma membrane from others in nerves or Schwann cells.

The morphological correlates of exocytosis have been investigated successfully in nonneural systems by attaching the

[1] A preliminary report of this work was presented at the Third Biophysical Discussions and appeared in the *Biophysical Journal* (19).

[2] Hooper, J. E., J. W. Deutsch, G. P. Miljanich, A. R. Brasier, and R. B. Kelly, manuscript in preparation.

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plasma membrane of a cell to a solid substrate and then removing the remainder of the cell by mechanical shear (28). This has the advantage of exposing the cytoplasmic face of the plasma membrane with its associated recognition and fusion sites. We have followed a similar approach in isolating the PSPM, with two modifications. First, large surface areas are needed to isolate enough membrane for biochemical analysis. For this reason we have used microscopic beads as the substrate for binding rather than flat surfaces (3). Secondly, instead of using nonspecific charge interactions to bind the synaptosomes to the beads we have used antibodies that specifically bind to nerve terminals, in a modification of the procedure of Ito and Palade (14).

The antibodies used for specific immunoadsorption were those raised to synaptic vesicles purified from the electric organ of *Narcine brasiliensis* (2). The anti-synaptic vesicle (anti-SV) antibodies after suitable adsorption, bind selectively to synaptic vesicle determinants. They bind to antigens inside the nerve terminal of the resting frog neuromuscular junction but not to the outside surface (30). After extensive exocytosis, vesicle antigens can be detected on the junctional nerve terminal surface. In contrast, preparations of intact electric organ synaptosomes, not purposely stimulated, bind anti-SV antibodies.[2] The binding of antibodies from the anti-SV serum to the surface of synaptosomes could be accounted for if the synaptic vesicle preparation contained antigens also present on the outside of nerve terminals of resting electric organ. Alternatively, antibodies could specifically recognize only synaptic vesicle antigens, but some of these are transferred to the outside of the nerve terminal by exocytosis during synaptosome isolation (11). Nonetheless, as will be demonstrated, the binding of antibodies to the outside of synaptosomes makes possible the *selective* adsorption of synaptosomes to the surfaces of beads modified to recognize and bind antibodies.

We confirm here that anti-SV does bind to the outside of synaptosomes by demonstrating that membrane-bounded soluble components of the synaptosomal cytoplasm become bead-bound after immunoadsorption. The immunoadsorption procedure has an advantage over nonselective binding procedures, using lectins or cationic surfaces for example, in that it also purifies the synaptosomes away from contaminating membranes. Lysis of the synaptosomes, monitored by release of soluble contents, is effected by osmotic shock and sonication. We present evidence that the resulting membrane fraction is enriched in the presynaptic plasma membrane (PSPM) of the nerve terminal. We also demonstrate that this membrane fraction is an effective immunogen, giving rise to antibodies that bind to antigens on the outer surface of nerve terminals.

MATERIALS AND METHODS

Preparation of Antisera

Rabbit anti-SV antiserum was obtained from rabbits that had been intradermally injected with highly purified electric organ synaptic vesicles emulsified in Freund's adjuvant as described in detail elsewhere (1). Rabbit anti-PSPM antiserum was obtained in a similar fashion, except that emulsified, bead-bound plasma membrane was used as the immunogen.

Solid Phase Tray Assay of Antibody Titer

The titers of antisera against a variety of antigens were determined by the solid phase tray assay (15, 24) incorporating the modifications of Carlson et al. (1).

Adsorption of Antisera

Before use, anti-SV antiserum was adsorbed with resuspended pellet material from the first (10,000 *g*) centrifugation step of the electric organ synaptic vesicle preparation scheme (2). This pellet material was resuspended in an approximately equal volume of homogenization buffer (0.4 M NaCl, 10 mM HEPES, 10 mM EGTA, 0.02% NaN_3, and a saturating amount of the antioxidant 3,5-di-tert-butyl-4-hydroxylbenzyl ether) and stored frozen. First, several ratios of 10,000*g* pellet material to anti-SV antiserum were subjected on a small scale to the adsorption procedure described below for larger amounts. The ratio of 10,000-*g* pellet to anti-SV antiserum which gave the maximum ratio of synaptic vesicle titer to non-synaptic vesicle membrane ("excluded material," see reference 2) titer while maintaining a high titer to synaptic vesicles was scaled up for the adsorption of large amounts of anti-SV antiserum. Typically, for the large-scale adsorptions, 25 ml of the 10,000-*g* pellet suspension was washed twice with homogenization buffer by centrifugation (12,000 *g*, 30 min) and resuspended. 5 ml of anti-SV antiserum or preimmune serum was then added to the pellet suspension and the mixture rotated overnight at 4°C. The suspension was then centrifuged at 12,000 *g* for 30 min and the supernatant collected. The pellet was resuspended in homogenization buffer and recentrifuged, and the supernatant was collected and combined with the first. The IgG fraction of the combined supernatants was purified on a column of protein A (*Staphylococcus aureus*) linked to Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (bed volume, 5 ml). The bound IgGs were washed with ~50 ml of homogenization buffer, then were eluted with 0.1 M acetic acid, 0.35 M NaCl, pH 4.0. The IgGs were collected until no more protein could be detected (~50 ml). The solution was concentrated to ~5 ml in an Amicon ultrafiltration cell over an XM-100A filter. The concentrated solution was washed twice by filtration with homogenization buffer and filtered to a final volume of 5 ml. 1-ml aliquots were stored frozen. Immediately before use, the adsorbed antiserum was centrifuged at 12,000 *g* for 15 min in a Brinkmann microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY) to remove small amounts of suspended aggregates.

For the immunoadsorption experiment discussed in Fig. 7, the anti-PSPM antiserum and the corresponding preimmune serum were preadsorbed with disrupted synaptic vesicles to remove anti-SV antibodies. Synaptic vesicles were subjected to three cycles of freezing and thawing and to eight 15-s periods of sonication by placing the tube against the probe of a Branson W185 sonifier (Branson Sonic Power Co., Danbury, CT) at a power setting of 7 (output ~100 W) while immersed in an ice bath. 100 μg of vesicle protein was added to 100 μl of serum, and the mixtures were rotated overnight at 4°C. The mixtures were centrifuged at 178,000 *g* in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) for 1 h. The clear supernatants were collected. Solid phase tray radioimmunoassay showed virtually zero titer against synaptic vesicles for both preadsorbed sera.

Synaptosome Preparation

Synaptosomes from the electric organ of *Narcine brasiliensis* or, occasionally, *Torpedo californica*, were prepared by a modification of the procedures of Israel et al. (13) and Michaelson and Sokolovsky (18) which will be described in detail elsewhere.[2] Briefly, the fish was anesthetized and perfused, and the electric organs were removed as described previously (2). The organs were weighed and minced. The tissue was homogenized in ~10-g batches with an equal volume of homogenization buffer in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT) for four periods of 15 s. The homogenates were centrifuged for 10 min at 1,100 *g*. The supernatants were gravity filtered through cheesecloth, and the filtrates were centrifuged for 15 min at 31,000 *g*. The supernatants were removed and the pellets resuspended to 40 ml with homogenization buffer and homogenized again for 10 s in a Waring blender. The homogenates were again centrifuged at 31,000 *g* for 15 min, the supernatants discarded and the pellets resuspended in ~6 ml each of homogenization buffer. The suspensions were further homogenized with three strokes of a motor-driven, Teflon-glass conical homogenizer. The homogenates were each applied to a 3–20% Ficoll continuous gradient in homogenization buffer and centrifuged at 83,000 *g* for at least 3 h in a Beckman SW27 swinging bucket rotor. The white synaptosome-enriched band at a density of ~1.04 g/ml was the second band from the top of the gradient and was just below the fine, light band, as reported in other methods of preparation (13, 18). This band was collected from each gradient and diluted ~1:1 with homogenization buffer and centrifuged at 31,000 *g* for 30 min. The supernatants were discarded and pellets resuspended in a total pooled volume of ~1 ml of homogenization buffer. The synaptosome suspension was washed twice by centrifugation at 12,000 *g* for 15 min in a Brinkmann microcentrifuge. The final pellet was resuspended to ~1 ml in homogenization buffer. The synaptosome fractions from six gradients normally yielded several milligrams of protein.

Immunoadsorption of Synaptosomes and PSPM to Beads

The scheme for the anti-SV antiserum binding of synaptosomes to polyacrylamide beads and the subsequent preparation of presynaptic plasma membrane

by hypotonic lysis and sonication is summarized pictorially in Fig. 1. An aliquot of the concentrated synaptosome suspension was mixed with an equal volume of 1% bovine serum albumin (BSA) in homogenization buffer (1% BSA buffer) followed by the addition of anti-SV antiserum that had been adsorbed as described above. The ratio of synaptosomal protein to IgG protein was normally 1:1. The mixture was rotated for 2 h at 4°C, then brought up to 1 ml with 1% BSA buffer and centrifuged at 12,000 *g* for 15 min in a Brinkmann microcentrifuge. The supernatant was removed and the pellet resuspended with 1 ml of 1% BSA buffer, centrifuged again, the supernatant removed, and resuspended to 0.25 ml of 1% BSA buffer. A suspension of 10 mg/ml (dry wt) goat anti-rabbit Ig Immunobeads (Bio-Rad Laboratories, Richmond, CA) in 1% BSA buffer was added to the synaptosome suspension and the combined suspension rotated for ~2 h at 4°C. The ratio of synaptosomal protein to dry wt of beads was usually 1:5–10. After incubation, the suspension was brought to 0.8 ml with homogenization buffer and underlaid with 0.2 ml 10% Ficoll in 0.1% BSA buffer. This suspension was centrifuged at 4°C at 2,000 *g* for 20 min in the swinging bucket rotor of a Sorvall GLC-4 tabletop centrifuge; the supernatant was then removed; and the bead pellet was resuspended to 0.8 ml with 0.1% BSA buffer, again underlaid with 10% Ficoll in 0.1% BSA buffer, and centrifuged, and the supernatant was removed. The pellet was resuspended with 1 ml of homogenization buffer and centrifuged at 2,000 *g* for 20 min, and the supernatant was discarded. The pellet was washed again as above and the final pellet resuspended to a convenient volume (usually <1 ml) with homogenization buffer.

To remove cytoplasmic contents, the bead-bound material was subjected to hypoosmotic shock and brief sonication. An aliquot of the bead-bound synaptosome suspension was added to five times its volume of 10 mM HEPES, pH 7.0, in a 1.5-ml Brinkmann polypropylene centrifuge tube, mixed, then sonicated by placing the tube against the probe of a Branson model W185 sonifier at a power setting of 7 (output ~100 W), while immersed in an ice bath. Sonication consisted of four cycles of 15 s on and 15 s off. The sonicated bead suspension was centrifuged at 2,000 *g* for 15 min, the supernatant removed, and the pellet resuspended with 0.8 ml of 10 mM HEPES buffer. This suspension was underlaid with 0.2 ml of 10% sucrose in 10 mM HEPES buffer and centrifuged at 2,000 *g* for 20 min. The resulting pellet was washed twice with 1 ml of homogenization buffer by 20-min, 2,000-*g* centrifugations, and the final pellet was resuspended to a convenient small volume.

Protein Assay

Protein concentrations were determined by the amido schwarz staining procedure (22) using BSA as a standard. A modification in the preparation of samples of bead-bound material was necessary. To each 50-μl bead-bound sample (and serum albumin standards, also) was added 200 μl of 2% SDS, 1% 2-mercaptoethanol. The samples were placed in boiling water for 5 min and centrifuged in a Brinkmann microcentrifuge for 5 min. The supernatants were removed and the pellets were resuspended in an additional 250 μl of 2% SDS, 1% 2-mercaptoethanol. The samples were again placed in boiling water for 5 min and centrifuged for 5 min, and the supernatants were collected and combined with the corresponding first supernatants. 120 μl of 60% TCA was added to these combined supernatants, which were then applied to the Millipore filter (Millipore Corp., Bedford, MA) and treated as the other samples in the assay. Because the beads themselves contributed significantly to the protein signal, a bead-containing blank was assayed in parallel. In addition, the bead concentration varied somewhat from sample to sample, so it was necessary to determine the bead concentration relative to the bead-containing blank for each suspension being assayed. This was accomplished by adding 5 μl of 10% SDS to 40 μl of bead suspension, then drawing duplicate 10-μl aliquots into 10-μl capillary pipettes and plugging the bottoms with modeling clay. Each capillary pipette was immersed in water up to the suspension meniscus in a conical centrifuge tube. The tubes were centrifuged for 5 min at 2,000 *g* and the heights of the bead pellets measured. These heights were used to normalize the blank value, which was subtracted from the samples.

ATP Assay

ATP was assayed by the luciferin-luciferase method (27) with the minor modification noted in Carlson et al. (2). The presence of beads in samples had no effect on the assay.

Choline Acetyltransferase Assay

Choline acetyltransferase (CAT) activity was determined by the rate of synthesis of [^{3}H]acetylcholine by a modification of the method of Fonnum (9) as described in detail by Hooper et al.[2]

Acetylcholinesterase Assay

Activity was measured by the method of Ellman (8). Virtually all esterase activity was inhibitable by 10 μM of the specific acetylcholinesterase inhibitor, 1,5-*bis*-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (Burroughs-Wellcome, Greenville, NC).

Na,K-ATPase Assay

Mg^{++}-dependent, ouabain-sensitive adenosine triphosphatase was measured by the spectrophotometric method of Medzihradsky et al. (17).

5′-Nucleotidase Assay

5′-Ribonucleotide phosphohydrolase activity was assayed by measuring the rate of hydrolysis of [^{3}H]adenosine monophosphate to [^{3}H]adenosine according to Gentry and Olsson (10) with a slightly modified reaction mixture containing 334 mM NaCl, 62 mM Tris, 2.25 mM $MgCl_2$, 22.5 μM [^{3}H]5′-AMP (5 mCi/mmol), 0.5% Nonidet P-40, 0 or 40 nM α,β-methylene adenosine diphosphate (AOPCP), pH 9.0. Only hydrolysis inhibitable by AOPCP was attributed to the enzyme.

Acetylcholine Receptor Assay

Binding of ^{125}I-α-bungarotoxin was used to quantitate nicotinic acetylcholine receptor (23). The assay was modified to accommodate bead-bound samples. After the usual 30-min incubation with iodinated toxin, the samples (100 μl) were centrifuged 5 min in a Brinkmann microcentrifuge and 90 μl of each supernatant

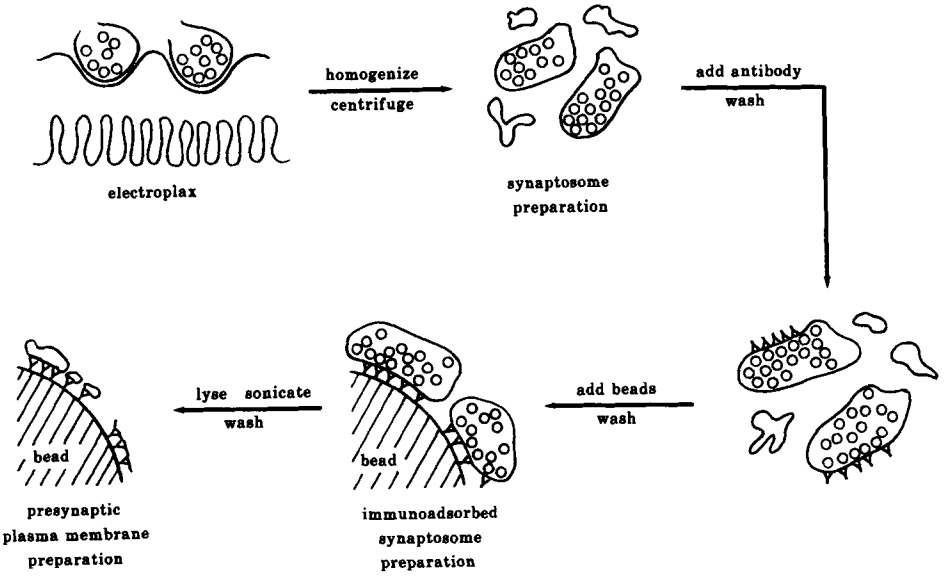

FIGURE 1 Scheme for the immunoadsorption of synaptosomes and PSPM. Electric ray electric organ is homogenized and subjected to three velocity sedimentation centrifugation steps and an equilibrium density gradient centrifugation to produce the synaptosome preparation. Anti-SV antiserum is then added and the synaptosomes washed of unbound antiserum. Anti-rabbit Ig antibody-coated polyacrylamide beads are added and the beads washed of unbound material. (Note that for simplicity the explicit schematic representation of anti-rabbit Ig antibodies linked to the beads has been omitted.) The bead-bound material is then subjected to hypotonic lysis and sonication and washed free of unbound material.

was carefully removed and placed on a Whatman DE81 filter circle. The remainder of the samples were each resuspended with fresh 1% Triton X-100, 20 mM $PO_4^=$, pH 7.2, and centrifuged again. 90 μl of each supernatant was carefully removed and transferred to a Whatman DE81 filter circle. The filters were washed and the filters and pellets counted in the usual ways. Bead-containing blanks were included to control for nonspecific toxin binding to beads.

Radioiodination

^{125}I iodination of membranes was performed by a modification of the procedure of Cohn and Hubbard (4), which employs lactoperoxidase and glucose oxidase. When necessary, membranes were disrupted with 0.5% nonionic detergent, Nonidet P-40, before iodination.

Two-dimensional PAGE

Two-dimensional gel electrophoresis was performed essentially by the method of O'Farrell (20). The first dimension was isoelectric focusing, spanning the range pH 3–10, and the second dimension was a 12% polyacrylamide SDS gel. Intensifying screens (Cronex; DuPont Co., Wilmington, DE) were used to hasten the exposure of autoradiograms.

Electron Microscopy

Bead-bound membranes were fixed in suspension with 2% glutaraldehyde in homogenization buffer for 10 min on ice, then centrifuged 5 min in a Beckman microcentrifuge. The pellet was resuspended in fixative and drawn into agar tubes preformed on capillary pipettes; the tubes were sealed with agar and replaced in fresh glutaraldehyde solution for 30 min at 4°C. The tubes were washed 30 min in homogenization buffer and then postfixed with 2% OsO_4 in 0.1 M sodium cacodylate pH 7.3 for 1 h. After one wash in 0.1 M cacodylate and two washes in veronal acetate pH 5.5, the tubes were stained with 1% uranyl acetate in veronal buffer for 1 h at room temperature. The tubes were dehydrated in a graded series of ethanol, followed by propylene oxide, propylene oxide:araldite 1:1, and embedded in araldite. Thin sections were cut and stained with uranyl acetate/lead citrate and viewed.

RESULTS

Isolation of Nerve Terminals by Immunoadsorption

Synaptosomes from the electric organ of *Narcine brasiliensis* can be specifically immunoprecipitated by antiserum raised to purified synaptic vesicles that had been adsorbed to remove nonspecific antibodies. The synaptosomal fraction was prepared by density gradient centrifugation by a modification[2] of an earlier procedure (12). Synaptosomes were monitored in these experiments by measuring CAT, ATP, or both. When the synaptosomal fraction was coated with increasing amounts of anti-SV antibodies, synaptosomal contents could be immunoprecipitated by the addition of excess amounts of polyacrylamide beads coated with goat anti-rabbit Ig. The immunoprecipitation was specific since equivalent amounts of preimmune serum gave very little binding (Fig. 2). A ratio of 1 μg of immune IgG to 1 μg of synaptosomal protein was sufficient to sediment the maximum amount of synaptosomes. Usually ~40–60% of the CAT activity was adsorbed to beads initially, but a variable fraction was desorbed by extensive washing of the beads. All CAT activity that did not bind initially or that desorbed was membrane-bounded.

The specificity of the anti-SV serum with respect to the preimmune serum can be improved by reducing the amount of goat anti-rabbit polyacrylamide beads. Using a fixed ratio of antibody to synaptosomes but varying the bead concentration (Fig. 3) it was found that 1 μg of beads (dry wt) was sufficient to give maximal adsorption when 1 μg of synaptosomal protein was used. At the lower bead concentrations, the selectivity of anti-SV serum over preimmune serum was ~100 to 1. In this experiment only ATP was used as a marker of synaptosomal contents since in these experiments and those described elsewhere[2] the behavior of synaptosomal ATP always parallels that of CAT. Note also that under the conditions used, no synaptosomes pelleted in the absence of beads.

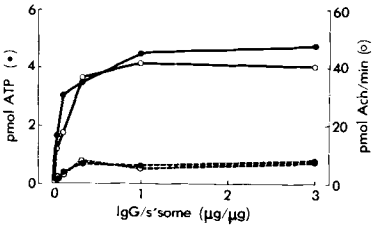

FIGURE 2 ATP (●) and choline acetyltransferase activity (○) bound to beads vs. the ratio of IgG protein to synaptosomal protein. Solid lines are for samples with the preadsorbed anti-SV antiserum and the dashed lines are for samples with preadsorbed preimmune serum. Transferase activity is expressed as pmoles of acetylcholine formed per minute. Each point represents synaptosomes immunoadsorbed by 50 μg of anti-rabbit Ig antibody-coated polyacrylamide beads (dry wt). The ratio of micrograms of beads to micrograms of *Torpedo* synaptosomal protein was 10:1.

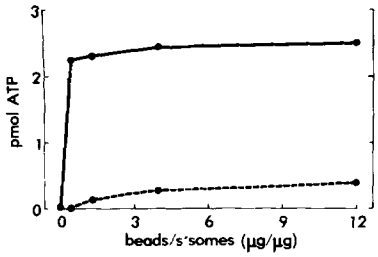

FIGURE 3 ATP bound to beads vs. the ratio of beads (dry wt) to synaptosomal protein. The solid line is for samples with adsorbed anti-SV antiserum and the dashed line is for samples with adsorbed preimmune serum. Each point represents a sample that began with 40 μg of *Narcine* synaptosomal protein. The IgG protein to synaptosomal protein was 10:1.

The specific activities of CAT and ATP, the two synaptosomal markers used in these experiments, typically increased about 1.5- to 2.5-fold by the immunoadsorption step. If the anti-SV serum were specific for nerve terminals then this result would imply that 40–67% of the protein in the synaptosomal fraction was associated with nerve terminals. By comparing synaptosomal fractions from normal and denervated electric organ, Hooper et al.[2] have estimated that 74 ± 17% of the protein in the synaptosomal fraction was indeed due to nerve terminals.

The anti-SV serum recognizes antigens enriched on nerve terminals but not on the innervated and noninnervated faces of the postsynaptic electroplaque. The innervated face of the electroplaque is rich in acetylcholine receptor (AChR) and the noninnervated face in Na^+,K^+ATPase. Both these markers and the nerve terminal marker, CAT, can be found in the crude P2 synaptosomal fraction (Table I). Fractionation of the P2 material on Ficoll gradients increased the CAT specific activity ninefold, but not the specific activity of the postsynaptic membranes. Although they are not enriched in synaptosomal fractions, the postsynaptic membranes are readily detectable contaminants and so provide a convenient measure of the specificity of the immunoadsorption procedures. In the experiment shown, immunoadsorption reduced the specific activity of the Na^+,K^+-ATPase by a factor of three, and the AChR by a factor of 70. These results indicate that the anti-SV antibodies bind selectively to nerve terminal membranes.

Although their precise distributions among the basal lamina and the presynaptic and postsynaptic membranes are not unequivocally known, acetylcholinesterase and 5′-nucleotidase (6) are thought to reside in the synaptic cleft and play a role in hydrolyzing the material released from the nerve terminal. Immunoadsorption produced little change in their specific activities (Table I).

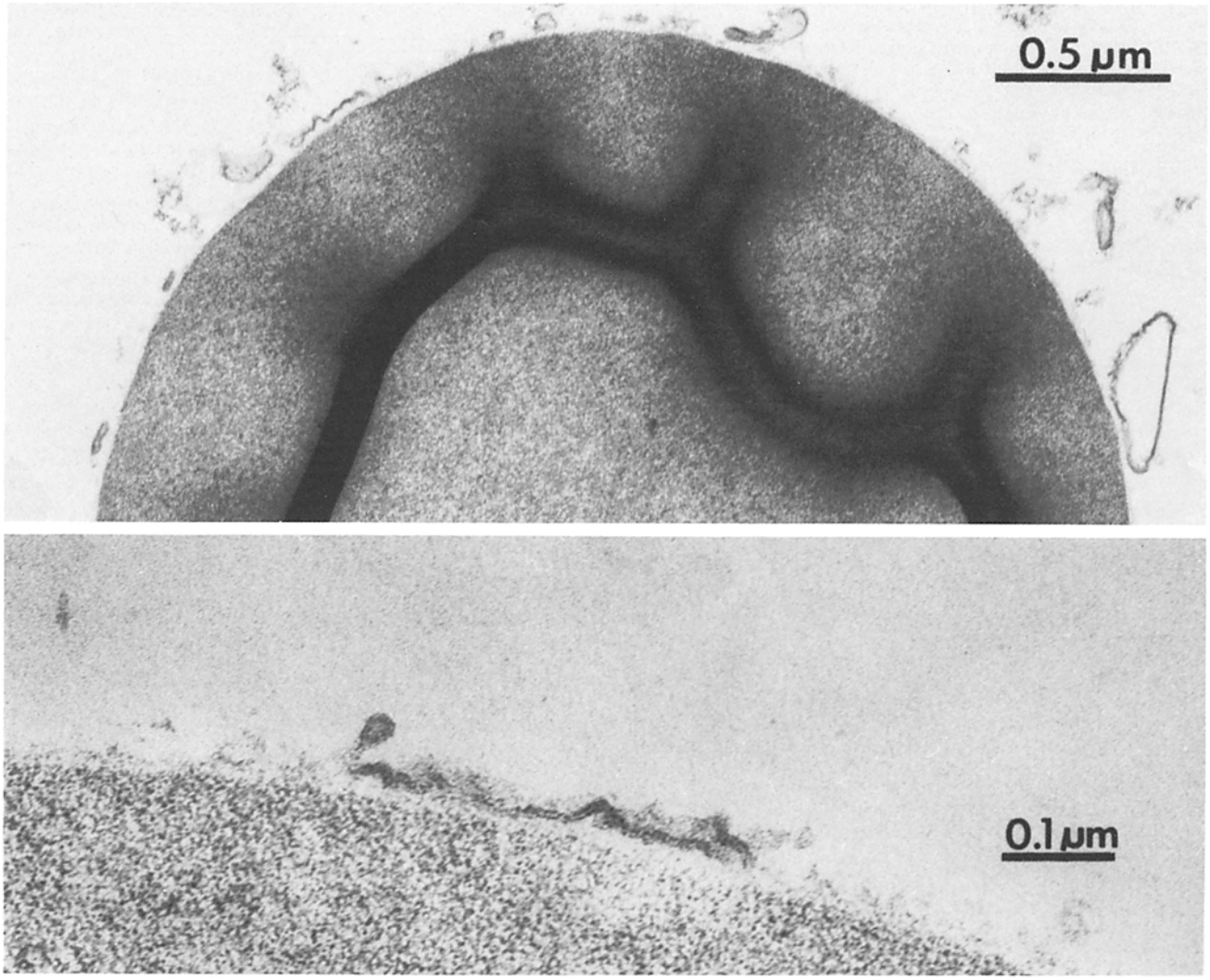

FIGURE 4 Electron micrographs of the bead-bound PSPM preparation.

TABLE I

Specific Activities of Synaptosomal Preparations

	Crude synaptosomes	Gradient synaptosomes	Adsorbed synaptosomes	Lysed synaptosomes*	Adsorbed/gradient	Lysed/adsorbed	% Activity bound after lysis
Choline acetyltransferase, *nmol/min/mg*	3.1	27.5	43.2	0.3	1.6	1/144	0.01
ATP, *nmol/mg*	2.2	13.8	23.5	0.3	1.7	1/78	0.03
5′-nucleotidase, *nmol/min/mg*	0.1	1.8	1.4	0.7	1/1.3	1/2.0	1
Na,K-ATPase, *nmol/min/mg*	3.3	3.0	1.0	0.2	1/2.9	1/4.9	0.4
Acetylcholine receptor, *nmol/mg*	0.2	0.1	<0.01	1/70	<0.01	<5	<10
Acetylcholinesterase, *µmol/min/mg*	35.5	21.5	25.1	24	1.2	1.0	2
^{125}I surface label	—	—	—	—	—	5	10
Anti-SV antibody, *mg of IgG/mg*	—	—	0.07	2.0	—	29	60
Total protein	—	—	—	—	—	—	2

* Presynaptic plasma membrane (PSPM) preparation.

Isolation of Presynaptic Plasma Membrane

If CAT and ATP are truly markers of synaptosomal contents then lysis of the bead-bound synaptosomes should release them. When bead-bound synaptosomes were exposed to hypotonic lysis and sonication, only 2–3% of the protein remained bead-bound. The specific activity of the residual CAT was 144-fold lower than that of the starting material and the residual ATP 78-fold lower (Table I). The most likely conclusion is that the bulk of these markers is inside the synaptosomal plasma membrane and is released by lysis.

Lysis of the synaptosomes would be expected to release from the beads both synaptosomal contents and that fraction of the plasma membrane not tightly associated with the beads (Fig. 4). To monitor the recovery of plasma membrane after lysis and sonication, intact synaptosomes were surface labeled with ^{125}I by lactoperoxidase before immunoadsorption. Typically, following lysis and sonication, ~10% of the ^{125}I surface label remained on the beads. Since only ~2% of the total protein remained, this indicates a fivefold enrichment in the bead-bound surface membrane relative to that in the synaptosomes. This result would also imply that ~20% of the protein in the synaptosomes is associated with the surface membrane.

At least 90% of the AChR was also removed after lysis and sonication (Table I), so that the AChR could only account for <0.3% of the bead-bound membrane proteins. The specific activity of the Na^+,K^+-ATPase dropped a further fivefold upon lysis.

Pieces of synaptosomal membrane linked directly to the beads via anti-SV antibodies might also be shaken off during the lysis procedures. To attempt to monitor the recovery of the attached membranes, synaptosomes were first coated with ^{125}I-labeled anti-SV antibodies and washed extensively to remove unbound antibody. Recovery of synaptosomal membrane containing vesicle antigens could then be followed using the radioactive antibody. After lysis and sonication, typically 50–70% of the radioactivity remained with the beads. Since only ~2–3% of the protein remained, this implies that the antibody-binding regions of the surface membrane were enriched 20- to 30-fold compared with the whole synaptosomes. This assumes that all the label that is recovered is taken to represent antigenic membrane. However, we have not eliminated the possibility that some antibodies dissociate during the lysis procedure and then bind to the beads.

Since 10% of the surface membrane is retained on the beads after lysis a similar recovery would be expected for an enzyme marker on the surface membrane. The recoveries of 5′-nucleotidase, acetylcholine esterase, and Na^+,K^+-ATPase are considerably <10% (Table I) but are considerably more than those of ATP and CAT. Intermediate recoveries of this sort could arise if a marker is both intraterminal and on the surface. Alternatively, they could be on contaminating membranes nonspecifically adsorbed to the beads that are desorbed from the beads by the lysis procedure.

Polypeptide Composition of Synaptosomal Membranes

The proteins in the PSPM preparation obtained by lysis of bead-bound synaptosomes were compared with those in synaptic vesicles, bead-bound synaptosomes, and the starting synaptosome preparation. The synaptosome fraction from the Ficoll gradient was iodinated using lactoperoxidase, before and after lysis with detergent. The iodinated polypeptides were separated first by isoelectric point and then by molecular weight using the conventional two-dimensional gel electrophoresis procedure (20). The autoradiogram of the two-dimensional gel of the synaptosome preparation labeled after lysis in detergent (Fig. 5 *A*) showed a multitude of spots most of which were not present when the intact synaptosomes were labeled (Fig. 5 *B*). Since many synaptosomal polypeptides are protected from labeling in the absence of detergent lysis, this implies that a considerable fraction of the synaptosomes are indeed intact, as suggested by their CAT content.

Synaptic vesicle polypeptides can be identified on these gels. Synaptic vesicle protein has been estimated to contribute ~8% to the total protein in a synaptosome preparation.[2] The proteins in a purified synaptic vesicle preparation that were iodinated after lysis in detergent are shown in Fig. 5 *E*. Spots correspond-

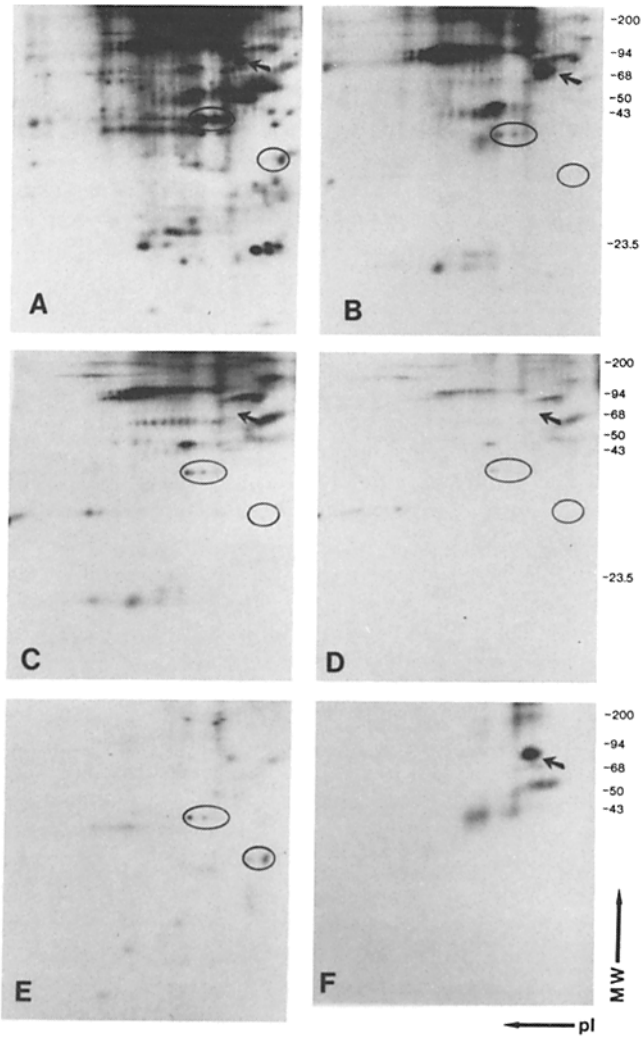

FIGURE 5 Two-dimensional PAGE autoradiograms of (*A*) a synaptosome-enriched fraction purified by density gradient centrifugation and labeled in detergent with ^{125}I by the lactoperoxidase method; (*B*) the synaptosome preparation as in *A*, except surface-labeled while intact with ^{125}I; (*C*) synaptosomes from the preparation in *B* after immunoadsorption by anti-SV antiserum and anti-rabbit Ig antibody-coated polyacrylamide beads; (*D*) the same preparation as in *C* after hypotonic lysis and sonication (PSPM preparation); (*E*) highly purified electric organ synaptic vesicles labeled in detergent with ^{125}I; (*F*) density gradient purified AChR-rich membranes surface-labeled with ^{125}I. See text for explanation of symbols. From left to right is the isoelectric focusing dimension and from top to bottom is the SDS dimension. (Autoradiograms were exposed with the aid of intensifying screens.)

ing to almost all of the synaptic vesicle proteins can be identified in the two-dimensional gel of detergent-lysed synaptosomal protein (Fig. 5 *A*). Some of these are circled. Some vesicle proteins can also be identified in the surface-labeled, intact synaptosomes (Fig. 5 *B*), suggesting that synaptic vesicle proteins are exposed on the outer surface of the nerve terminal. This is not unexpected in light of the ability of anti-SV antiserum to mediate the immunoadsorption of intact synaptosomes. Not all of the synaptic vesicle spots are represented in the autoradiogram of the gel of the surface-labeled synaptosomes however, suggesting that some are only accessible from the cytoplasmic surface. Prominent examples of surface-labeled synaptic vesicle polypeptides are circled in Fig. 5 *B* (large circle). Examples of the absence of labeled synaptic vesicle polypeptides that might represent ones exposed only to the cytoplasm are also noted in Fig. 5 *B* (small circle).

Another set of spots that might be identified are those associated with AChR-rich membranes. Two-dimensional analysis of surface-labeled AChR-rich vesicles (prepared by the method of Duguid and Raftery [7]) is shown in Fig. 5 *F*. In addition to the four known subunits of the receptor, α, β, γ, and δ, a spot thought to correspond to the Na^+,K^+-ATPase (arrow) was readily detected. The AChR subunits were scarcely detectable in the synaptosomal fractions labeled either before or after detergent lysis (Fig. 5 *A* and *B*). The putative Na^+,K^+-ATPase spot is, however, quite visible in both preparations, especially when the synaptosomes are labeled before lysis.

After immunoadsorption of the surface labeled synaptosomes (Fig. 5 *C*), the polypeptide composition was qualitatively similar to that before immunoadsorption (Fig. 5 *B*) with the major difference being that the spot corresponding to the Na^+,K^+-ATPase was virtually absent. The synaptic vesicle spots, as expected, were still present. After hypotonic lysis and sonication (Fig. 5 *D*), the pattern of polypeptide spots was similar to that of the intact surface labeled synaptosomes, confirming that few if any of the cytoplasmic proteins were labeled. Again, some of the synaptic vesicle proteins were readily detectable in the lysed membranes. The procedure is not yet sufficiently quantitative to know if vesicle proteins are enriched upon lysis. What is striking, however, is that the polypeptide composition of synaptic vesicles and PSPM are quite different. This demonstrates that the anti-SV antibodies are not just selecting occasional free synaptic vesicles from the synaptosomal preparation and that the anti-SV antibodies are not simply purifying patches of synaptic vesicle membrane from the PSPM.

Generation of Antibodies to PSPM

The criteria used here to show that a PSPM has been purified are that: (*a*) markers for the major contaminating membranes have been drastically reduced, (*b*) the preparation has been enriched in plasma membrane derived from bead-bound synaptosomes that approach total purity, and (*c*) inserted vesicle membrane components, presumably marking the PSPM, are increased dramatically. Such indirect methods are necessary because no specific biochemical or morphological marker is known for PSPM. To strengthen our claim that the purified membrane does indeed contain PSPM elements, we have used an immunological approach. To raise antibodies to antigenic sites in the PSPM preparation, the bead-bound PSPM was injected directly into rabbits. The resulting antiserum (anti-PSPM) showed a high titer against electric organ synaptosomes, relative to preimmune serum. When microtiter wells saturated

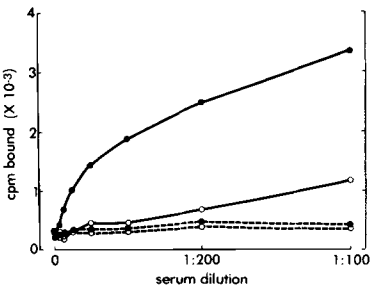

FIGURE 6 Solid-phase tray assay of anti-PSPM antiserum binding to synaptosomes and synaptic vesicles. Before being assayed, immune and preimmune sera were adsorbed against homogenized, washed *Torpedo* tail muscle. Adsorbed anti-PSPM antiserum (solid lines) and adsorbed preimmune serum (dashed lines) were assayed against gradient purified electric organ synaptosomes (●) and electric organ synaptic vesicles (○). Suspensions of synaptosomes at 25 μg/μl and synaptic vesicles at 2 μg/μl were used to coat the wells with antigen. *cpm bound* refers to the amount of second antibody, ^{125}I-labeled goat anti-rabbit-IgG IgG which was bound to each well.

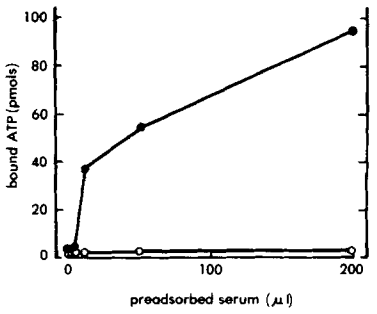

FIGURE 7 Immunoadsorption of synaptosomes by anti-PSPM antiserum preadsorbed with synaptic vesicles. Shown are the amounts of pelleted ATP immunoadsorbed by serial dilutions of anti-PSPM antiserum (●) and preimmune serum (○). Before the immunoadsorption procedure, both sera were adsorbed with disrupted synaptic vesicles to remove anti-SV antibodies. The ratio of serum to synaptic vesicles was (1 μl of serum):(1 μg vesicle protein). Solid-phase tray assays of both preadsorbed sera showed virtually zero titer against synaptic vesicles.

with synaptosomal membranes were incubated with different antiserum concentrations, binding of adsorbed anti-PSPM was well above background at serum dilutions <1:3,200 (Fig. 6), whereas binding of adsorbed preimmune serum was scarcely detectable. Synaptic vesicles adsorbed to the wells did not bind anti-PSPM as well as synaptosomes. The initial slope of specific binding was >20-fold higher against gradient purified synaptosomes than against synaptic vesicles. This contrasts strongly with the specificity shown by anti-SV serum, which, under the same conditions, binds synaptosomes ~0.8 times as well as synaptic vesicles.[3] The anti-PSPM serum appears, therefore, to recognize in synaptosomal preparations antigens other than those on synaptic vesicles.

Since the synaptosomal preparation is not pure, the antigens recognized by the anti-PSPM in the solid-phase radioimmune assay need not be on nerve terminals. To show that nerve terminal antigens were being recognized, intact synaptosomes were coated with anti-PSPM and then adsorbed to goat anti-rabbit Ig coated beads, as before. The synaptosomes coated with anti-PSPM bound to the beads just as readily as when coated with anti-SV antibodies, as demonstrated by the pelleting of ATP. This was true even when the anti-PSPM serum was adsorbed extensively with disrupted synaptic vesicles to remove antibodies directed at synaptic vesicle antigens (Fig. 7). Thus, anti-PSPM contains antibodies against determinants on the outside of the nerve terminal that are not

[3] It is likely that a significant fraction of synaptosomes are lysed upon adsorption to the wells, so that synaptic vesicle antigens are exposed for anti-SV binding.

contained in synaptic vesicles. We conclude that the PSPM preparation is a valuable immunogen with which to produce antibodies to nerve terminal membranes. The cross-reactivity of this serum with the neuromuscular junction of higher vertebrates and its ability to recognize antigens restricted to the external surface of the junctional nerve terminal will be documented later.[2]

DISCUSSION

Evidence has been presented that antigens recognized by anti-SV antibodies are present on the outside of nerve terminals from *Narcine* electric organ. The key argument is that two known markers of the nerve terminal cytoplasm, CAT and ATP, can be immunoprecipitated by the anti-SV antibodies and beads coated with secondary antibody. It is conceivable that the ATP results from the immunoprecipitation of free synaptic vesicles and the CAT from nonspecific adsorption of the enzyme to membranes. The anti-SV antibodies can, of course, immunoprecipitate synaptic vesicles[4] and it is known that CAT has a predilection for sticking to membranes. We believe that the binding to the beads of free synaptic vesicles in the synaptosomal preparation is negligible for three reasons. Free vesicles would be washed away at every step of the immunoadsorption procedure before addition of the beads (Fig. 1), the polypeptide compositions of vesicles and the PSPM are quite different from each other (Fig. 5), and antibodies generated to the PSPM preparation recognize antigens on the PSPM surface that are different from those recognized by antibodies to synaptic vesicles (Fig. 7). In addition, it is highly unlikely that the amount of nonspecifically adsorbed CAT could approach the levels present in intact synaptosomes and that it could also be present in precisely the same stoichiometric ratio to ATP as is found in purified synaptosomes.

The anti-SV antibodies appear to recognize antigenic determinants present on the outside of nerve terminals but absent from postsynaptic membranes. Thus, upon immunoprecipitation, the specific activities of the synaptosomes increased to a level that may be close to that of pure synaptosomes,[2] whereas the AChR-rich and Na^+,K^+-ATPase-rich postsynaptic elements were markedly reduced in specific activity. The use of Na^+,K^+-ATPase as a marker is of course less than optimal since it should be a constituent of all plasma membranes, including the PSPM. The decrease in specific activity upon immunoadsorption and upon synaptosomal lysis suggests that most of the enzyme in the original synaptosomal preparation is due to contaminating membranes. The lack of enrichment of this enzyme in the synaptosomal fraction (Table I) is consistent with this conclusion. It would appear that the PSPM is not nearly as rich in Na^+,K^+-ATPase as the noninnervated electroplaque membrane. Acetylcholinesterase, most of which is probably membrane associated under the conditions used here (29), behaved somewhat similarly to the Na^+,K^+-ATPase (Table I). It would appear to be present in the PSPM but not enriched there.

The 5′-nucleotidase activity also appears not to be enriched in the PSPM preparation. This was a surprising observation since 5′-nucleotidase has been considered a marker for plasma membrane. It is thought to be utilized in the synaptic cleft for the hydrolysis to adenosine of the cleavage products of ATP (6).

Importantly, the specific activity of anti-SV-binding determinants on the surface of bead-bound synaptosomes was increased 20- to 30-fold after the lysis procedure (Table I). This is strong evidence that the bead-bound membrane preparation is enriched in inserted synaptic vesicle membrane. Presumably, this inserted vesicle membrane marks the PSPM.

Two-dimensional gel electrophoresis showed that the polypeptide composition of the synaptic vesicles is quite unlike that of the PSPM preparation although the PSPM fraction does seem to contain a small amount of vesicle proteins (Fig. 5). In addition, antibodies to the PSPM recognize surface determinants of the synaptosome other than those of the synaptic vesicle (Fig. 7). If the compositions of the PSPM and the synaptic vesicle membrane are indeed different as these data suggest, then a molecular mechanism must exist to segregate the membranes during membrane recycling at the nerve terminal.

Differences between the membranes of synaptic vesicle preparations and PSPM preparations have been reported before, as have similarities (for a review, see reference 25). The value of these reports is critically dependent on the purity of the PSPM preparation, which is difficult to determine in the absence of specific markers for PSPM and synaptic vesicle membranes. In the best of these, pure synaptic vesicles from *Torpedo marmorata* were compared with a membrane fraction obtained by lysing a synaptosome preparation and isolating from a 105,000-*g* pellet a band at a 0.58–0.7 M sucrose interface of a subsequent equilibrium density gradient (26). The evidence that this material was indeed the PSPM was that it contained 5′-nucleotidase, acetylcholine esterase, and Na^+,K^+-ATPase, none of which have been demonstrated to be enriched in PSPM. The membrane fraction did not resemble synaptic vesicles upon analysis by two-dimensional gel electrophoresis.

One potential marker for a PSPM from electric organ is the muscarinic AChR (16). We have confirmed the presence of [^{3}H]quinuclidinyl benzilate binding in our synaptosomal preparation (~65 fmol/mg protein), but the amounts were too low to be of value in assaying the purification of PSPM on the scale utilized here.

The electric organ is an obvious source of large amounts of synaptic elements. Fortunately, elements purified from the electric organ such as the AChR or synaptic vesicles appear to contain antigenic determinants that are conserved in higher vertebrates (12, 21). Here we show that it is possible to use antibody technology to bootstrap from one pure element, the synaptic vesicle, to the purification of another, the PSPM, which is biochemically much less well defined. The PSPM preparation itself is capable of generating the production of antisera directed at the outside of nerve terminals, in the fish electric organ as well as the frog neuromuscular junction.[4] Given the complexity of the PSPM composition, the value of a conventional antiserum is largely to show that unique determinants exist. These determinants must then be identified either by conventional biochemistry or by techniques using monoclonal antibodies from hybridomas. In preliminary observations, we have used the criterion of immunoprecipitation of intact synaptosomes to show that several monoclonals from the library raised to an electric organ synaptosome preparation, by Drs. Kushner and Reichardt of the University of California, San Francisco, bind to the outer surface of electric organ nerve terminals.

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[4] G. P. Miljanich, unpublished results.

line receptor membranes, and Leslie Spector for typing the manuscript.

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