PARTIAL PURIFICATION OF ACTIVE ZONES OF PRESYNAPTIC PLASMA MEMBRANE BY IMMUNOADSORPTION

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Understanding the molecular mechanisms of membrane fusion and its regulation is one of the foremost goals of membrane biology. If secretory vesicles and the appropriate secretory plasma membrane could be isolated, it might then be possible to investigate membrane fusion by reconstituting exocytotic release in vitro. Highly purified preparations of several types of secretory vesicles have now been obtained, but comparable preparations of the corresponding plasma membrane have been elusive. Among those vesicles which have been purified to near homogeneity are the synaptic vesicles of the nerve terminals of the electric organ of the marine ray (Carlson et al., 1978; Wagner et al., 1978). A highly specific antiserum has been obtained against these vesicles which contains antibodies to antigenic sites on both the inner and outer surfaces of the vesicle membrane (anti-SV) (Carlson and Kelly, 1980). Since the antigens on the inner vesicle surface are exposed on the outer surface of the nerve terminal following vesicle exocytosis (von Wedel et al., 1981), the anti-SV antibodies serve as affinity ligands with which to purify, by immunoadsorption, the secretory regions, or "active zones," of the presynaptic plasma membrane (PSPM).

MATERIALS AND METHODS

Pinched-off nerve terminals, or synaptosomes, were isolated from homogenized marine ray electric organ by a density gradient centrifugation procedure. Synaptosomes were incubated ~ 2 h with the IgG fraction of anti-SV (1 μg synaptosomal protein to 1 μg IgG protein). The unbound antibodies were washed from the synaptosomes and bound antibodies by centrifugation and resuspension. Microscopic polyacrylamide beads (Bio-Rad Laboratories, Richmond, CA) covalently coated with anti-IgG antibodies were then added to the suspension of anti-SV-coated synaptosomes (1 μg synaptosomal protein to 1 μg beads by weight) and incubated for ~ 2 h. The immunoadsorbed, bead-bound material was washed free of unbound material by centrifugation through a 10% Ficoll solution. The pellet was resuspended and diluted at least five-fold in hypotonic solution and sonicated in an ice bath by touching the tube containing the suspension against the probe of a Branson sonifier for four 15-s bursts (100W output). Material still immunoadsorbed to the beads was washed free of unbound material by centrifugation and resuspension.

RESULTS AND DISCUSSION

This lysed, sonicated, immunoadsorbed preparation, the immunoadsorbed, whole synaptosomes, and the gradient-purified synaptosomes were each subjected to a variety of biochemical analyses. The results for a representative set of preparations are summarized in Table I. Typically, upon immunoadsorption the specific activity of the nerve terminal cytoplasmic marker, cholineacetyltransferase, increased 1.5–2.5-fold, indicating a purification of synaptosomes away from contaminating structures. The specific activity of ATP, the bulk of which is contained in synaptic vesicles, paralleled that of the transferase. The activity of 5′-nucleotidase, commonly employed as a plasma membrane marker, changed little upon immunoadsorption. Na/K ATPase specific activity decreased by approximately threefold, indicating a reduction of contamination by membranes of the noninnervated face of the postsynaptic cell, which is highly enriched in this enzyme. A drop in acetylcholine receptor, a postsynaptic membrane marker, was also observed. The specific activity of acetylcholinesterase, probably present in the postsynaptic membrane and basal lamina, increased slightly after adsorption. To obtain a preparation enriched in plasma membrane, the immunoadsorbed synaptosomes were subjected to hypotonic lysis and sonication. As expected, well over 99% of the cytoplasmic and vesicle markers, cholineacetyltranferase and ATP, was removed from the adsorbed material. At the same time ~ 2% of the total protein remained bead-

TABLE I
ACTIVITIES OF VARIOUS PREPARATIONS

	Adsorbed S.A.* / Gradient S.A.*	Lysed S.A.* / Adsorbed S.A.*	Lysed S.A.* (per mg total protein)	% Bound After lysis
ATP (nmol/mg)	1.7	1/60	0.4	0.04
Cholineacetyltransferase (nmol/min/mg)	1.6	1/110	0.4	0.02
5′ nucleotidase (nmol/min/mg)	1/1.3	1/1.5	0.9	1.5
Na/K ATPase (nmol/min/mg)	1/2.9	1/3.6	0.3	0.6
Acetylcholine receptor (nmol/mg)	1/70	≤5	≤0.01	≤10
Acetylcholinesterase (μmol/min/mg)	1.2	1.3	32	2.9
^{125}I-surface label	—	4.9	—	11
Anti-vesicle antibody (mg/mg)	—	28	4.3	61
Total Protein	—	—	—	2.2

*S.A. is specific activity per mg total protein.

bound. About 2% of the total 5'-nucleotidase activity also remained bound. Although 5'-nucleotidase is conventionally used as a plasma membrane marker, we have found that the specific activity of nucleotidase is 30–40 times greater in highly purified synaptic vesicles than in synaptosomes. Thus, it appears that the bulk of total nucleotidase activity in the nerve terminal resides in the synaptic vesicles and not in the PSPM. Lysis and sonication further removed Na/K ATPase activity and removed at least 90% of the receptor activity, leaving < 0.3% of the total bound protein as receptor. Esterase specific activity was unchanged. To monitor recovery of plasma membrane, synaptosomes were surface-labeled with ^{125}I by lactoperoxidase prior to immunoadsorption. Typically, following lysis and sonication, 10–20% of the ^{125}I remained bead-bound. About 2% of the total protein remained adhered subsequent to lysis and sonication, indicating a 5–10-fold enrichment in surface-labeled membrane. When ^{125}I-labeled anti-SV was used for immunoadsorption, 50–70% of the radioactivity remained bead-bound after lysis and sonication. This implies a ~ 20–30-fold enrichment of anti-SV-binding membrane. Presumably these are the regions of the PSPM containing the active zones for exocytosis.

Electron micrographs of immunoadsorbed synaptosomes and of lysed and sonicated membranes corroborate the biochemical assays. The immunoadsorbed synaptosome preparation is dominated by nerve terminal-like structures attached to beads and containing the characteristics synaptic vesicles and glycogen granules. Micrographs of lysed and sonicated material show numerous, irregular vesicles, usually < 2,500 Å across, and less numerous, flattened membrane sheets adhering to beads.

Protein compositions of ^{125}I-surface-labeled gradient-purified synaptosomes, immunoadsorbed synaptosomes, and lysed and sonicated synaptosomes as well as purified synaptic vesicles iodinated in detergent, were analyzed by two-dimensional gel electrophoresis. The synaptosomes contain approximately eight major labeled polypeptides and many minor ones. As expected, some of these spots coincide with the major spots observed in the synaptic vesicle gel. However, the general pattern of spots is quite different from the vesicle gel pattern. A spot which corresponds to Na/K ATPase is prominent in the synaptosome gel. The pattern of spots for the immunoadsorbed synaptosomes is qualitatively very similar to that of the synaptosomes. An exception is the absence of the Na/K ATPase spot. Many spots, especially the minor ones, in the immunoadsorbed synaptosome pattern are absent in the plasma membrane pattern.

Lysed and sonicated immunoadsorbed membranes were used to raise an antiserum against the PSPM. This antiserum (anti-PSPM) possesses a 22-fold higher titer against synaptosomes than against synaptic vesicles. Anti-PSPM is capable of mediating the immunoadsorption of synaptosomes, even after preadsorption with synaptic vesicles to remove anti-SV antibodies from the serum. This antiserum also binds specifically to microtome sections of nerve terminals of the frog cutaneous pectoris neuromuscular junction, just as anti-SV does (von Wedel et al., 1981). In addition, anti-PSPM binds to the outer surface of intact, unstimulated nerve terminals, in contrast to anti-SV which binds to intact terminals only after stimulation (von Wedel et al., 1981).

In conclusion, biochemical markers, electron microscopy, anti-SV binding, gel electrophoresis, and the production of a specific anti-PSPM antiserum demonstrate that we have isolated a membrane preparation which is enriched in the PSPM of the nerve terminal and its active zones. Furthermore, these analyses clearly show that the PSPM contains both synaptic vesicle and nonsynaptic vesicle elements. The immunoadsorbed sheets of active zones are of potential value for reconstituting an in vitro system of exocytosis that can be used to study the molecular events of exocytosis. The anti-PSPM antibodies could be of use in this regard. Finally, the scheme for isolation of nerve terminals and nerve terminal plasma membrane presented here should have general applicability to purification of other cells and organelles.

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