Transfer of synaptic vesicle-antigens to the presynaptic plasma membrane during exocytosis*

(immunofluorescence/nerve terminals/neuromuscular junction/lanthanum/β-bungarotoxin)

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ABSTRACT We have utilized immunofluorescence techniques to look for synaptic vesicle antigens on the plasma membrane of resting and active nerve terminals. Rabbit antiserum was raised against purified cholinergic synaptic vesicles from the electric organ of Narcine brasiliensis, a marine electric ray. Antibodies to synaptic vesicles were shown to bind selectively to nerve terminals in cryostat sections of frog nerve–muscle preparations. Binding was demonstrated indirectly by using fluorescein-labeled goat anti-rabbit antibodies. Structures in cross sections that bound antiserum were identified as nerve terminals because of their size, shape, and position and because they coincided with sites that bound rhodamine-conjugated α-bungarotoxin and had acetylcholine esterase activity. Presumably, sectioning gave antibodies access to binding sites within the nerve terminal. However, when antibodies to synaptic vesicles were added to the bathing medium of intact neuromuscular preparations prior to sectioning, antibody binding was marginal or undetectable, suggesting that few vesicle antigens were normally accessible on the outer surface of resting nerve terminals. When intact preparations were stimulated to release their vesicular acetylcholine by the addition of 1 mM LaCl3, antibody binding to the intact nerve terminals became striking. These findings suggest that the synaptic vesicle membrane and the synaptic terminal plasma membrane differ in composition. They also provide further support for the exocytotic hypothesis of neurotransmitter release, which predicts that vesicle markers should be exposed on the outside of nerve terminals when vesicles fuse with the plasma membrane during stimulation.

The availability of pure cholinergic synaptic vesicles from electric organs of the marine ray Narcine brasiliensis (1) has enabled us to raise, in rabbits, anti-synaptic vesicle antibodies that recognize vesicle-specific antigens (2). At least some of these specific antigens are evolutionarily conserved, because antibodies in rabbit synaptic vesicle antiserum bind to nerve terminals in cryostat sections of rat skeletal muscle (3) and to a subclass of nerve terminals in the rat brain (4). However, it had not been determined whether the conserved antigens were restricted to synaptic vesicles or were also present in the plasma membrane of the terminals. This is an important point because there have been conflicting claims about whether the synaptic vesicle membrane and the plasma membrane are biochemically distinct (5). If transmitter is released by exocytosis, then synaptic vesicle and plasma membrane must mix at least transiently. If they can do so yet remain structurally dissimilar, then there must be some way of segregating the components after mixing.

We have studied this question, using the cutaneous pectoris (CP) muscle from the frog. The neuromuscular junctions of this muscle are well characterized both anatomically and electrophysiologically, particularly in regard to studies of exocytosis (6–8). In addition, the CP muscle is thin and flat, which should favor the penetration of antibody into synaptic regions. In this investigation we have confirmed that antibodies raised to Narcine brasiliensis synaptic vesicles bind exclusively to nerve terminals in cryostat sections of the frog muscle. Under these conditions, the antibodies are likely to have access to intraterminal antigens. We have then sought to determine if the antibodies could recognize vesicle antigens on the extracellular surfaces of living nerve terminals. Finally, we have made use of the observation that lanthanum causes massive quantal release of acetylcholine from the frog nerve terminal (9, 10) to determine if transmitter release is associated with the appearance of synaptic vesicle antigens on the plasma membrane.

MATERIALS AND METHODS

Preparation of Synaptic Vesicle Antisera. Rabbit antiserum was raised against highly purified vesicle from Narcine and adsorbed overnight at 4°C with sonicated electric organ membranes devoid of synaptic vesicles as described (2). The resulting antiserum is specific for synaptic vesicles in crude homogenates of the electric organ (2) and selectively labels nerve terminals in cross sections of vertebrate skeletal muscle (3). For control experiments, either preimmune serum or the immune serum adsorbed with an excess of sonicated synaptic vesicles was used. In all cases both control sera gave similar results.

Labeling of Nerve Terminals in Intact Muscle. For each experiment, a pair of CP muscles was dissected intact with its nerve trunk from 3- to 5-cm Rana pipiens frogs in phosphate-free (PF) Ringer solution (116 mM NaCl/2 mM KCl/3 mM d-glucose/5 mM Heps, pH 7.3/1.8 mM CaCl2). One muscle was incubated 8–12 hr at 4°C in 200 µl of PF Ringer solution containing a 1:50 dilution of rabbit anti-synaptic vesicle antiserum, 50 mM rhodamine-conjugated α-bungarotoxin (RoBtx), and 1% bovine serum albumin. The second muscle of each pair was incubated under the appropriate control conditions. Purified α-bungarotoxin (from Zach Hall, University of California, San Francisco) was conjugated with tetramethylrhodamine isothiocyanate (Cappel Laboratories, Cochranville, PA), and the monoconjugated form (RoBtx) was isolated by column chromatography by the method of Rudin and Axelrod (11). An IgG fraction of rabbit antiserum raised to chicken gizzard actin was obtained from J. Ramachandran, University of California, San Francisco.

Stimulation Procedures. Stimulated preparations were obtained by incubating the muscles in 1 mM LaCl3/PF Ringer so-

Abbreviations: CP, cutaneous pectoris; PF, phosphate-free; P/NaCl, phosphate-buffered saline; RoBtx, rhodamine conjugated α-bungarotoxin; β-Btx, α-bungarotoxin; Fl-CAR, fluorescein-conjugated goat anti-rabbit IgG.

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† Miledi, R., Molenaar, P. C. & Polak, R. L. (1979) Seventh Meeting of the International Society for Neurochemistry, Jerusalem, Israel, Sept. 2–6, 1979, p. 100 (abstr.).
solution (1 ml) for 1–2 hr at 23°C prior to incubating with rabbit anti-synaptic vesicle antiserum and RaBTx. The rapid increase in transmitter release under these conditions (9, 12) was confirmed by standard electrophysiological recording. By 2 hr, little spontaneous release remained. Unstimulated preparations, used as controls, were maintained in 14 mM MgCl₂/0.9 mM CaCl₂ PF Ringer solution to suppress spontaneous transmitter release (13) both prior to and during the incubation with antiserum and RaBTx. For some experiments, normal CP nerve-muscle preparations were incubated with β-bungarotoxin (β-BTX) from Bungarus multicinctus (Boehringer Mannheim) for 1 hr at 23°C with β-BTX at 10 μg/ml (50 nM) in normal frog Ringer solution and washed prior to the routine whole mount incubation with antiserum and RaBTx as described above.

Preparation of Frozen Muscle Sections. After the incubation with antiserum and RaBTx, each CP muscle was washed for 30 min in PF Ringer solution. The control and experimental muscles of each pair were sandwiched together between thin sheets of sartorius muscle such that the innervated regions of both CP muscles could be viewed in the same cross sections for comparison. The sandwich was quick frozen in liquid nitrogen, and 6- to 8-μm serial cross sections were cut at ~22°C in an IEC-CTD refrigerated microtome cryostat. As cutting proceeded, specimens were checked (every 8–10 sections) for the presence of neuromuscular junctions in the CP muscles by staining for acetylcholine esterase activity with the method of Karnovsky and Roots (14).

**Immunofluorescence Histochemistry.** Unfixed sections were dipped into phosphate-buffered saline (P/NaCl; 20 mM NaH₂PO₄ pH 7.5/150 mM NaCl/1 mM ethylene glycol bis(β-aminoethoxy)-N,N,N',N'-tetraacetic (EGTA)/0.02% NaNO₃ containing 1% bovine serum albumin (P/NaCl/albumin) and then incubated in a humidified box for 30 min at 23°C with fluorescein-conjugated goat anti-rabbit IgG (Fl-GAR, Cappel Laboratories) diluted to 1:100 in P/NaCl/albumin. After a 1-min wash in P/NaCl/albumin and then in P/NaCl, the slides were mounted under coverslips with 90% glycerol/10% (vol/vol) P/NaCl. In order to minimize background fluorescence caused by nonspecific binding, the Fl-GAR (1:100) in P/NaCl/albumin was preadsorbed with P/NaCl/albumin-washed muscle tissue for 1 hr at 4°C before it was applied to sections for staining. Muscle sections that were to be labeled with antiserum after sectioning were incubated with adsorbed antiserum (1:180) in P/NaCl/albumin for 30 min at 23°C, washed in P/NaCl/albumin, and then stained with the Fl-GAR second antibody as described above. Stained sections were examined with a Zeiss Photomicroscope Mark III equipped with epifluorescence optics (interchangeable filters for fluorescein and rhodamine), a built-in camera, and an automatic exposure meter. For most experiments, clusters of endplates were located by the RaBTx binding, then viewed and photographed with both fluorescein optics and rhodamine optics to demonstrate the coincidence of binding of RaBTx and synaptic vesicle antiserum at synapses. Acetylcholine esterase staining of near-consecutive serial sections confirmed the positions of neuromuscular junctions.

**RESULTS**

Antiserum binding to sections of frog muscle

We had previously shown that adsorbed rabbit anti-synaptic vesicle antiserum bound selectively to nerve terminals in sections through the neuromuscular junctions of rat diaphragm (3). To confirm this result for the frog CP muscle, cryostat sections through the innervated regions of the muscle were simultaneously labeled with antiserum and RaBTx, washed, and then exposed to the fluorescein-conjugated second antibody (Fl-GAR). Appropriate filter combinations on the epifluorescence photomicroscope allowed us to excite and view fluorescein and rhodamine selectively. We could thus compare the distribution of vesicle antiserum binding to that of RaBTx, which binds tightly and specifically to the acetylcholine receptors in the postsynaptic membrane.

Comparison of synaptic vesicle antiserum and RaBTx binding to cryostat sections is shown in Fig. 1. Antiserum-labeled structures were a few micrometers in diameter, positioned on the periphery of muscle fibers, and present only when sections were cut through the innervated regions of the CP preparation. The immunoreactive sites were usually found in clusters of up to a dozen, with each site often appearing to be uniquely associated with a single muscle fiber. In longitudinal sections of antiserum-labeled frog muscle, immunoreactivity was restricted to thin strips running along a few individual muscle fibers (Fig. 2a), consistent with the notion that terminal membranes were being labeled. Intermuscular nerve bundles, however, were not stained by the adsorbed antiserum. Because nerve terminals in frog skeletal muscle are relatively small com-

![Fig. 1. Antibodies to Narcine synaptic vesicles bind to nerve terminals in cryostat sections of frog CP muscle. Cross sections (6 μm) were incubated with antiserum and RaBTx, washed, and then treated with Fl-GAR to localize the synaptic vesicle antibodies. Sections photographed in fluorescein optics and rhodamine optics demonstrate that the immunofluorescent labeling of nerve terminals coincides with RaBTx labeling of neuromuscular junctions in these double-stained sections. (a) Fluorescein optics. Synaptic vesicle antiserum binding to terminals (arrow). When antiserum is applied to sections of unfixed tissue, nonspecific binding to skin (lower left) and some punctate staining of myofibrils occurs. (b) Rhodamine optics. RaBTx binding to endplates (arrow) in the same section. (c) Bright-field optics. Acetylcholine esterase staining of synapses (arrow) in a near-consecutive section.](image1.png)

![Fig. 2. Longitudinal section of frog neuromuscular junctions, showing nerve terminals labeled by indirect immunofluorescence with antibodies to Narcine synaptic vesicles. A frog sartorius muscle section was incubated with antiserum and RaBTx, washed, and then treated with Fl-GAR. (a) Fluorescein optics. Antiserum binding to sectioned terminals. (b) Rhodamine optics. RaBTx binding to postsynaptic membrane in the same cryostat section. The scale bar is 100 μm.](image2.png)
pared to the thickness of the section, the immunofluorescent target sites completely overlapped the toxin-labeled endplate regions on the muscle fiber surfaces, both in cross sections and in longitudinal sections. Virtually no specific antibody binding at nerve terminals was observed with preimmune serum or with immune serum preadsorbed with sonicated synaptic vesicles.

The localization of acetylcholine esterase activity provided another criterion by which to correlate immunoreactive sites with synapses in near-consecutive cross sections of antisera-labeled CP preparations (Fig. 1c). Generally, esterase staining patterns for synapse distribution could be readily matched to the staining patterns obtained for RaBTx-labeled endplates and antisera-labeled sites in adjacent sections.

**Antiserum binding to intact frog muscle**

In order to detect antigens on the external surface of the nerve terminal, prior to sectioning, intact CP muscles were incubated for 8–12 hr at 4ºC in a mixture of adsorbed antiserum and RaBTx. After washing, the preparations were sectioned and the sections were incubated with Fl-GAR. Nerve terminal-rich regions were localized by using RaBTx binding and acetylcholine esterase staining. In contrast to the cross section staining results, synaptic vesicle antiserum binding on the surfaces of intact nerve terminals was much less pronounced and often undetectable (Figs. 3c and 4c). Of 230 synapses examined in five experiments, 50 (22%) had a barely detectable level of antiserum binding and the remainder showed none. The negative result was not due to an absence of nerve terminals or synaptic vesicles inside them, because intraterminal antigens could be strongly labeled by applying vesicle antiserum to the junctions after sectioning (Fig. 4e).

The above result would be consistent with the relative lack of synaptic vesicle antigens on the plasma membrane of the resting nerve terminal. Assuming this were so, and synaptic vesicle antigens were transferred to the nerve terminal by exocytosis, then stimulation of transmitter release might induce antiserum binding to the nerve terminals of intact preparations. Heuser and Miledi (15, 16) have shown that 1 mM La³⁺ causes a massive increase in the frequency of miniature endplate potentials with concomitant depletion of synaptic vesicles. We have confirmed both the electrophysiological and the ultrastructural effects of lanthanum on this preparation. We have now demonstrated that exocytosis induced by lanthanum permits vesicle antibody binding to nerve terminals of intact preparations of the frog CP.

For these stimulation experiments, pairs of muscles were dissected, and one was exposed for 1–2 hr to 1 mM LaCl₃/1.8 mM CaCl₂, while the other remained in 14 mM MgCl₂/0.9 mM CaCl₂ or ordinary PF Ringer solution as a control. Both muscles were then incubated overnight in a mixture of adsorbed vesicle antiserum and RaBTx. Cryostat sections, cut simultaneously through the innervated regions of both muscles, were then incubated with Fl-GAR. The results (Figs. 3a and 4a) show synaptic vesicle antibody binding to nerve terminals in the La³⁺-treated fractions, but not the unstimulated controls (Figs. 3c and 4c). In 286 synapses (five experiments) detected by RaBTx staining, 221 (77%) also showed antiserum binding after La³⁺ stimulation. Because it is unlikely that lanthanum changes the permeability of the preparation to antibody, the negative result in the control experiments must mean that synaptic vesicle antigens are not present in significant amounts in the plasma membrane of the resting nerve terminal.

Does the exposure to lanthanum induce binding by stimulating exocytosis or could the lanthanum be breaking down the membrane permeability barriers? Morphologically, the plasma membrane appears intact after the nerve terminal is depleted of vesicles by lanthanum (D. A. Riley, personal communication). In addition, others have shown that nerve terminals lose about 85% their acetylcholine content after exposure to lanthanum but...
lipase of the transfer nerve terminals /3-BTx (17). We have found by coated rotoxin preparations in sections, it can restore it to normal or greater than normal levels within several hours. Indeed, such behavior would not be expected of disrupted nerve terminals. We have shown that lanthanum does not simply cause widespread membrane damage by repeating the above experiments with anti-actin antibody. Although this antibody binds extensively to the inside of muscle fibers in frozen sections, it shows no intracellular penetration with intact preparations in either the presence or the absence of lanthanum.

The ability of antibodies to gain access to the synaptic regions of intact neuromuscular preparations was confirmed in another way. Treatment of CP preparations with the presynaptic neurotoxin β-BTx has been shown to cause an unusual accumulation of coated vesicles at the plasma membrane (8) followed at late times by selective disruption of the nerve terminal membrane (17). We have found that prior exposure of the intact muscle to β-BTx (10 μg/ml) also permitted selective antibody binding to nerve terminals (Fig. 4i). Whether this is due to exocytotic transfer of vesicle antigens to the surface or to permeabilization of the nerve terminal plasma membrane by the toxin's phospholipase activity (17) is not known.

FIG. 4. Comparison of experimental and control results obtained by incubating intact CP preparations with synaptic vesicle antiserum and RaBTx. Synaptic vesicle antigens become accessible to antibodies in the bathing medium only when terminals are stimulated with Le3+ or exposed to β-BTx. In all cases, experimental and control CP muscles were incubated with RaBTx and the appropriate serum, quick frozen, and sectioned. Sections were then stained with Fl-I GAR. Panels in the left column were photographed with fluorescein optics to show details of immunofluorescent antiserum-labeled structures. The corresponding panels to the right of each of the fluorescein panels are with rhodamine optics to show RaBTx-labeled endplates present in the same section. (a) Le3+-stimulated nerve terminals bind synaptic vesicle antibodies on their outer surfaces; (b) RaBTx. (c) Resting, unstimulated preparations bind little or no antiserum on their surfaces; (d) RaBTx. (e) Control, an adjacent section to c/d (unstimulated) incubated in cross section with antiserum (as in Fig. 1) to demonstrate the nerve terminals that were present but in which synaptic vesicle antigens had remained inaccessible to antibodies in the bathing medium; (f) RaBTx. (g) Le3+-stimulated control preparation exposed to preimmune serum rather than immune serum, micrograph overexposed to emphasize the absence of any detectable signal; (h) RaBTx. (i) β-BTx-treated nerve terminals also bind synaptic vesicle antibodies in whole mount; (j) RaBTx. The scale bar in j is 100 μm for all micrographs in this figure.

DISCUSSION

We have made use of the antigens shared by synaptic vesicles from electric organ and vertebrate nerve terminals to explore changes in membrane antigen distribution upon nerve stimulation. It had seemed likely that antigenic sites on synaptic vesicles would be useful markers and that some of the antigens, those on the inner surface of the vesicles, would be transferred to the outer surface of the nerve terminals as a result of vesicle fusion with the plasma membrane. From data presented here, we know that, in the absence of exocytotic activity, synaptic vesicle antigens are normally inaccessible or are at a very low concentration on the external surface of the plasma membrane. We have now shown that, concomitantly with lanthanum-induced exocytosis, previously concealed synaptic vesicle antigens become accessible to antibodies in the bathing medium of intact frog nerve–muscle preparations. Our data could be explained if at least one of the antigens shared by the frog nerve terminals and the fish synaptic vesicles is on the inner surface of the frog synaptic vesicle and if this antigen is transferred to the outer surface
of the nerve terminal during the exocytic release of transmitter. If this explanation is correct, then the shared internal antigen must be selectively removed from the plasma membrane after exocytosis, because it is present in such low amounts on the surfaces of resting terminals.

While this is an appealing interpretation, one obvious criticism is the use of nonphysiological conditions to stimulate exocytosis. In our initial studies, we found that transmitter release induced by extensive tetanic electrical stimulation did not result in appreciable antibody labeling of terminals in intact frog nerve-muscle preparations. We suspect that during normal release the time a synaptic vesicle membrane remains in the plasma membrane is very short (7) compared to the time required for antigen–antibody interaction in the synaptic cleft and that too little antigen would be exposed on the terminal at one time to later give a detectable immunofluorescent signal in cross section. In order to maximize the transfer of vesicle antigens to the plasma membrane for detection with immunofluorescence, we chose to use La^{3+} stimulation, which triggers an enormous increase in spontaneous mini-endplate potentials, leading to a depletion of nerve terminal vesicles (15). The depletion appears to be irreversible and is accompanied at early times by an expansion of the plasma membrane as a consequence of the addition of synaptic vesicle membrane by exocytosis (16). During the period of incubation with vesicle antibodies, La^{3+}-treated nerve terminals apparently then retrieve much of their synaptic vesicle membrane by the formation of large vacuoles, although or-6-

To arrive at the conclusion that, after extensive exocytosis, previously concealed synaptic vesicle antigens become accessible to antibodies on the outside of the nerve terminal. Although we have no direct evidence to indicate that exocytosis per se causes the antigens to become exposed, the indirect evidence reported here is compelling. The trivial explanation that lanthanum disrupts the plasma membrane and allows antibodies to reach synaptic vesicles is unlikely because the electron microscope morphology of the treated terminals (refs. 15 and 16; D. A. Riley, personal communication), their ability to restore their acetylcholine levels to normal, and the evidence against permeabilization presented here all indicate that the terminals are intact.

Although we know that vesicle antiserum specifically binds to synaptic vesicles from electric organ and to vetebrate nerve terminals, we do not yet know if the antibodies bind to vertebrate synaptic vesicles. The data presented here, showing the transfer of antigens to the external surface of frog terminals during exocytosis, are certainly consistent with that possibility. If we are indeed observing exocytotic transfer of synaptic vesicle membrane to the plasma membrane, then some conclusions follow. The possibility has been raised that the bulk of the vesicles in the nerve terminal is not involved in exocytosis but has a storage function. Transmitter release is claimed to involve a saturable "operator unit" different from the synaptic vesicle (18). Our data do not favor this claim unless the putative operator contains a molecule or molecules antigenically similar to those in the synaptic vesicle. The same conclusion applies if the true exocytotic vesicle is the small, denser vesicle proposed by Suszkiew and Whittaker (19). Further, the data suggest antigenic and, therefore, biochemical differences between the resting plasma membrane and the plasma membrane during exocytosis. If exocytosis introduces novel antigenic determinants to the outside of the nerve terminal, then these determinants must be selectively removed. To confirm this suggestion, we need to use more physiological stimulation conditions for promoting exocytosis such that the plasma membrane could return to its original resting state. Furthermore, the vesicle antigen transfer experiments need to be repeated with antibodies directed against individual vesicle membrane components to identify which vesicle antigens appear on the plasma membrane.

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