EFFECTS OF CALCIUM-CONTAINING FIXATION SOLUTIONS ON CHOLINERGIC SYNAPTIC VESICLES

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ABSTRACT

Calcium (Ca)-containing fixation solutions applied to slices of electric organ of the electric ray, *Narcine brasiliensis*, have been shown to have three distinct ultrastructural effects on cholinergic synaptic vesicles of the nerve terminals.

(a) An electron-dense particle (EDS) is observed within the vesicle; the particle is seen in unosmicated, unstained tissues and can be removed from thin sections by Ca-chelating agents. It is concluded that the EDS represents Ca bound by the vesicle. It is suggested that the bound ATP of the vesicle provides anionic Ca binding sites.

(b) The vesicle membrane tends to 'crinkle' or collapse depending on the concentration of the other components of the fixative solution. The 'crinkling' or collapse are largely reversed by a wash step in the absence of Ca.

(c) The presence of Ca results in the appearance of a population of vesicles which form characteristic fusions or 'tight' junctions with the terminal membrane. This appears to be morphological evidence for the proposal, which has been frequently put forward, that Ca facilitates such a fusion before discharge of vesicle-bound transmitter.

With the discovery that the use of Ca-containing fixatives leads to the demonstration of a subpopulation of synaptic vesicles fused to the terminal membrane, we are led to propose that this is the ultrastructural location of the newly synthesized acetylcholine which has been shown by others to be preferentially released by stimulation.

Recently, we (4) described fixation conditions which result in the appearance of a single, dense particle in many of the cholinergic vesicles of the neuroplaque junction of the electric ray, *Narcine brasiliensis*. Now we are presenting additional findings including the demonstration that calcium is necessary in the fixative solutions, if the particle is to be visualized.

Oschman and Wall (29) have suggested that calcium-containing fixatives may facilitate the ultrastructural identification of calcium binding sites, which then appear as spots or plaques of high electron density. These electron-dense spots (EDS) were regarded as Ca binding sites in insect intestinal membranes because (a) they occurred only when Ca was included in the fixation solutions, and (b) they were visible in sections from aldehyde fixed, unosmicated tissues, indicating that the EDS have an intrinsic electron density. Hillman and Llinás (15) and Oschman et al. (28) have recently analyzed Ca-dependent EDS in the membranes of the squid giant axon by energy dispersive X-ray
analysis. The results showed that high concentrations of Ca (15, 28) and phosphorus (28) were present in the region of the electron densities. Politoff et al. (32) have recently reported that synaptic vesicles of the frog neuromuscular junction show EDS when fixed in Ca-containing solutions; they concluded that the vesicles have a Ca binding site.

Heuser and Reese (14) and Holtzman et al. (17) have provided evidence that, during stimulation of the frog (14) and lobster (17) neuromuscular junction, vesicular membrane becomes incorporated into nerve terminal membrane and is subsequently recovered into 'coated' vesicles which move from the terminal membrane to the cytoplasm. Coated vesicles then fuse to form cisternae from which new naked vesicles are generated. One of the aims of the present study was to establish which of these structures displayed EDS when fixed in Ca-containing solutions.

The presence of Ca is essential for the depolarization-coupled release of acetylcholine (ACh) at cholinergic nerve terminals (20, 21). Elevation of intraterminal Ca has been shown to increase the frequency of spontaneous miniature end-plate potentials (MEPP's) in the frog neuromuscular junction (26). Although the precise role(s) of Ca in these processes has remained obscure, it has been hypothesized that divalent cations may facilitate the fusion of vesicles with the presynaptic membrane, as a prerequisite for vesicular transmitter release (2). Heuser et al. (13) did not obtain fusions between vesicles and the nerve terminal membrane in frog neuromuscular junction when the preparations were incubated for several hours in isotonic (83 mM) Ca followed by fixation in 10 mM Ca-containing media, but fusions were seen after prolonged incubation in solutions containing 83 mM magnesium (13). Although the effects of calcium and magnesium on MEPP's may not be identical in both the ray neuroplaque and the frog neuromuscular junctions, we can now report that Ca-dependent vesicle fusions to the terminal membrane can be seen in the neuromuscular preparation.

Elevation of intraterminal Ca concentration is thought to immediately precede transmitter release (22, 24). In our previous report (4), we noted that synaptic vesicles in slices of N. brasiliensis electric organ sometimes appeared to breach the terminal membrane when the excised tissues were directly immersed in a Ca-containing fixation solution. We have now compared the morphology resulting from the presence of Ca, Mg, or Na in the fixation solutions. These experiments were carried out in the hope that they might shed some light on the early morphological changes that could be induced by the elevation of intraterminal Ca concentrations.

MATERIALS AND METHODS

Tissues

Electric organ slices: Rays were killed by chilling to 4°C or were anesthetized by placing them in 2 liters of sea water containing 0.05% of ethyl m-amino- benzoate methanesulfonate (MS-222). During removal of the fish from the aquarium and placement in a pan for cooling or anesthesia, discharge of the electric organ often occurred; therefore, the nerve terminals described here probably do not represent the 'resting' state. Cylinders of tissue were punched out of electric organs in situ, inserted in a Riggins-Stadie microtome, and sliced in a horizontal plane. Thin sections of tissue less than 1 mm thick and containing 10-20 stacks of electric cells were immersed in primary fixative. After washing (see below for solution composition), individual polygonal stacks were teased apart and immersed in the postfixative. After postfixation, the tissues were washed with the same wash solution used before postfixation, dehydrated, and embedded in Epon or Epon-Araldite. The flat stacks of cells were then oriented so that thin sections could be cut in a transverse plane.

Vesicle isolation: Isolated nerve terminal vesicles were obtained and prepared for electron microscopy as described previously (4).

Chemicals: Glutaraldehyde (EM grade) was obtained from Taab Laboratories, Reading, England; paraformaldehyde from Eastman Kodak, Rochester, N. Y.; sodium cacodylate, sodium citrate, and sodium ferrocyanide from Baker Chemical Co., Phillipsburg, N. J.; calcium chloride, magnesium chloride, sodium chloride, and ethylenediaminetetraacetic acid (disodium salt) (EDTA) from Fisher Chemical Co., Fairlawn, N. J. The anesthetic agent MS-222 was obtained from Aldrich Chemical Co., Milwaukee, Wis.

Fixatives: The osmolarity of Torpedo ray body fluids is 870-1.098 mosmol l⁻¹ (36, 16). The osmolarity of buffer and salts in the fixation and postfixation solutions used in the present experiments was either slightly hypo- or approximately isotonic as described below. In addition, all fixation solutions contained 4% formaldehyde and 5% glutaraldehyde.

Low osmolarity (700 mosmol l⁻¹): 0.075 M sodium cacodylate, pH 7.2, 0.14 M NaCl together with either 0.09 M CaCl₂, or 0.09 M MgCl₂, or 0.0135 M NaCl.

High osmolarity (1,000 mosmol l⁻¹): 0.3 M sodium cacodylate pH 7.2, 0.133 M sucrose together with either 0.09 M CaCl₂, or 0.09 MgCl₂, or 0.135 M NaCl. Washing solutions had the same salts and buffer as the primary fixative but lacked the aldehydes.

Boyne et al. Calcium Effects on Vesicle Morphology 781
POSTFIXATION: Previous fixations (4) were carried out using buffered osmium tetroxide as postfixative. A substantial improvement in final tissue preservation and contrast in thin sections was observed when sodium or potassium ferrocyanide was included in the postfixation solution (19). The following postfixation solutions were therefore used in the studies reported herein. For low-osmolarity fixation, a solution calculated to have an osmolarity of approximately 700 mosmol 1⁻¹ was made up as follows: 1.5% OsO₄, 0.035 M Na₄Fe(CN)₆ and 0.075 M sodium cacodylate together with either 0.09 M CaCl₂ or 0.09 M MgCl₂ or 0.135 M NaCl. The postfixation solution used with the high-osmolarity fixation had a composition identical to the wash solution with the addition of 1.5% OsO₄ and 0.135 M Na₄Fe(CN)₆. In some experiments, a hypo-osmotic postfix was used after high-osmolarity primary fixation and wash steps. The hypo-osmotic postfixation solution was composed of 1.5% OsO₄; 0.035 M K₄Fe(CN)₆; 0.075 M sodium cacodylate, pH 7.2, and either 0.09 M CaCl₂, or 0.09 M MgCl₂, or 0.045 M MgCl₂ together with 0.045 M CaCl₂. Primary fixation was for 2-3 h; washing was carried out over 1-2 h. Primary fixations were carried out at 0°C or 23°C; washing and postfixation were carried out at 23°C.

MICROSCOPY: Thin sections (silver to gray sections) were cut on an LKB microtome. Staining was carried out for 6 min in uranyl acetate followed by 45 s in lead citrate. Longer times in lead citrate resulted in the disappearance of the intravesicular particles (see Results).

QUANTITATION: The frequency of 'tight' junction formation between synaptic vesicles and nerve terminal membrane was estimated as follows. Silver sections (about 70 nm thick) were obtained from blocks of electric organ fixed as described above. The sections were picked up on 300-mesh naked copper grids and stained. They were scanned at × 5,000 magnification in a JEM 100 B electron microscope until a region of the section free of holes and supported on four sides by grid bars was located. The magnification was then raised to × 20,000 and consecutive photographs were taken of the nerve terminals along two adjacent electric cells. In this manner, 20-25 nerve terminals were photographed from each block. The negatives were printed to give a final image of × 45,000. A Dietzgen Plan Measure (Model No. 1718) was used to measure the lengths of nerve terminal membrane apposed to the electric cells and the number of vesicles fused to that membrane were counted. The general ultrastructure of the electric organ corresponded to that previously described (35). Nuclei, mitochondria, and fibrillar material were seen in the generally translucent cytoplasm of the flat electric cells. The innervated dorsal surfaces of these cells were increased in area by extensive systems of canaliculi. Nerve terminals were applied profusely to the ventral surface. The space between the neuroplaque units contained nerve fibers, collagen fibers, and elongated cells with presumed pinocytotic vesicles.

Calcium Dependence of the Intravesicular Particles

The appearance of nerve terminals in slices fixed with the low osmolarity solutions containing 90 mM CaCl₂ is shown in Fig. 1. Both intravesicular

RESULTS

Fixation Conditions for N. brasilienis Electric Organ

Our initial fixation of electric organ tissue was carried out using the solution recommended by Karnovsky for mammalian tissue (18) but at 0°C rather than room temperature. The nerve terminals were predominantly ruptured and were devoid of synaptic vesicles. Although this fixative has been used successfully at room temperature on electric ray electric organ (41), it appeared that the low osmolarity of the 0.045 M CaCl₂ and 0.08 M sodium cacodylate (295 mosmol 1⁻¹) relative to the osmolarity of the Torpedo ray body fluids (870-1,098 mosmol 1⁻¹) (36, 16) resulted in the development of osmotic stress at 0°C. This interpretation was supported by an experiment in which the combined osmolarity of the buffer and added 0.09 M calcium chloride were set at 420, 600, and 870 mosmol 1⁻¹ in three fixatives which were used at 0°C. After use of the 420-mosmol solution, no vesicles were preserved in the nerve terminals; after the 600-mosmol solution, a sparse population of vesicles was seen; after the 870-mosmol mixture, nerve terminal vesicles were preserved in abundance. When fixation was carried out at room temperature (about 23°C), preservation of vesicles was seen to be less dependent on the osmolarity of the fixative solution. Therefore, a routine procedure of fixation at room temperature in solutions of 700-1,000 mosmol 1⁻¹ was adopted. These are referred to respectively as low- and high-osmolarity fixation solutions in the subsequent text (see Materials and Methods section for details of their composition).

The mean diameter of synaptic vesicles in these terminals is 84 nm (35, 40); profiles of vesicles with diameters of less than 40 nm were not included in the counts. The frequency of the fusions per square micron of nerve terminal membrane was then calculated assuming that the sections represented a 70 nm width of the terminal membrane.

This report is based on studies of electric organs from 18 rays in which approximately 90 slices of tissue were processed and examined in the electron microscope.
FIGURE 1 Nerve terminal from *N. brasiliensis* electric organ fixed in low-osmolarity fixative containing 90 mM Ca. Prominent EDS can be seen in most of the vesicles; note that the vesicle membranes appear to be crinkled. Three fusions between vesicles and the terminal membrane opposite the electric cell membrane can be seen (arrows). × 60,000. Inset: higher magnification view of a vesicle fused to the terminal membrane. × 140,000. Scale bar = 100 nm.

and extravesicular dense particles can be seen. The intravesicular particles can also be clearly demonstrated in pellets of isolated fixed vesicles as shown in Fig. 2. Both the intra- and extravesicular particles can be seen in unstained sections as well as after staining with uranyl acetate and lead citrate as described in the Materials and Methods section.

If the CaCl₂ in the fixation solutions is replaced by 90 mM MgCl₂ or 135 mM NaCl, the intravesicular particle disappears except for occasional vesicles wherein a particle of relatively low electron density can be observed (Figs. 14, 15). If 10 mM CaCl₂ together with 120 mM NaCl was used as the cation addition in the fixative, dense intravesicular particles were still observed although less frequently than they were seen in blocks from the same fish fixed with 90 mM Ca (cf. Figs. 1, 3 and Table I). When a mixture of 45 mM CaCl₂ and 45 mM MgCl₂ was utilized in the fixation solutions, the intravesicular particle was apparent again but with reduced electron density. The appearance of the EDS was not dependent on the temperature of fixation between 0°C and 23°C.

EDS of a similar size to the intravesicular particles but of an apparently lower electron density could be discerned in un-osmicated, unstained sections of tissues fixed in 90 mM Ca solutions (Fig. 7). After staining such sections, the apparent density of the EDS increased, and a vesicular membrane could be seen around them (Fig. 8). The Ca-dependent intravesicular particles, therefore, have an electron density that is not dependent on osmication or staining but may be enhanced by these procedures.

Chelation Experiments with Thin Sections

When thin sections (on copper grids) of tissues that had been fixed in the presence of Ca were floated on 0.12 M sodium citrate, or 50 mM EDTA at pH 7.0 for 10 min, the intravesicular particle disappeared and spots of low electron density appeared. These spots remained electron lucent after staining. Control sections floated on 0.1 M NaCl containing 10 mM sodium cacodylate, pH 7.0, retained the particle. The results of these treatments are shown in Figs. 9–11. If tissues floated on either of the chelating agents were
subsequently floated on a 90 mM CaCl₂ solution, the leaching out of the intravesicular particle was not reversed.

Effect of Divalent Cations on Vesicle Shape

From Figs. 1 and 3, it can be seen that the presence of Ca at 10 mM–90 mM concentrations in the fixative of low-osmolarity produces a 'crinkling' distortion of most vesicle membranes as compared with the generally smooth curvatures of vesicles fixed in the presence of Na and Mg (Fig. 4). Some 'crinkling' is seen in occasional vesicles in all the fixation conditions employed, but the unique Ca influence on the process is quite clear when fixation is carried out in the high-osmolarity fixative (cf. Figs. 12, 14, 15). When tissue slices fixed in primary fixatives containing Ca were divided into two pieces, and one piece given a Ca wash while the other received Na wash, then, in the Na washed tissue, (a) the intravesicular particle...
was not observed, and (b) the vesicular wrinkling (in the low-osmolarity medium) or collapse (in the high-osmolarity medium) was not present (Figs. 6, 16).

Effect of Divalent Cations on Vesicle Interactions with Presynaptic Membrane

When fixation was done in the absence of divalent cations, many vesicles were found in close proximity to the preterminal membrane, and occasionally vesicles appeared to touch the preterminal membrane (Figs. 5, 14). In tissues fixed in the presence of Ca and/or Mg, characteristic and extensive vesicle attachments to the terminal membrane were also seen (Figs. 1, 3, 12). As illustrated in Figs. 1, 3, and 4 (insets), fusion of the two unit membranes occurs, and the vesicle contour bulges into the neurolaque cleft. The mean number of fusions seen in tissues fixed in 0, 10, and 90 mM Ca and in 90 mM Mg were measured as described in the Materials and Methods section; the values are presented in Table I. When primary fixation was carried out in the presence of Ca, and washing and postfixation were conducted in the absence of divalent cations, large numbers of fusions between vesicles and the preterminal membranes were still observed (Figs. 6, 16).

Components of the Hypothesized Vesicle Membrane Cycle

Well-defined omega figures suggestive of exocytotic vesicular discharge were too rare to permit
statistical analysis. In the outermost cells of several blocks of Ca-fixed material, electron-dense deposits were seen along membranes of the nerve terminals and nerve fibres. These densities were also seen (though infrequently) in the membrane of the innervated face of the electric cell. They were much more numerous in the membrane of the noninnervated face (Fig. 17).

These deposits were only observed in the membranes of the outermost 5–10 layers of cells in each block of tissue. They were completely absent from nerve terminals and electric cells in the interiors of the blocks (from which most of the electron micrographs presented here were derived).

Coated vesicles were observed at the terminal surface, in the cytoplasm (Fig. 18) and in association with cytoplasmic cisternae (Fig. 19). None of these structures contained EDS. The membranes of cisternae also lacked EDS (e.g. Fig. 3).

DISCUSSION
The above experiments demonstrate that the final appearance of the previously reported (4) intravesicular EDS in cholinergic vesicles of *N. brasiliensis* electric organ is modified by the presence and the concentration of Ca in the fixative. To summarize the various experimental results: after using Ca-containing fixatives, vesicular EDS can be seen in thin sections of unstained, unosmicated tissues; when such sections are stained or when the tissue has been postfixed in a solution containing osmium (4) or a mixture of osmium and ferrocyanide (the present results), then a more electron-dense EDS is observed; EDS can be removed from thin sections by chelating agents known to have a high affinity for Ca. It therefore seems reasonable to conclude that these cholinergic vesicles have the capacity to bind Ca. Although the presence of calcium in buffered aldehyde fixation and wash solutions is a sufficient condition for the appearance of vesicular EDS, the increase in electron density seen after staining such sections, and in postfixed tissues, may be due to a secondary attachment or staining of the preserved particles by the heavy metals used in these procedures.

Our results support the recent conclusions of Politoff et al. (32), in their study, in the frog neuromuscular junction, that cholinergic vesicles have a Ca binding capacity. Differences between the results in these two cholinergic systems may be noted. In the frog neuromuscular junction, the vesicle size is about 50 nm in diameter; the EDS were 5–7 nm in diameter and of similar electron density, whether fixation was carried out in 5 or 90 mM Ca. The vesicles in the neuroplaque junction have a larger diameter (about 84 nm); the EDS in this case show a size, electron density, and frequency dependence on the Ca concentration in the fixative. After the use of 90 mM Ca fixatives, EDS are present in about 50–60% of the vesicles; these EDS have a diameter of about 25 nm and a fairly uniform electron density. After the use of 10 mM Ca fixatives, the frequency of vesicular EDS is greatly reduced (see Table I); the diameter of those EDS which are seen varies from 5–25 nm, and the electron density also shows a much wider range (see Fig. 3 and Table I).

Politoff et al. concluded that the EDS was intravesicular but probably attached to the vesicle membrane, since it can be seen to contact the membrane in many vesicles (32). These workers then noted that, "If the site marked by these particles plays a role in ACh release, it would be necessary to consider that the vesicles are polarized, anisotropic structures and that a specific region on their surface has to be in contact with the presynaptic membrane in order to release its contents." This interpretation is dependent on the conclusion that the Ca binding site is a unique part of the vesicle membrane. However, the nature of the binding site itself has not been determined. Alternative explanations for the Ca-binding phenomenon deserve consideration; one such possibility is discussed below.

Dowdall et al. (10) have shown that cholinergic vesicles isolated from the electric organ of *Torpedo marmorata* contain ATP: the same has been established also for *N. brasiliensis* (4). The molar ratio of ACh to ATP in these vesicles is about (10) 5:1. Hence, from estimates of ACh concentrations within cholinergic vesicles (about 0.6 M [40]), it is possible to estimate that ATP is present at a concentration of about 0.1 M. This high concentration of negatively charged phosphate residues may be responsible for the localization of Ca reported here.

If the EDS does represent a complex of Ca and vesicle-bound ATP, it does not seem essential to infer that the vesicles are anisotropic; a particle within the vesicle core may come to rest against the vesicle membrane, unless a specific structure were to keep it suspended. While it remains to be proven that the vesicles fused to the terminal membrane in...
TABLE I

Effect of Divalent Cations on the Frequency of Vesicular EDS and Fusions to the Nerve Terminal Membrane

| Fish | Fixative | Vesicles† with EDS | Frequency§ of fusions per μm² | Blocks analyzed |
|------|----------|--------------------|------------------------------|-----------------|
| 1    | 0 mM Ca  | 0                  | 0                            | 3               |
| 1    | 10 mM Ca | 26.6 ± 5.0         | 3.7 ± 2.0                    | 3               |
| 1    | 90 mM Ca | 47.7 ± 4.4         | 9.6 ± 1.5                    | 3               |
| 1    | 90 mM Mg | 0                  | 8.0                          | 1               |
| 2    | 0 Ca     | 0                  | 0                            | 3               |
| 2    | 90 mM Ca | 64.6 ± 4.0         | 8.2 ± 1.0                    | 2               |

* Results are values obtained from tissues fixed in the low-osmolarity fixative described in the methods section.
† Based on counts of 700-1200 vesicles in each block.
§ Expressed as mean ± standard deviation of results for three blocks of tissue, or range for two blocks of tissue.

The electric organ are involved in transmitter release, the present morphological results do not support the hypothesis that a specific orientation occurs between vesicular EDS and sites of fusion (see Figs. 1 and 3).

Similar explanations for Ca binding may be advanced for the 5-HT granules of platelets which are known to contain ATP (9) and for which Martin et al. (25) have recently argued that high concentrations of Ca are present. Koko and Barret (23) have described fixation conditions which preserve electron-dense contents in otherwise clear synaptic vesicles both with and without the use of added cations. These workers concluded that a negatively charged species was a constituent of many usually clear vesicles in several locations in mammalian central nervous system. In cholinergic vesicles, bound ATP can now be recognized as a possible source of these negative charges, as discussed above. It must nevertheless be noted that a complete analysis of synaptic vesicle constituents may disclose other possible sources and mechanisms of cation binding.

The present experiments have shown that the Ca binding in neuroplaque vesicles can be reversed by washing the tissues in a solution containing only sodium as added cation. This suggests that the binding affinity is not high. The possibility is therefore open that some vesicles contain bound cations in vivo which are leached out during the usual fixation procedures for electron microscopy. This interpretation would be consistent with the occasional appearance of vesicular EDS in tissues fixed without added Ca (Fig. 14, 15). In this connection, it should be emphasized that the present experiments provide a very limited test of the specificity of the binding site; Ca-containing fixatives gave rise to EDS, Mg-containing fixatives did not. Politoff et al. reported that the binding site in the frog cholinergic vesicles will also bind cobalt (32).

The demonstrations of nerve membrane EDS and noninnervated face membrane EDS (Fig. 17) are of interest with respect to the reports of Ca-EDS in the membranes of insect gut (29) and squid giant axon (15, 28). The presence of these membrane deposits only in the outer layers of slices of electric organ is reminiscent of the diffusion artifacts encountered in histochemical

FIGURES 12-16 These micrographs illustrate the same sequence of cation effects as in Figs. 3-6, but the fixations were carried out in the high-osmolarity fixatives. The tissues were taken from the same ray as Figs. 1 and 3-6. All are at x 35,000 except Fig. 13 which is at x 80,000. Scale bar = 100 nm.

FIGURE 12 Fixative contained 90 mM Ca. Note vesicle flattening and vesicular EDS. Arrows indicate vesicles fused to the terminal membrane.

FIGURE 13 Higher magnification of flattened vesicles.

FIGURE 14 Fixative contained 90 mM Mg. Flattening effect is not apparent; EDS are absent except for occasional faint particles (arrow).

FIGURE 15 Fixative contained no divalent cations. Flattening effect is not apparent; occasional EDS of relatively low density are observed (arrow).

FIGURE 16 Primary fixative contained 90 mM Ca; wash and postfix contained Na as the only cation. Compare with Fig. 12 and note the reversal of flattening and the loss of EDS. Arrows indicate vesicles fused to the terminal membrane.
Figure 17. Electron-dense deposits in outermost layers of tissue slices. Low-osmolarity fixation with 90 mM Ca. Note deposits in nerve terminal membrane (NT), nerve fiber membrane (NF) and electric cell canaliculi membrane (EC). Fixation is probably not optimal in this region adjacent to the cut edge of tissue. × 35,000. Scale bar = 1,000 nm.
procedures (12). Since the vesicular EDS are seen, and adequate fixation is obtained throughout the tissue blocks, it is unlikely that diffusion of the aldehydes or Ca is restricted to the edge of the tissue slices. A possible explanation of the phenomenon is provided by the recent suggestion of Oschman et al. (28): aldehyde-calcium fixation may localize sites of ATPase activity by using endogenous ATP as the substrate and Ca from the fixative as the trapping agent to precipitate inorganic phosphate as it is released. Such a reaction occurring in freshly excised electric organ slices would be expected to give rise to membrane EDS along cells which contained adequate levels of endogenous ATP. The lack of membrane EDS in the interior of the blocks could then be explained by the possible exhaustion of the endogenous ATP and dispersion of inorganic phosphate by the time that the Ca in the fixative had penetrated deeply into the tissue slice. In contrast, vesicle-bound ATP would not be free to diffuse away before the fixative had penetrated.

Effects on Vesicle Shape

Several workers have reported fixation and wash conditions which result in flattening of some vesicle populations (37, 3, 38). This has been suggested as a basis for distinguishing different types of nerve terminal. The usefulness of these procedures would be greatly enhanced if the forces responsible for flattening or collapse were understood and could be manipulated. The present results show that the presence of 90 mM Ca has an effect on cholinergic vesicle membranes which appears as 'crinkling' in hypotonic fixation solutions and as collapse in hypertonic solutions. These distortions are not due to simple osmotic effects, since they are not observed in equivalent Na- or Mg-containing fixatives. Furthermore, they are reversible by wash solutions of similar osmolarity but free of Ca. Further experiments will be required to clarify the mechanisms producing these effects.

Fate of the Vesicular EDS

The 50% loss of vesicle profiles in stimulated electric organ (41) is consistent with the hypothesized exocytotic discharge (6, 14, 34, 41) and incorporation of vesicle membrane into nerve terminal membrane. What happens to the EDS of such vesicles? EDS were absent from nerve terminal membranes deep in the tissue blocks. Furthermore, EDS do not appear in the nerve terminal membrane when electric organs are stimulated in vivo and the vesicle population is dramatically depleted (Alan F. Boyne, Timothy P. Bohan, Terence H. Williams, unpublished results). Therefore, even if vesicles do fuse with the
terminal membrane, the Ca-binding capacity does not appear to become incorporated into that membrane. It is of interest that binding capacity is not evident in coated vesicles either at the terminal membrane or in the cytoplasm (Figs. 18, 19). Neither did cisternae show EDS (Fig. 3). Such disappearance of EDS would be expected if vesicular ATP were responsible for the binding, since exocytosis of vesicular contents would be expected to remove the ATP. Zimmerman and Whittaker (41) have demonstrated that the loss of vesicle profiles in electric organs stimulated to fatigue in vivo is associated with a fall in vesicle-bound ATP. This has been confirmed by ourselves.

Possible Physiological Significance of the Vesicular 'Tight Junctions'

Vesicle fusions to the terminal membrane of electric organ have previously been demonstrated using freeze-etched samples of resting nerve terminals (27). The present results confirm this interpretation of the morphology in thin sectioned material and show that Ca can facilitate the fusion process.

While this work was being prepared for publication, Pfenninger and Rovainen reported the results of a study on specialized structures interpreted as vesicle attachment sites (VAS) in freeze-cleaved profiles of presynaptic membranes in the central nervous system of the sea lamprey (31). They showed that after aldehyde fixation in a medium containing 56-120 mM K and 5 mM Ca, there was a 4.4-fold increase in the frequency of VAS relative to control tissues fixed in high K, Ca-free media. They were also able to show that electrical stimulation increased the frequency of VAS in tissues fixed in low K and 5 mM Ca. Their experiments led them to the conclusion that the VAS represent a morphologically distinct, Ca-dependent stage of transmitter release. Although we first recognized the induction of fusions between vesicles and the terminal membrane after fixation of electric organ in the presence of 90 mM Ca, it is clear that large numbers of fusions are also observed in 10 mM Ca; this 9-fold reduction in Ca concentration produced only a 2.6-fold reduction in fusion frequency (see Table I). These results suggest that the fusion frequency may have reached a maximum in 90 mM Ca or Mg. The facilitation of fusions by Ca or Mg is consistent with the work of Blioch et al. (2), who showed that polyvalent cations have a general property of facilitating fusions of artificial lipid membranes. These workers and other (13) have also shown that both Ca and Mg (in a Ca-free medium) can elevate the frequency of MEPPs in the frog neuromuscular junction. Blioch et al. proposed that the level of intraterminal cations controls the adhesion of vesicles to the nerve terminal membrane and thus the frequency of MEPPs (2).

The blood Ca in N. brasiliensis is 6 mM (16); the fixations in 10 mM Ca are therefore in a reasonably close approximation to the expected physiological levels of Ca. In assessing the potential physiological significance of the Ca-dependent vesicle fusions, however, and particularly the relationship between physiological frequencies of fusion and the frequencies seen after fixation in 10-90 mM Ca, the following considerations may be important. The repeating layers of densely innervated, thin electric cells, which provide an ideal situation for morphometric analysis, also constitute a substantial diffusion barrier (11). This is unlike the situation in the frog neuromuscular junction preparation (20) or the lamprey spinal cord (31) where fairly rapid exchange of ions occurs between bathing medium and tissue. The Ca concentration in immersion fixation solutions can therefore be expected to exert a significant control over, not only the final concentration of Ca achieved, but also the rate at which Ca will penetrate the tissue slice and the nerve terminals themselves. Knowing that fusions can nevertheless be demonstrated after immersion in 10 mM Ca fixatives, it does not seem unreasonable to regard the present morphological evidence as support for the hypothesis that vesicles close to or touching the terminal membrane can be induced to form fusions with that membrane by the rapid influx of Ca during physiological depolarization. Further work will be required to substantiate this conclusion; experiments to test whether the frequency of fusions can be altered by electrical stimulation or depolarizing levels of K during fixation in this tissue are being planned.

It must be acknowledged that the preservation of tissues for ultrastructural study involves a succession of unphysiological treatments which may produce artifactual relationships between cellular organelles. The present results are particularly suggestive, however, because they provide morphological support for a hypothesis which has been derived from electrophysiological studies of living tissues (2, 26). If the protruding vesicle fusions do represent a stage in physiological transmitter release, as Pfenninger and Rovainen have suggested for the VAS in central synapses (31),
two speculative mechanisms of ACh discharge can be considered. If the area of fusion were to break down, an exocytotic release of all the vesicular contents would occur and the vesicle would be eliminated as a separate organelle. Several studies have suggested that vesicle membrane does become incorporated into the terminal membrane during stimulation of the nerve (6, 14, 34, 41). However, several workers have shown that newly synthesized ACh is preferentially released on subsequent nerve stimulation (1, 7, 8). Each of these groups proposed that a population of transmitter-depleted vesicles may be situated near the terminal membrane (1, 7, 8). Such a hypothetical population of vesicles would be ideally suited to take up ACh newly synthesized from incoming choline, and the same vesicles would be well placed to discharge during subsequent depolarizations. Furthermore, in a recent study of electric organs stimulated to fatigue, it was found that the order of loss of cholinergic vesicular constituents in the isolated vesicles was ACh > ATP > bound nucleotide > vesicular protein (5, 41). Such a pattern is not consistent with the simple exocytotic model, and these authors suggested that there may be a preferential discharge of vesicles 'supercharged' with ACh.

An alternative possibility, which is consistent with all the above experimental findings, is that the fused membranes may have a high permeability to small molecules. An example of such a process is provided by the fused membranes at electrotonic synapses (30), which may not, however, be functionally and morphologically identical to the fusions observed herein. The process of vesicular bulging into the neuroplaque cleft reported here provides a large surface area of fused membrane and could therefore facilitate a rapid exchange of small molecules between the extracellular environment and the vesicle core. In vitro studies of isolated vesicles have demonstrated that selective release of ACh with complete retention of ATP can occur after phospholipase A treatment (5). Osmotic shock and detergent treatment result in a more rapid release of ACh than ATP (5). It therefore seems feasible that cations entering vesicles fused to the terminal membrane could selectively displace the more weakly bound ACh out of the vesicles and into the terminal cleft. This hypothesis would thus explain the preferential loss of ACh over other vesicle constituents in the stimulated electric organ (41).

If the increase in permeability were then terminated, perhaps by reversal of the fusion, a population of ACh-depleted but otherwise intact vesicles would be available near the nerve terminal membrane (1, 7, 8). Poste and Allison have reviewed current knowledge of the forces involved in the close approach of two charged membranes and the potential for rupture, stabilization, or reversal of the fusions (33). Subsequent cycles of uptake and release of ACh by this hypothetical population could explain the preferential release of newly synthesized ACh as discussed above. After several cycles, 'aged' vesicles may undergo an exocytotic discharge by perforation of the fusion and thus would begin the cycling of vesicle membrane proposed by others (14, 17).

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REFERENCES

1. BARKER, L. A., M. J. DOWDALL, and V. P. WHITTAKER. 1972. Choline metabolism in the cerebral cortex of guineapigs. Stable bound acetylcholine. Biochem. J. 130:1063.

2. BLOCH, Z. L., I. M. GLAGOLEVA, E. A. LIBERMAN, and E. A. NENASHEV. 1968. A study of the mechanism of quantal transmitter release at a chemical synapse. J. Physiol. 199:11.

3. BODIAN, D. 1966. Electron microscopy: Two major synaptic types on spinal motoneurons. Science (Wash. D. C.). 151:1093.

4. BOHAN, T. P., A. F. BOYNE, P. S. GUTH, Y. NARAYANAN, and T. H. WILLIAMS. 1973. Electron dense particle in cholinergic synaptic vesicles. Nature (Lond.). 224:82.

5. BOYNE, A. F., M. J. DOWDALL, and V. P. WHITTAKER. 1973. The structural and chemical
properties of synaptic vesicles. In Proteins of the Nervous System. D. J. Schneider, R. H. Angeletti, R. A. Brandshaw, A. Grasso, and B. W. Moore, editors. Raven Press, New York. 155. (This reference also appears in The Society of General Physiologists Series. Vol. 28: Synaptic Transmission and Neuronal Interaction. M. V. L. Bennett, editor. Raven Press, New York. 217. in the chapter entitled “Biochemical studies on cholinergic synaptic vesicles.”)

6. Ceccarelli, B., W. P. Hurlbut, and A. Mauro. 1973. Depletion of vesicles from frog neuromuscular junction by prolonged tetanic stimulation. J. Cell Biol. 67:499.

7. Chakrin, L. W., R. M. Marchbanks, J. F. Mitchell, and V. P. Whittaker. 1972. The origin of the acetylcholine released from the surface of the cortex. J. Neurochem. 19:2727.

8. Collier, B. 1969. The preferential release of newly synthesized transmitter by a sympathetic ganglion. J. Physiol. 205:341.

9. Da Prada, M., and A. Pletscher. 1968. Isolated 5-hydroxytryptamine organelles of rabbit blood platelets: physiological properties and drug induced changes. Br. J. Pharmacol. Chemother. 34:591.

10. Dowdall, M. J., A. F. Boyne, and V. P. Whittaker. 1974. Adenosine triphosphate. A constituent of cholinergic synaptic vesicles. Biochem. J. 140:1.

11. Dunant, U., J. Gautron, M. Israel, B. Lesbats, and R. Manaranche. 1972. Acetylcholine compartments in stimulated electric organ of Torpedo marmorata. J. Neurochem. 19:1987.

12. Fahimi, H. D. 1973. Diffusion artefacts in the cytochemistry of catalase. J. Histochem. Cytochem. 21:999.

13. Heuser, J. E., B. Katz, and R. Miledi. 1971. Structural and functional changes of frog neuromuscular junction in high calcium solutions. Proc. R. Soc. Lond. B Biol. Sci. 178:407.

14. Heuser, J. E., and T. S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57:315.

15. Hillman, D., and R. Llinás. 1974. Calcium-containing electron dense structures in the axon of the squid giant synapse. J. Cell Biol. 61:146.

16. Hoar, W. S., and D. J. Randall, editors. 1969. In Fish Physiology. Academic Press, Inc., New York. 1:40–41.

17. Holtzman, E., A. R. Freeman, and L. A. Kashner. 1971. Stimulation dependent alterations in peroxidase uptake at lobster neuromuscular junctions. Science (Wash. D. C.) 173:733.

18. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137A–138A.

19. Karnovsky, M. J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Eleventh Annual Meeting of The American Society for Cell Biology, New Orleans, Louisiana. Abstract no. 284.

20. Katz, B., and R. Miledi. 1965. The effect of calcium on acetylcholine release from motor nerve terminals. Proc. R. Soc. Lond. B Biol. Sci. 161:496.

21. Katz, B., and R. Miledi. 1967. Ionic requirements of synaptic transmitter release. Nature (Lond.). 215:651.

22. Katz, B., and R. Miledi. 1969. Tetrodotoxin resistant electric activity in presynaptic terminals. J. Physiol. 203:459.

23. Koko, A., and R. J. Barnett. 1971. Dense contents in synaptic vesicles produced by sequential cation binding, alcohol treatment and osmium tetroxide fixation. In Progress in Brain Research. Vol. 34: Histochemistry of Nervous Transmission. O. Eranko, editor. Elsevier Scientific Publishing Company, Amsterdam. 319.

24. Llinás, R., J. Blanks, and C. Nicholson. 1972. Calcium transient in presynaptic terminal of squid giant synapse: detection with aequorin. Science (Wash. D. C.). 176:1127.

25. Martin, J. H., F. L. Carson, and F. J. Race. 1974. Calcium containing platelet granules. J. Cell Biol. 60:775.

26. Miledi, R. 1973. Transmitter release induced by injection of calcium into nerve terminals. Proc. R. Soc. Lond. B Biol. Sci. 183:421.

27. Nickel, E., and L. T. Potter. 1970. Synaptic vesicles in freeze etched electric tissue of Torpedo. Brain Res. 23:95.

28. Oschman, J. L., T. A. Hall, P. D. Peters, and B. J. Wall. 1974. Association of calcium with membranes of squid giant axon. J. Cell Biol. 61:156.

29. Oschman, J., and B. Wall. 1972. Calcium binding to intestinal membranes. J. Cell Biol. 55:58.

30. Pappas, G. D., and M. V. L. Bennett. 1966. Specialized junctions involved in electrotonic transmission between neurons. Ann. N. Y. Acad. Sci. 137:495.

31. Pfenninger, K. H., and C. M. Royainen. 1974. Stimulation- and calcium-dependent of vesicle attachment sites in the presynaptic membrane; a freeze-cleave study on the lamprey spinal cord. Brain Res. 72:1.

32. Poltioff, A. L., S. Rose, and G. D. Pappas. 1974. The calcium binding sites of synaptic vesicles of the frog sartorius neuromuscular junction. J. Cell Biol. 61:318.

33. Poste, G., and A. C. Allison. 1973. Membrane fusion reaction: A theory. Biochim. Biophys. Acta. 300:421.

34. Pesh, J. J., and R. G. Wiley. 1974. Synaptic vesicle depletion and recovery in cat sympathetic ganglia.
electrically stimulated in vivo. Evidence for transmitter secretion by exocytosis. J. Cell Biol. 60:365.

35. SHERIDAN, M. J. 1965. The fine structure of the electric organ of Torpedo marmorata. J. Cell Biol. 24:129.

36. SMITH, H. C. 1936. Biol. Rev. (Camb.). 11:49.

37. UCHIZONO, K. 1965. Characteristics of excitatory and inhibitory synapses in the CNS of the cat. Nature (Lond.). 207:642.

38. VALDIOIA, O. 1971. Methods of fixation and morphology of synaptic vesicles. J. Comp. Neurol. 142:257.

39. WHITTAKER, V. P., M. J. DOWDALL, and A. F. BOYNE. 1972. The storage and release of acetylcholine by cholinergic nerve terminals: Recent results with non-mammalian preparations. Biochem. Soc. Symp. 36:49.

40. 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 Torpedinidae. Biochem. J. 128:833.

41. ZIMMERMAN, H., and V. P. WHITTAKER. 1974. Effect of electrical stimulation on the yield and composition of synaptic vesicles from the cholinergic synapses of the electric organ of Torpedo: A combined biochemical, electro-physiological and morphological study. J. Neurochem. 22:435.