CHANGES IN CHOLINERGIC SYNAPTIC VESICLE POPULATIONS AND THE ULTRASTRUCTURE OF THE NERVE TERMINAL MEMBRANES OF *NARCINE BRASILIENSIS* ELECTRIC ORGAN STIMULATED TO FATIGUE IN VIVO

ALAN F. BOYNE, TIMOTHY P. BOHAN, and
TERENCE H. WILLIAMS

From the Department of Anatomy, University of Iowa, Iowa City, Iowa 52242, and the Department of Pharmacology, Tulane Medical School, New Orleans, Louisiana 70112. Dr. Boyne's present address is the Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611.

ABSTRACT

Narcine brasiliensis electric organ was stimulated to fatigue in vivo. Electrical display of organ output and biochemical assay of bound acetylcholine (ACh) and ATP in isolated vesicles were used to assess the state of fatigue relative to denervated control organs of the same fish. A morphometric analysis of the fate of the synaptic vesicle populations in the nerve terminals was carried out. Statistically significant morphological changes in vesicle populations and plasma membranes were observed between control and fatigued electroplaque stacks from individual fish. Pooled data from several fish were used to evaluate the possible role of the different vesicle types in neurotransmission. Fatigue resulted in the loss of 49% of the total vesicle population and a 76% loss of vesicles with bound calcium (Ca). An approximately equivalent increase in the nerve-terminal plasma membrane area was measured. This was predominantly in the form of fingerlike protrusions and/or invaginations of the terminals which were present in the control organs but which were significantly increased by stimulation. Vesicle attachments to the nerve terminal membrane were reduced by 90%. This suggests that the failure in transmission may be due to a reduction in the number of vesicles which are loaded with transmitter and can attach to the terminal membrane. The Ca-binding capacity of the lost vesicles was not transferred to the plasma membranes. This result was interpreted as support for the hypothesis that vesicle-bound ATP provides the Ca-binding site.

Cholinergic synaptic vesicles of the frog neuromuscular junction (15) and the electric organ of *Narcine brasiliensis* (6) have been shown to have a Ca-binding capacity. The appearance of Ca-dependent, bulging, pentalaminar attachments between vesicles and nerve-terminal plasma membrane has also been described, and it was suggested that this may represent the first step in Ca-dependent neurotransmitter release (6).

The effect of stimulation on the biochemistry and ultrastructure of *Torpedine* ray electric organ have been studied by Dunant et al. (8), who used

pieces of excised tissue, and by Zimmerman and Whittaker (21), who carried out in vivo stimulation. No loss of vesicles was observed when excised tissue was stimulated (8). On the other hand, in vivo stimulation produced a progressive fall in ACh, ATP, total nucleotide, and vesicle protein in the isolated vesicles, along with an eventual 50% loss of vesicle profiles in fatigued nerve terminals (21). The latter results were consistent with other evidence that vesicle membrane is eventually incorporated into nerve terminal membrane or cytoplasmic cisternae during repetitive stimulation (10, 11, 16).

The fate of the vesicular Ca-binding site during the hypothesized cycle (10) of vesicle membrane is a question of some importance. If the Ca-binding capacity is due to vesicular ATP (6), it would be expected to be eliminated during stimulation as the level of vesicle-bound ATP falls. On the other hand, if the Ca-binding site is a specialized part of the vesicle membrane (15), it should be possible to follow it through the stages of the vesicle membrane "cycle" (10). Since in vivo stimulation of *Torpedine* ray electric organ apparently initiates the vesicle membrane cycle (20), we have used this preparation to examine the behavior of the vesicular Ca-binding site. Evidence for the fate of lost vesicle membrane was provided by morphometric analysis of rearrangements in the plasma membrane of the terminals.

In view of the suggestion that vesicles attached to the terminal membrane may be active in transmitter secretion (6), it was also of interest to determine how the frequency of these pentalaminar attachments was affected at the stage of fatigue when the total vesicle pool is reduced by about 50% (20).

MATERIALS AND METHODS

Fish

Specimens of *N. brasiliensis* were obtained from the Gulf of Mexico and were kept in laboratory aquaria at room temperature (about 23°C). Experiments were begun by transferring the fish to a bowl containing 2 liters of seawater. The anesthetic agent MS-222 (ethyl-*m*-amino benzoate, methane sulfonic acid salt) was then added to a concentration of 0.005%. After 30-60 min the rate of spiracle opening and closing was reduced from about 30-50 min^{-1} to 7-15 min^{-1}, and the fish were unresponsive to touch. They were transferred to a tray containing a sloping bed of wax, and the mouth, which has a roughly tubular configuration, was pulled forward

and tied around a Tygon tube attached to a Dynaflo aquarium filter pump. The seawater was pumped through the fish's mouth; it emerged from the ventral gill slits and trickled down the sloping wax surface. It then siphoned back into the Dynaflo apparatus. The rate of flow into the mouth was adjusted by a clamp on the Tygon tubing. Intradermal Grass platinum wire recording electrodes were placed in the ventral and dorsal skin of both the right and left electric organs and were connected to a Type 502 dual beam Tectronix oscilloscope. Tygon tubing was obtained from Norton Plastics, Inc., Akron, Ohio; the Dynaflo filter pump was supplied by Metaframe, Inc., East Patterson, N. J.; platinum wire recording electrodes were obtained from Grass, Inc., Quincy, Mass.; and the dual beam oscilloscope was purchased from Tectronix Corp., Beaverton, Ore.

The skin over the cartilaginous brain case was then reflected, and two platinum wire electrodes were plunged through two depressions in the cartilage which are present over the electric lobes of the central nervous system. The electrodes were adjusted so that they were several millimeters above the floor of the brain case and so that a substantial discharge of both electric organs could be recorded after single stimulating pulses; they were then cemented in place. Lower placement of the electrodes stimulates motor tracts of the spinal cord and muscular contractions are initiated.

Five nerves arise from the CNS and travel between the gill slits to reach the electric organs. The muscles around one side and the caudal surface of the cartilage were freed from their attachments so that all the nerves to one electric organ were exposed; they were then severed at the exit from the brain case.

The efficacy of denervation of the control organ was checked by a further single stimulating pulse. The seawater in the surgical apparatus was then replaced with anesthetic-free seawater which was allowed to circulate for 5-10 min. Biphasic square wave stimulation of 2 ms duration at 5 Hz from a Physiograph Rs 1-10 stimulator was then begun while the output voltage was monitored. The stimulating voltage was increased from about 5 V to about 20V as the output declined from 15 to 40 V down to about 25 mV over a 12-16-min stimulation period (see Results for a description of the observations which led to this mode of stimulation).

Morphology

Fixation and tissue processing were carried out as described previously (6). The primary fixative used here was the high osmolarity Ca-containing fixative. In one set of fixations, the Ca in the primary fixative was replaced by an equal concentration of Mg and primary fixation was followed by a Ca wash and postfix. This was carried out because Birks has reported the preservation of increased numbers of vesicles after Mg fixation (2) of cat superior cervical ganglion. Ca-EDS were not observed in the final sections. In this case, therefore,

morphometric measurements were confined to the total vesicle population and various categories of plasma membrane described below. In the postfixation step, potassium ferrocyanide was used rather than sodium ferrocyanide (12).

Morphometric measurements were carried out as follows. Tissue from three stacks of electric cells was sectioned from each control and each stimulated electric organ. Silver sections were obtained and stained in uranyl acetate (6 min) and lead citrate (45 s). Sections were scanned in a JEM microscope at × 5,000 until a region of the section supported on four sides by grid bars was observed. The magnification was raised to 20,000 and 10–12 negatives of consecutive sequences of nerve terminals were exposed. The negatives were printed to give a final magnification of 55,000 and the following measurements were made on the prints with a straight rule, a map measurer, and a planimeter. (a) Length of electric cell membrane was measured with a straight rule. Convolutions of the membrane were ignored so that this measure referred to the linear length of electric cell photographed. (b) Perimeter of nerve terminals applied to the electric cell ventral surface was measured with a map measurer. (c) Perimeter of cisternal membrane within nerve terminal cytoplasm. (d) Perimeter of membrane involved in interdigitations between adjacent nerve terminal profiles. (e) Area of the nerve terminals was measured with a planimeter. (f) The number of vesicles with EDS was counted together with separate counts of flattened vesicles without EDS, rounded vesicles without EDS, and vesicles which could not be included in any of these categories. (g) The number of vesicles forming bulging, pentalaminar attachments to the nerve terminal membrane were counted.

Expression of Data

The experimental measures 2–7 were computed per micrometer length of electric cell (measure 1). The counts of synaptic vesicles seen in the micrographs were corrected for the numbers of vesicles which could be seen in two successive sections. Since 44.6% of the vesicles seen in one section could also be seen in an adjacent section, the counts were reduced by 22.3% to give the number of vesicles whose centers were in the section being analyzed (10).

Statistics

Means and standard deviations of all the measurements made are presented in Tables I–III. Some quantitative differences in the extent of depletion between different parameters are considered in the discussion; the statistical significance of these differences was tested by applying student's t test. Since all of the categories of chemical and morphometric measurements were not made on each fish, only data from those fish in which both parameters had been measured were used in making these statistical evaluations.

Biochemical Analyses

As described previously (4), synaptic vesicles from the control and from the stimulated electric organ were isolated by extracting weighed portions of frozen, powdered tissue in 20% (wt/vol) sucrose-saline solution. Contaminating membranes were removed by centrifugation over a discontinuous sucrose gradient (see Fig. 1). After centrifugation, samples of free vesicles, which had not migrated in the centrifugal field, were obtained from the top of the gradients. Small volumes (0.1 ml) were removed for assay of the nonsedimentable protein (13) and a boiled acid extract was also prepared. This latter extract was stored at −20°C for 12–72 h, and the vesicular ACh was then assayed using the guinea pig ileum method (14) as applied by Barker et al (1). The remainder of the gradient sample was treated with an apyrase-myokinase cocktail for hydrolysis of free ATP (7). Vesicle-bound ATP was then assayed within 30 min using the firefly luciferase technique as described previously (7).

Analysis of entire sucrose density gradients prepared with paired control and fatigued organ extracts was carried out after fractionating the gradients with an Isco
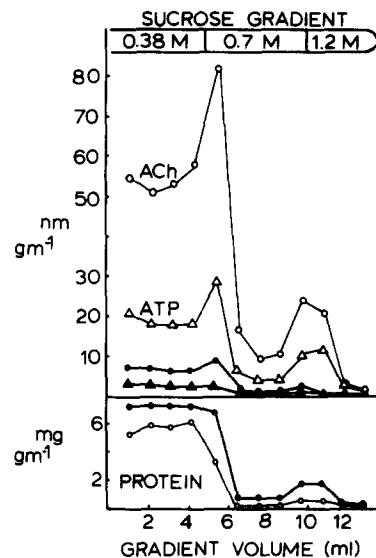

FIGURE 1 Distribution of vesicle-bound ACh, ATP, and protein after centrifugation of 0.38 M sucrose-saline extracts from paired denervated and fatigued electric organs. Centrifugation was at 113,500 g for 90 min in a Beckman SW-40 rotor. Free vesicles (see Fig. 2 in reference 6) were isolated from the top 3 ml of the gradient. Open symbols = control extract. Closed symbols = fatigued extract. Note that the ordinate is only directly quantitative for the free vesicle fraction; the other peaks have been concentrated from their starting volume and have not been corrected for this effect.

fractionator (model no. 183, Isco, Lincoln, Neb.). Vesicle-bound ACh, ATP, and total protein were assayed in each fraction.

Expression of Results

The amounts of vesicle-bound ACh and ATP in the free vesicles were calculated by measuring the concentrations in the samples taken from the first 3.0 ml of the density gradient. The values were then multiplied by the volume of extraction solution used and divided by the weight of tissue used. The final expression was therefore per gram wet weight of fresh tissue. The absolute values in the control organs from different fish varied widely (Table I, and see Discussion). As in the previous study by Zimmerman and Whittaker (20), the effect of fatigue was therefore expressed as the *percentage extent of depletion*. This was calculated by first subtracting the stimulated tissue values of vesicle-bound ACh or ATP from the control levels (i.e. the mean of front and back control values if the tissue had been analyzed in two parts); this *difference* was then expressed as a percentage of the control level.

RESULTS

Relation between Stimulus Voltage and Discharge of the Electric Organ

It was found that an initial stimulus of about 4 V was adequate to produce apparently maximal discharge of the organ (measured at 15–40 V). However, as the output voltage declined during repetitive stimulation, an increase in the stimulus voltage was found to increase the output voltage substantially. Since the purpose of the experiments was to compare the fully fatigued organ with the resting, denervated organ, the stimulating voltage was increased in steps from about 4 V to about 20 V over the stimulation period. At this point, organ discharge was in the range of 1–50 mV, and further increases in stimulating voltage did not bring the discharges above 100 mV. One fish was stimulated while seawater containing 0.05 g% MS-222 was circulated over the gills. A maximal discharge of 3.6 V was recorded from the stimulated organ. This was regarded as an inadequate response to stimulation and the stimulated organ was therefore discarded. The control organ was analyzed to provide additional data for Fig. 9. In all the other fish, anesthesia was induced with 0.005% MS-222, and stimulation was carried out while anesthetic-free water circulated over the gills. Initial discharges of 15–40 V were then recorded.

Biochemical Measurements

VESICLE-BOUND ACH AND ATP: The extraction, isolation of vesicles, and assays of vesicle-bound ACh and ATP were carried out on the control organ from the anesthetized fish described above and on successfully fatigued and control electric organs from four fish. The bound ACh and ATP in the isolated vesicle fraction of the gradient were assayed routinely so that the data would be comparable to the work of Zimmerman and Whittaker who followed the isolated vesicles on zonal density gradients (20). In order to evaluate the reproducibility of the procedures, the electric organs were divided into anterior and posterior "halves" in three of the fish. These were weighed, extracted, and assayed separately. The results are shown in Table 1. As reported in other work (20), the highly significant ($P < 0.001$) depletion in vesicular ACh and ATP did not seem to be due to differential extraction of control vs. stimulated organs since the protein extracted was similar in both cases. Furthermore, the reduction in bound chemicals occurred throughout the present density gradients, as it did in the zonal gradients (20) (see Fig. 1).

Morphometric Measurements

The normal fine structure of the electric organ has been well described (18) and confirmed in our previous studies (4, 5). Control tissue morphology of the experimental animals was very similar to that found in a previous study (5). In particular, the Ca-dependent distortion of vesicles in an isotonic fixative was readily apparent (Fig. 2). The thin flat electric cells contain relatively infrequent organelles; occasional nuclei and mitochondria are present in a cytoplasm containing mainly scattered fibrillar material. A dense network of canaliculi is present in the dorsal aspect of the cells, and a network of nerve terminals is applied almost continuously over the ventral surface. The terminals contain numerous synaptic vesicles and extravesicular dense granules which may be glycogen. Mitochondria are fewer than in neuromuscular nerve terminals (5). Stimulation to fatigue had two morphological consequences which were seen in virtually every microscope field: (*a*) the canaliculi in the dorsal surface of the electric cells had become narrower, longer and less convoluted (Fig. 3); and (*b*) in the nerve terminals, there was a major reduction in total vesicle numbers together with a more extensive elimination of Ca-EDS-con-

TABLE I

Effect of Fatigue on the Levels of Vesicle Bound-ACh and ATP

	ACh nm g^{-1}		ACh mean depletion	ATP nm g^{-1}		ATP mean depletion
	Control	Fatigued		Control	Fatigued	
Ray 1* Whole organ	(149.6)	—	—	(47.0)	—	—
Ray 2						
Front	77.8	8.9		30.2	3.3	
Back	82.9	3.9	−91.9%	33.9	3.4	−89.5%
Ray 3						
Front	193.0	77.5		22.5	4.6	
Back	201.0	93.0	−56.8%	22.0	4.1	−80.4%
Ray 4						
Front	64.9	15.3		16.6	3.7	
Back	56.0	10.1	−78.8%	17.9	3.3	−79.7%
Ray 5						
Whole organ	53.8	6.3	−88.2%	17.9	2.1	−88.2%
SD of duplicate extracts	±5.4%	±24.2%		±6.9%	±2.1%	
% depletion‡ mean ± SD			−78.9 ± 15.8			−84.5 ± 5.1

Assays were performed on free synaptic vesicle fractions isolated on sucrose density gradients. Each value refers to results obtained with a separate extraction and fractionation. The soluble protein extracted from control organs was 6.86 ± SD 1.90, and from fatigued organs 7.04 ± SD 2.34 mg g^{-1} tissue (n = 6 in each case).

* This fish was deeply anesthetized and did not give an adequate initial discharge of its electric organ. Morphometric measures were carried out on the control organ to provide an additional point for the graph in Fig. 8.

‡ Depletion was calculated by subtracting the value in the fatigued organ from the value in the control. This difference was then expressed as percent of the control value.

taining vesicles. Scanning of the stimulated tissue revealed frequent interdigitations or double profiles of the unit membrane (Fig. 4), often in a circular profile within an elliptical profile of a nerve terminal. Layered accumulations of membrane within the terminals were also seen (Fig. 5). Many of the remaining EDS-containing vesicles in stimulated tissues were found within the very extensive interdigitations (Fig. 4). These vesicles were never seen to form attachments to the plasma membrane of the interdigitation. Occasional clusters of round vesicles without EDS were seen in stimulated tissues (Fig. 6).

Morphometric measurements were carried out on the four pairs of control and stimulated organs, portions of which had been analyzed biochemically, and on a fifth pair which had been successfully fatigued as judged by the decay in electric discharges. The control organ of the deeply anes-

thetized fish was also analyzed morphometrically (fish 1, Table 1). The numbers of vesicles in the control organ of the Mg-fixed fish were 50% higher than the mean of the other controls, while the numbers in the fatigued organ were very similar to the others. This is consistent with Birk's findings (2), but since it was not possible to see Ca-EDS after a Ca wash of the Mg-fixed tissue, the use of Mg was not studied further in the present experiments. The presence of discrete electron densities in vesicles binding Ca greatly facilitated the counting of this population of vesicles; perhaps for this reason, statistically significant ($P < 0.05$ in each case) depletion in these vesicles was measured in each fish fixed in a Ca-containing fixative. Pooled data for changes in the synaptic vesicle populations are presented in Table II. The analysis showed that there had been a 75.6% depletion of vesicles with EDS. This was partially balanced by significant
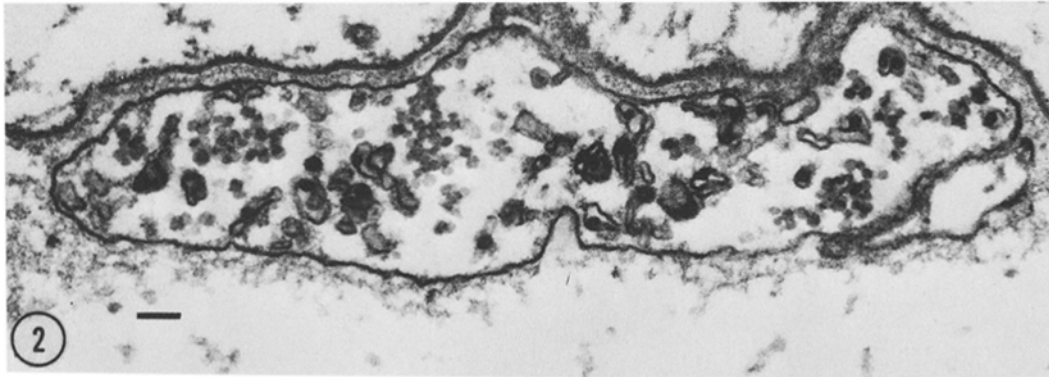
FIGURE 2   Nerve terminal from denervated electric organ fixed in 1,000 m′ osmol l⁻¹ fixative containing 90 mM Ca. Note the high frequency of electron-dense structures within collapsed vesicle membranes. Magnification = 55,000. Scale = 100 nm.
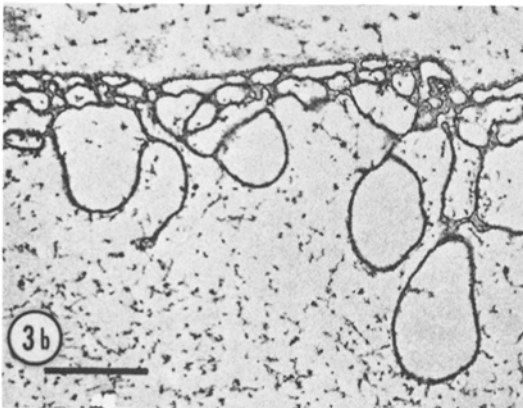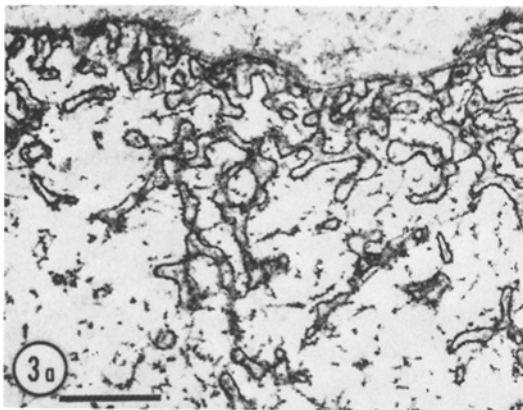

FIGURE 3   (a) Noninnervated dorsal region of electrocyte from denervated organ. Extensive canaliculi open into the intercellular space. Magnification = 13,700. Scale = 1 μm. (b) Similar view of electrocyte from fatigued electric organ of the same fish. The canaliculi are now much narrower. Magnification = 13,700. Scale = 1 μm.

increases in the numbers of round and flat vesicles without EDS, so that the net change in the total vesicle population was a 48.6% depletion.

The effect of fatigue on the various categories of plasma membrane and on the terminal cross-sectional areas is presented in Table III. The only significant change was in the perimeter of plasma membrane involved in interdigitations (Fig. 4); once again this change was significant in each of the fish examined ($P < 0.05$ in each case).

DISCUSSION

Biochemical Results

The swinging bucket system used here to isolate free vesicles offered the opportunity to prepare one or more extracts of both control and stimulated tissues and to analyze these simultaneously. It was found that the overall reproducibility of the extractions and assays was high (Table I). The variation in the extent of ACh depletion was only ±6.3% in the two portions of the organ. The variation in the extent of depletion (in the two portions of one organ) of vesicle-bound ATP was ±0.8%. These results showed that the front and back regions had become similarly fatigued relative to their control organs. The morphometric measurements were expressed as the means of the measures from two stacks of electroplaques in the front of the organ and one from the back.

VARIABILITY BETWEEN FISH: Since the extractions and assays gave highly reproducible results within a given fish, it seems unlikely that the large chemical differences between control organs of different fish could be due to differences
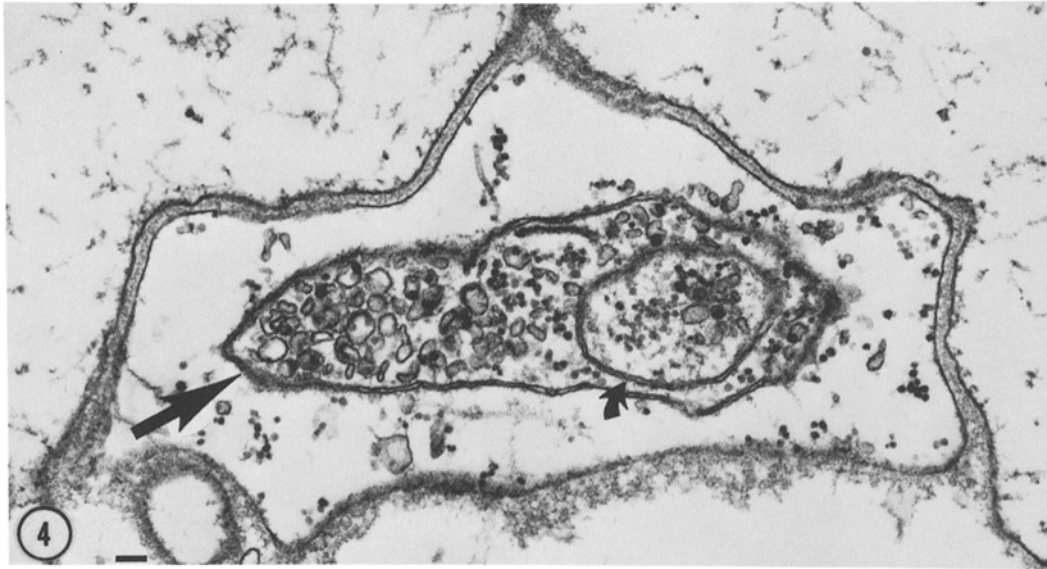
FIGURE 4 Fatigued terminal. The outer region of the terminal is depleted of all vesicle forms but a large elliptical central region (large arrow) is bounded by two separate layers of unit membrane and contains synaptic vesicles. A second, roughly circular region (small arrow) lies within the ellipse; this region is also surrounded by two separate layers of unit membrane and contains synaptic vesicles. Magnification = 44,000. Scale = 100 nm.
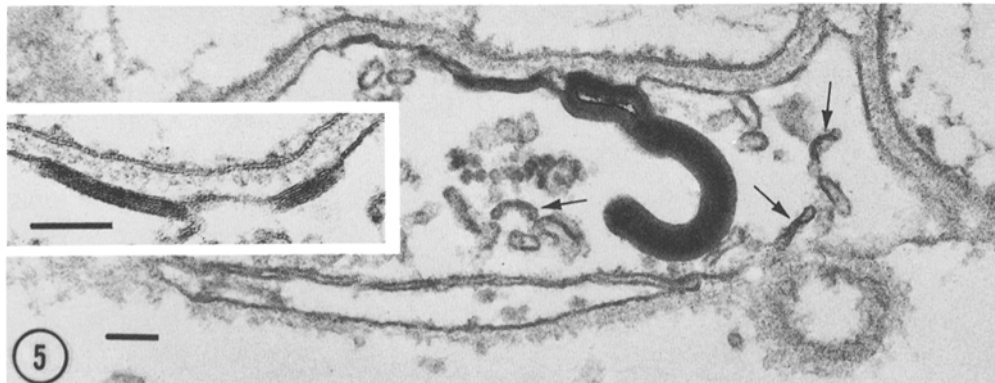

FIGURE 5 Fatigued terminal. Regions of the terminal plasma membrane facing the electric cell are multilayered and are connected to a multilamellar membranous accumulation within the nerve terminal. Note flattened vesicles (arrows). Magnification = 65,000. Scale = 100 nm. *Inset:* Higher magnification (100,000) of layers of nerve terminal unit membrane facing the electric cell membrane.

in extraction efficiency (Table I). It has been shown that the *Torpedo* electric organ takes several days to recover from fatigue (21). Since the fish used for the present experiments had spent varying times in the laboratory aquaria (2-14 days), it may be that they were in different stages of recovery from discharges occurring during transportation and laboratory maintenance.

COMPARISON WITH PREVIOUS BIO-CHEMICAL RESULTS: The figures for extent of depletion of vesicular ACh and ATP can be compared with the values quoted by Zimmerman and Whittaker for fatigued *T. marmorata* electric organ (20). They observed a 90% ACh depletion and 80% ATP depletion in the peak tubes of the vesicle fraction after zonal centrifugation (from

Fig. 6, of reference 20). We observed a 78.9% depletion in ACh and an 84.5% depletion in ATP in the four fish which were both successfully fatigued and assayed biochemically. Our data are therefore consistent with those of Zimmerman and Whittaker and stand in contrast to the results of Dunant et al. who found little change in vesicular ACh
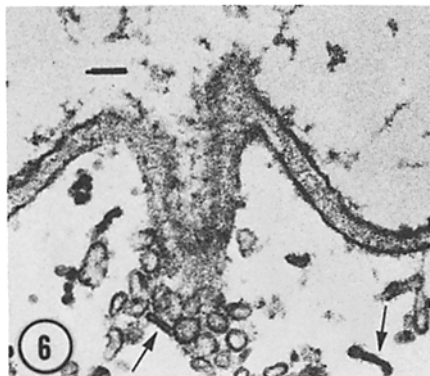

FIGURE 6 Fatigued terminal. An accumulation of round vesicles without EDS is present at a site of invagination of the nerve terminal by the electric cell. Note flat vesicles without EDS (arrows). Magnification = 55,000. Scale = 100 nm.

levels of pieces of electric organ stimulated to fatigue in vitro (8). Although it seems likely that the lack of a blood supply during in vitro stimulation leads to an unphysiological response, it would be of interest to determine the precise reasons for this maintenance of vesicular ACh levels in the in vitro preparations.

The range of values for vesicle-bound ATP showed no overlap between control and stimulated tissues. This was not true for vesicle-bound ACh. Thus, ray 3, with the highest control values (193.0 and 201.0 nm g^{-1} in paired extractions) gave fatigued terminals with values of 77.5 and 93.0 nm g^{-1} (paired extractions), and these were higher than the control values in two other fish (see Table I). By the criteria of vesicle content and vesicle-bound ATP, the fatigued organ of ray 3 appeared very similar to the other fatigued organs (Fig. 9 and Table I). This result serves to emphasize that as yet we do not have a functional explanation for the wide variations in the ratios of vesicle-bound ACh and ATP in different fish (7, 20).

Morphological Results

VESICLE DEPLETION: The net depletion of all synaptic vesicle profiles in fatigued organs

TABLE II

Effect of Fatigue on Vesicle Populations (Vesicles per Micrometer of Electric Cell + SD)

	Total vesicles	Vesicles + EDS	Round vesicles – EDS	Flat vesicles – EDS	Undefined vesicles – EDS	Vesicles attached to membrane
	$n = 5$	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 5$
Control	14.05 ± 3.4	9.11 ± 1.6	0.34 ± 0.10	0.55 ± 0.26	2.85 ± 2.3	0.41 ± 0.11
Fatigue	7.22 ± 0.6	2.22 ± 0.7	0.78 ± 0.21	1.76 ± 0.43	2.38 ± 0.4	0.04 ± 0.04
Difference as % of control	−48.6%	−75.6%	+129.4%	+220.0%	−16.4%	−90.2%
Significance	$P < 0.01$	$P < 0.001$	$P < 0.01$	$P < 0.01$	NS	$P < 0.001$

TABLE III

Effect of Fatigue on Nerve Terminal Plasma Membran and Area

	Perimeter*	Cisternae*	Invaginations*	Aggregates*	Area $\mu m^2 \mu m^{-1}$
Control	2.21 ± 0.27	0.035 ± 0.014	0.028 ± 0.02	0.148 ± 0.12	0.284 ± 0.064
Fatigue	2.53 ± 0.42	0.129 ± 0.098	0.780 ± 0.16	0.406 ± 0.55	0.361 ± 0.113
Difference as % of control	+14.4%	268.6%	+2686.0%	+174.3%	+27.1%
Significance	NS	NS	p < 0.001	NS	NS

* Membrane lengths are μm per μm electric cell.
$n = 5 ± SD.$

measured in this study with *N. brasiliensis* was 48.6 ± 16.4% ($n = 5$). This is very similar to the 50% depletion reported by Zimmerman and Whittaker in *T. marmorata* (20). In neither case did this dramatic loss in total vesicles show a simple correlation with the more extensive depletion of vesicular ACh and ATP (see Table I). It is therefore of interest that, in the present study, the 74.6 ± SD 8.8% depletion of vesicles with Ca-binding sites was significantly greater than the 41.6 ± 16.0% depletion of total vesicles measured in the same four fish ($P < 0.01$). Furthermore, this 74.6% depletion in vesicles with EDS correlates more closely with the 78.9–84.5% depletions in vesicle-bound ACh and ATP, respectively.

The demonstration of vesicles attached to the nerve terminal membrane has been shown to depend upon the use of divalent cations in the fixation solutions, and it has been suggested that such attachments may form as a normal stage in Ca-dependent neurotransmission (6). It is therefore of particular interest that fatigue caused a fall in the frequency of attachments which was significantly greater than the fall in the total number of vesicle profiles in the same fish ($P < 0.001$; $n = 5$; Table II). The vesticles that can attach to the membrane may belong to a particular subpopulation which was more extensively depleted than the overall vesicle population, and/or biochemical changes in the nerve terminal may have limited the frequency of attachments. The failure in transmission may therefore be due to a reduction in vesicles which are loaded with transmitter and can attach to the terminal membrane.

The entrapment of vesicles with EDS in interdigitations may account for the persistence of vesicle-bound ACh and ATP in isolated vesicles of fatigued organs (see Table I), at a time when the electrical discharge of the organ had become negligible. It seems likely that vesicles in this compartment are not able to secrete ACh onto the electric cell but they may well be recovered on the sucrose density gradients.

FATE OF THE VESICULAR MEMBRANE: The loss of synaptic vesicle profiles (corrected as described in Materials and Methods) was 5.29 μm^{-1} of electric cell. If the vesicle diameter is taken as the mean of the inner and outer diameters, then the equivalent surface area of unit membrane can be calculated as πD^2. The diameter of these vesicles is 76 nm (20) and the lost surface area of unit membrane is therefore 0.096 ± SE 0.022 μm^{-1} electric cell.

In a study of the morphological effects of prolonged stimulation on frog neuromuscular junction, Heuser and Reese reported that an early loss of vesicle membrane was correlated with a nearly equivalent increase in nerve terminal membrane (10). Pysh and Wiley reported that vesicle depletion in stimulated preganglionic synapses of the cat superior cervical ganglion is correlated with an increase in nerve terminal circumference (16) and that this causes an increase in the area of apposition of pre- and post-synaptic processes. Zimmerman and Whittaker reported that nerve terminals of fatigued *T. marmorata* electric organ became segmented to give smaller and more frequent terminals than in the control organs (20). Although a quantitative description of membrane loss and gain was not reported, their observations were consistent with a net increase in terminal membrane area with fatigue (20).

The present results illustrate an alternative means whereby added surface membrane may be accommodated in a stimulated nerve terminal. Although increases were measured in the perimeter of the terminals, cisternal membrane, and intracytoplasmic membrane accumulations, these changes were not statistically significant (see Table III). On the other hand, a major and significant increase in apparent interdigitations between nerve terminals was measured (Table III, Fig. 5). This evidently increased the total plasma membrane area of the outer terminal which had received the interdigitation and also of the terminal from which the process had originated. (A longitudinal section of interdigitating terminals is illustrated in Fig. 6 of reference 6); possible three-dimensional interpretations of the morphology have been illustrated elsewhere [5]). Assuming that the silver sections were 70-nm thick, the increase was equivalent to a gain in plasma membrane surface area of 0.052 μm^2 μm^{-1} electric cell. This rearrangement alone thereby accounts for 55% of the lost vesicular membrane. The mean net increase in terminal membrane in all categories (from Table III) was 0.099 ± 0.019 μm^2 μm^{-1} electric cell. The loss in vesicle membrane therefore corresponded within 5% to the gain in terminal membrane.

The possibility of electric organ fatigue in free-living *Torpedine* rays is not established and there is therefore no guarantee that the same extent of morphological rearrangement could

occur under natural conditions. Since low frequencies of "interdigitations" were seen in control electric organs, however, it seems possible that lower frequencies and/or shorter periods of physiological discharge may correlate with smaller increases in the surface area of plasma membrane involved in these structures.

Although cytoplasmic membrane accumulations are sometimes referred to as "myelin bodies" and regarded as a fixation artefact, it is of interest that in the case of the stimulated electric organ, they could sometimes be seen attached to the terminal membrane (Fig. 5). Birks has observed increases in the presence of "diffuse myelin bodies" in stimulated cat superior cervical ganglion (3) and it seems possible that they represent a form of membrane storage.

Why did we find a means of membrane growth different from that found by Zimmerman and Whittaker (20) using a similar preparation? Although the species difference between the rays used may be significant in this regard, it also seems feasible that the physiological states of the electric organs were different in the two experimental series. Possible origins of such differences may have been (a) the removal of the anesthetic before stimulation (the present work), or (b) our use of a graded stimulation from 4 to 20 V rather than a constant 10 V stimulus (20). The dramatic alteration in the dorsal surface of the electrocytes which we observed (Fig. 3), but which were not seen by Zimmerman and Whittaker (20), may provide a clue to these differences. Further work with electric organ may make it possible to recognize biochemical or physiological correlates of the morphology illustrated in Fig. 3.

FATE OF VESICULAR CA BINDING SITES: No EDS were seen in any of the membranous structures which showed increased profiles after vesicle depletion (Figs. 4–7). This result argues strongly that Ca is bound by a vesicular component such as ATP (6), which is lost during stimulation (Table I and reference 14), rather than by a unique protein in the vesicle membrane (15).

INCREASES IN VESICLES WITHOUT EDS: The present experiments clearly show that round and flat vesicles without EDS accumulate in fatigued nerve terminals ($P < 0.01$; Table III). At present we can only suggest possible explanations.

We have previously proposed that vesicles attached to the terminal membrane may secrete ACh without structural breakdown (6), i.e. that a cycle
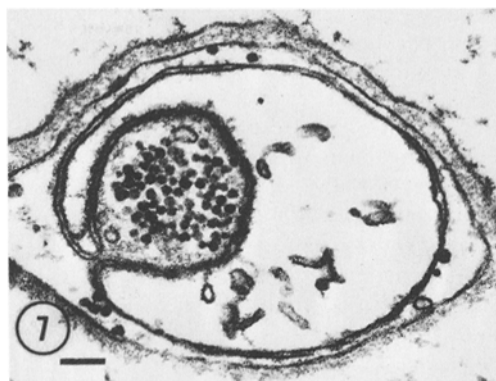

FIGURE 7 Fatigued terminal. A plasma membrane-bound structure within the nerve terminal has almost completely encircled a portion of the main terminal cytoplasm. The many dense particles in this region are probably glycogen granules. The encirclement is not complete at the lower left of the picture. Magnification = 55,000. Scale = 100 nm.

of transmitter release and reloading may precede the vesicle membrane cycle (10). Chemical data suggest that there is also a gradual loss of ATP (20). This may correlate with the loss of Ca-binding ability and may explain the increase in vesicle profiles without EDS.

Recovery of vesicle membrane from the plasma membrane has been proposed to occur via coated vesicles (10). Although we (6) and others (20) have reported the presence of coated vesicles in electroplaque nerve terminals, they are rather rare. A previous study of recovery from fatigue in electric organ noted the presence of rows of naked vesicles lying along the terminal membrane (21). It was suggested that they represented a form of membrane retrieval. Some of the vesicles without EDS in the present study were observed to be clustered at sites of invagination of the nerve terminal membrane by the electrocyte (Fig. 6). There is some similarity between this morphology and that described by Heuser and Reese in the frog neuromuscular junction (10). In that preparation, invaginations of Schwann cells into the nerve terminals were the sites of formation of coated vesicles (see Figs. 4 and 11; reference 10). The possibility that the vesicles without EDS arise in part from recovered terminal membrane is therefore apparent; the absence of "coats" may indicate that they are not essential to the process.

A third possibility is that stimulation has enhanced the migration of vesicle forms without

EDS along the axon to the terminals. The changes in this population might therefore be unrelated to the neurosecretion-induced changes in vesicle populations.

Further experiments will be required to elucidate the significance of the increases in vesicles without EDS.

LACK OF CORRELATION OF VESICULAR ATP WITH VESICLE NUMBERS: In a study of the recovery of fatigued *T. marmorata* electric organ, it was shown that morphological recovery of vesicle numbers precedes the chemical recovery of vesicular ACh and ATP by several days (21). It therefore appears that newly formed synaptic vesicles are reloaded relatively slowly. This conclusion is consistent with the relationship between the vesicle numbers and vesicle-bound chemicals seen in the control organs in the present work. As already noted, the levels of vesicle-bound ACh and ATP varied widely between different fish, but it is clear from Fig. 8 that this did not correlate with any trend in the numbers of vesicles. The simplest interpretation of this result is that morphological recovery from recent organ discharge had already occurred but that chemical recovery was in various stages of completion when the experiments were begun.
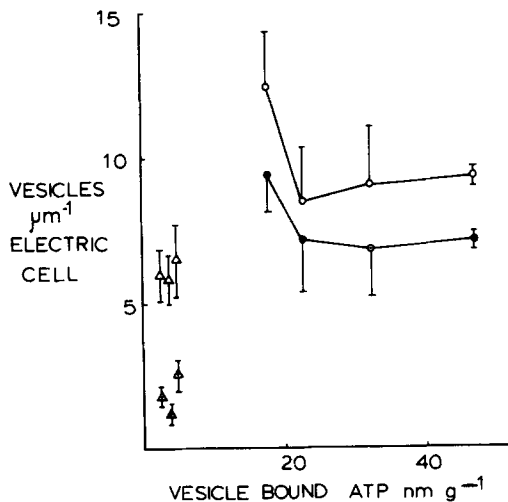

FIGURE 8 Plot of vesicle counts in nerve terminals as a function of the vesicle-bound ATP in isolated vesicles of the same electric organ. Points represent means of vesicle counts ± standard error of measures from three stacks of electric cells. Open symbols = total vesicle counts. Dotted symbols = vesicles with EDS. Circles = denervated control organs; triangles = fatigued electric organs.

The lack of correlation between vesicle-bound ATP and the numbers of vesicles with EDS seems incompatible with the idea that vesicular ATP provides the Ca-binding site. However, since a small fraction of the potential vesicular load of ATP may serve to bind Ca and produce EDS, then partially filled vesicles in one fish may have been counted as equivalent to more completely filled vesicles in another fish. This measure may not, therefore, be adequate to monitor the reloading of vesicles with ATP.

Since it has not been conclusively shown that the EDS represent Ca bound to ATP, it should also be noted that the above results could be similarly explained if another soluble component of the vesicles were responsible for binding Ca.

IMPLICATIONS FOR THE VESICLE HYPOTHESIS: The phenomenon of vesicle depletion with stimulation has now been reported in several systems (10, 11, 16) and has been interpreted as evidence for an exocytotic mechanism of vesicle discharge. In the cholinergic system, this interpretation has received additional support with the evidence that mammalian cholinergic nerve terminals release ATP during stimulation (19). In order to accommodate a variety of radiochemical results, we have presented an alternative hypothesis, that only aged vesicles undergo exocytosis and that newly formed vesicles are capable of several cycles of secretion and reloading before they become part of the terminal membrane (6). Evidence that vesicle loss occurs within minutes of the onset of stimulation (3, 9, 10, 17, 20) appears to contradict this latter point of view. However, Birks has now shown a 66% loss in synaptic vesicles in the cat superior cervical ganglion under conditions in which there is only a 15% release of ganglion ACh stores (3). Birks therefore suggested that much of the ganglionic ACh is vesicle bound at rest, but becomes cytoplasmic during stimulation and that secretion may derive from this cytoplasmic pool (3). An alternative possibility is that the Mg-fixed control tissues may contain a pool of transmitter-deficient vesicles; this possibility is being tested in the electric organ system.

REFERENCES

1. BARKER, L. A., J. AMARO, and P. S. GUTH. 1967. Release of acetylcholine from isolated synaptic vesicles. *Biochem. Pharmacol.* **16**:2181–2187.
2. BIRKS, R. I. 1971. Effects of stimulation on synaptic vesicles in sympathetic ganglia, as shown by fixation in the presence of Mg^{++}. *J. Physiol. (Lond.).* **222**:95–111.
3. BIRKS, R. I. 1974. The relationship of transmitter release and storage to fine structure in a sympathetic ganglion. *J. Neurocytol.* **3**:133–160.
4. BOHAN, T. P., A. F. BOYNE, P. S. GUTH, Y. NARAYANAN, and T. H. WILLIAMS. 1973. Electron dense particles in cholinergic synaptic vesicles. *Nature (Lond.).* **224**:32.
5. BOYNE, A. F. 1975. Nerve terminal plasticity in *Narcine brasiliensis* electric organ. Society for Neuroscience, Fifth Annual Meeting. New York. (Abstr.).
6. BOYNE, A. F., T. P. BOHAN, T. H. WILLIAMS. 1974. Effects of calcium-containing fixation solutions on cholinergic synaptic vesicles. *J. Cell Biol.* **63**:780–795.
7. DOWDALL, M. J., A. F. BOYNE, and V. P. WHITTAKER. 1974. Adenosine triphosphate. A constituent of cholinergic synaptic vesicles. *Biochem. J.* **140**:1–12.
8. DUNANT, U., J. GAUTRON, M. ISRAEL, B. LESBATS, and R. MANARANCHE. 1972. Acetylcholine compartments in stimulated electric organ of *Torpedo marmorata. J. Neurochem.* **19**:1987–2002.
9. FRIESEN, A. J. D., and J. C. KHATTER. 1971. Effect of stimulation on synaptic vesicles in the superior cervical ganglion of the cat. *Experientia (Basel).* **27**:285–287.
10. HEUSER, J. E., and I. S. REESE. 1973. Evidence for recycling of synaptic vesicle membrane during trans-

11. HOLTZMAN, E., A. R. FREEMAN, and L. A. KASHNER. 1971. Stimulation dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science (Wash. D.C.).* **1973**:733.
12. KARNOVSKY, M. J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Abstracts of the 11th Annual Meeting of The American Society for Cell Biology. 284.
13. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
14. MAGNUS, R. 1904. *Pfluegers Archiv. Gesamte. Physiol. Meuschen Tiere.* **102**:123.
15. POLITOFF, 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**:818–822.
16. PYSH, J. J., and R. G. WILEY. 1974. Synaptic vesicle depletion and recovery in cat sympathetic ganglia electrically stimulated *in vivo*. Evidence for transmitter release by exocytosis. *J. Cell Biol.* **60**:365–374.
17. PYSH, J. J., and R. G. WILEY. 1975. Ultrastructural evidence for acetycholine release by exocytosis in the cat superior cervical ganglion. Winter Conference on Brain Research. Steam Boat Springs, Colo.
18. SHERIDAN, M. J. 1965. The fine structure of the electric organ of *Torpedo marmorata. J. Cell Biol.* **24**:129–141.
19. SILINSKY, E. M. 1975. On the association between transmitter secretion and the release of adenosine triphosphate from mammalian motor nerve terminals. *J. Physiol.* **247**:145–162.
20. 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**:435–450.
21. ZIMMERMAN, H., and V. P. WHITTAKER. 1974. Different recovery rates of the electrophysiological, biochemical and morphological parameters in the cholinergic synapse of the *Torpedo* electric organ after stimulation. *J. Neurochem.* **22**:1109–1114.

mitter release at the frog neuromuscular junction. *J. Cell Biol.* **57**:315–344.