Structural Evidence that Botulinum Toxin Blocks Neuromuscular Transmission by Impairing the Calcium Influx that Normally Accompanies Nerve Depolarization

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ABSTRACT Taking advantage of the fact that nerve terminal mitochondria swell and sequester calcium during repetitive nerve stimulation, we here confirm that this change is caused by calcium influx into the nerve and use this fact to show that botulinum toxin abolishes such calcium influx. The optimal paradigm for producing the mitochondrial changes in normal nerves worked out to be 5 min of stimulation at 25 Hz in frog Ringer's solution containing five times more calcium than normal. Applying this same stimulation paradigm to botulinum-intoxicated nerves produced no mitochondrial changes at all. Only when intoxicated nerves were stimulated in 4-aminopyridine (which grossly exaggerates calcium currents in normal nerves) or when they were soaked in black widow spider venom (which is a nerve-specific calcium ionophore) could nerve mitochondria be induced to swell and accumulate calcium. These results indicate that nerve mitochondria are not damaged directly by the toxin and point instead to a primary inhibition of the normal depolarization-evoked calcium currents that accompany nerve activity. Because these currents normally provide the calcium that triggers transmitter secretion from the nerve, this demonstration of their inhibition helps to explain how botulinum toxin paralyzes.

According to present concepts of neuromuscular transmission, nerves discharge acetylcholine in response to depolarization by a mechanism which is activated by the entry of calcium into the presynaptic nerve terminal (23, 24, 38). This calcium entry triggers the exocytosis of acetylcholine-containing vesicles at specialized release sites named "active zones" (5, 14, 16, 18, 19). At the active zones of the frog neuromuscular junction, large intramembrane particles occur in characteristic double rows, which are so close to the sites of exocytosis that the idea has arisen that these large particles may be the sites of calcium channels in the presynaptic membrane (14, 18). We sought to prove this possibility by searching for an agent that would block the calcium channels by binding to them stereospecifically. One candidate for such a probe of the calcium channel is the neurotoxin produced by *Clostridium botulinum*. This is a 150,000 mol wt protein which blocks acetylcholine release at the neuromuscular junction without altering impulse conduction in the motor axon (3, 13) and without altering the acetylcholine sensitivity of the muscle membrane (4, 40, 41, 43). Previous physiological work has raised the possibility that this toxin does indeed block depolarization-induced calcium influx into the terminal (3, 13). Our previous autoradiographic and immunocytochemical studies demonstrated that the toxin binds preferentially to presynaptic membranes (21, 22) but unfortunately did not succeed in demonstrating a localized binding to the active zones of the synapse.

In the absence of more specific binding data, we have sought an alternate way to measure or visualize calcium influx into the nerve terminals to obtain indirect evidence for the possibility that this toxin blocks calcium channels. Our approach has been to study the mitochondria that abound in motor nerve terminals (15). It is well known that mitochondria can accumulate large amounts of calcium and deposit this calcium in electron-dense granules in their matrix (12, 28, 36). Mitochon-

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dria in nerve terminals have been shown to do this also (1). Several investigators have reported that repeated electrical stimulation induces nerve mitochondria to swell and to form dense granules in their internal compartments (16, 34, 35, 44). X-ray microanalysis also has provided evidence that these dense granules contain calcium (35). Unfortunately, to preserve these granules, it has generally been necessary to employ chemical fixatives containing high concentrations of ionic calcium, which raises questions of whether such calcium deposits were present during life or were deposited during fixation (33–35, 39, 44).

Recently, a less questionable method for preserving such deposits has been developed (31, 32). This involves quick-freezing followed by freeze-substitution in acetone containing osmium tetroxide as a fixative (17, 20) and oxalic acid as a capturing agent for the calcium (31, 32). With this technique, calcium does not have to be added to the fixative, and, hence, the question of artifactual deposition of calcium does not arise.

In this study, this improved method was used to preserve calcium deposits in mitochondria, and x-ray microanalysis was performed to confirm that the deposits contained calcium. The existence of a deposit of calcium in mitochondria was considered to be an indirect indication that a considerable amount of calcium had entered the nerve during stimulation. With this assumption in mind, the observed variations of the appearance of calcium deposits in normal versus botulinum-poisoned nerves could be taken as evidence that this toxin blocks the calcium entry that normally accompanies nerve stimulation.

MATERIALS AND METHODS

Botulinum Toxin

The botulinum type A crystalline toxin was provided by Dr. E. J. Schantz (University of Wisconsin) through Dr. M. Kitamura (National Institutes of Health in Japan). The neurotoxin, a toxic subunit of the type A crystalline toxin of 150,000 mol wt, was isolated from the crystalline toxin by DEAE-Sephadex chromatography and dialyzed against 10 mM phosphate buffer, pH 7.2, that contained 0.15 M NaCl.

About 0.2 ml of neurotoxin (containing ~1.0×10^6 mouse mean lethal dose) was injected into the peritoneal cavities of the smallest *Rana pipiens* frogs available. (Muscles from small frogs were easier to freeze; cf. reference 19.) After 24 h, the frogs became fully paralyzed. At this point, cutaneous pectoris nerve-muscle preparations were dissected from the animals, mounted in separate 5-ml Sylgard-bottomed (Dow Corning Corp., Midland, Mich.) petri dishes, and bathed in frog Ringer's solution (containing 116 mM NaCl, 2 mM KCl, 10 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 5 mM glucose, and 4 mM Hepes buffer, pH 7.2) before experimental treatment and quick-freezing.

When experimental treatment involved electrical stimulation, nerves were driven at 25 Hz for 5 min, with 0.3 ms 5 V supermaximal pulses delivered in 1.5-s trains interrupted by 1.5-s periods of rest. When experimental treatment involved black widow spider venom (BWSV), the venom was prepared fresh by homogenizing eight glands from four black widow spiders in 1.0 ml of frog Ringer's containing 5 mM $CaCl_2$. The homogenate was cleared by centrifugation at 3,000 rpm for 5 min and added in 100-μl aliquots to the 5-ml specimen chambers.

Immediately after treatment, muscles were mounted in the freezing machine described previously (19), and were dropped onto a pure copper block which had been precooled to 4° K by liquid helium. After freezing, tissues were placed in plastic scintillation vials on top of 10–20 ml of frozen acetone containing 5% OsO_4 and 25 mM oxalic acid (Sigma Chemical Company, St. Louis, Mo.) (31, 32), then allowed to warm to −93°C to melt the acetone, and were then left for 2–3 d to effect the freeze-substitution. Next, they were warmed to room temperature in steps, remaining at −20°C for 1–2 h and at 4°C for 2 h. Then they were washed in absolute acetone at room temperature, passed briefly through propylene oxide, and embedded in English Araldite. Thin sections were stained with uranyl acetate and lead citrate and observed in a JEM 100B electron microscope at 100 keV. Usually, sections were cut at two to three times normal thickness (200 nm) to promote retention of mitochondrial granules.

X-Ray Microanalysis

Thick and thin sections were analyzed in a JEM 100 CX electron microscope equipped with a scanning transmission capability and an energy dispersive x-ray analysis system (KEVEX 7000 Kevex Corp., Foster City, Calif.). Microprobe analysis was performed at 80 keV for 100 s and the size of the analyzing probe was ~30 nm.

Measurements

Micrographs were taken at 30,000 or 36,000 magnification and the short diameter of the mitochondria in nerve terminals was measured directly from negatives with a micrometer. Means and standard errors were determined for 64–100 mitochondria in each experiment (Table I).

Chemical Fixation

For comparative purposes, some muscles that had been subjected to the same experimental treatments were not frozen but were fixed by pouring on 1% glutaraldehyde and 2% formaldehyde in a 30 mM Hepes buffer which contained either 50 mM $CaCl_2$ or 50 mM $SrCl_2$, and leaving their fixative in place for 2 h. After washing with the same buffer, these muscles were postfixed with 2% OsO_4 in 30 mM barbital buffer, pH 7.2, which contained 50 mM NaCl and either 50 mM $CaCl_2$ or 50 mM $SrCl_2$, whichever had been used first. Then they were washed with the same barbital buffer, dehydrated with graded alcohols, passed briefly through propylene oxide, and embedded in Araldite. Thin sections were stained and examined as were the freeze-substituted muscles.

RESULTS

Nonintoxicated Neuromuscular Junctions

In this study, neuromuscular junctions were cut in cross section; and because mitochondria in the frog neuromuscular junction are tubular in shape and are oriented parallel to the long axis of the nerve terminal, they appeared as small round to oval profiles in cross section. In control nerves that received no electrical stimulation before quick-freezing and freeze-substitution, these mitochondrial profiles displayed a relatively electron-dense internal matrix, within which no dense granules could be seen (Figs. 1 and 4). In contrast, in nerves electrically stimulated for several minutes before quick freezing and freeze substitution, mitochondria were obviously swollen, pale, and filled with electron-dense granules (Figs. 2, 5, and 6). This illustrated that the new method of tissue preparation would be at least as good as older methods for displaying such mitochondrial changes.

To determine the optimal stimulation paradigm for producing these mitochondrial changes, we examined preparations after 1, 5, or 15 min of stimulation. After 1 min, changes were visible but only partially developed, but by 5 min, they were as severe as after 15 min of stimulation. Because we knew from

TABLE I

Changes of Mitochondrial Short Diameter by Various Treatments

Treatment	Average of mitochondrial short diameter ± SEM	Treatment	Average of mitochondria short diameter ± SEM
	nm		*nm*
No toxin No stimulation	127 ± 2.3	Botulinum toxin No stimulation	121 ± 1.9
No toxin Stimulation	161 ± 2.9	Botulinum toxin Stimulation	126 ± 1.6
No toxin 4-AP Stimulation	170 ± 3.4	Botulinum toxin 4-AP stimulation	147 ± 2.0

previous studies that the changes are slow to reverse after 15 min of stimulation (16), we chose 5 min for all subsequent experiments. To further characterize the mitochondrial changes, we stimulated nerves for 5 min in two different concentrations of calcium, 2 vs. 10 mM, and compared them with nerves stimulated for 5 min in Ringer's containing no calcium (with 10 mM magnesium instead). As expected, mitochondrial granules were more obvious in nerves stimulated in

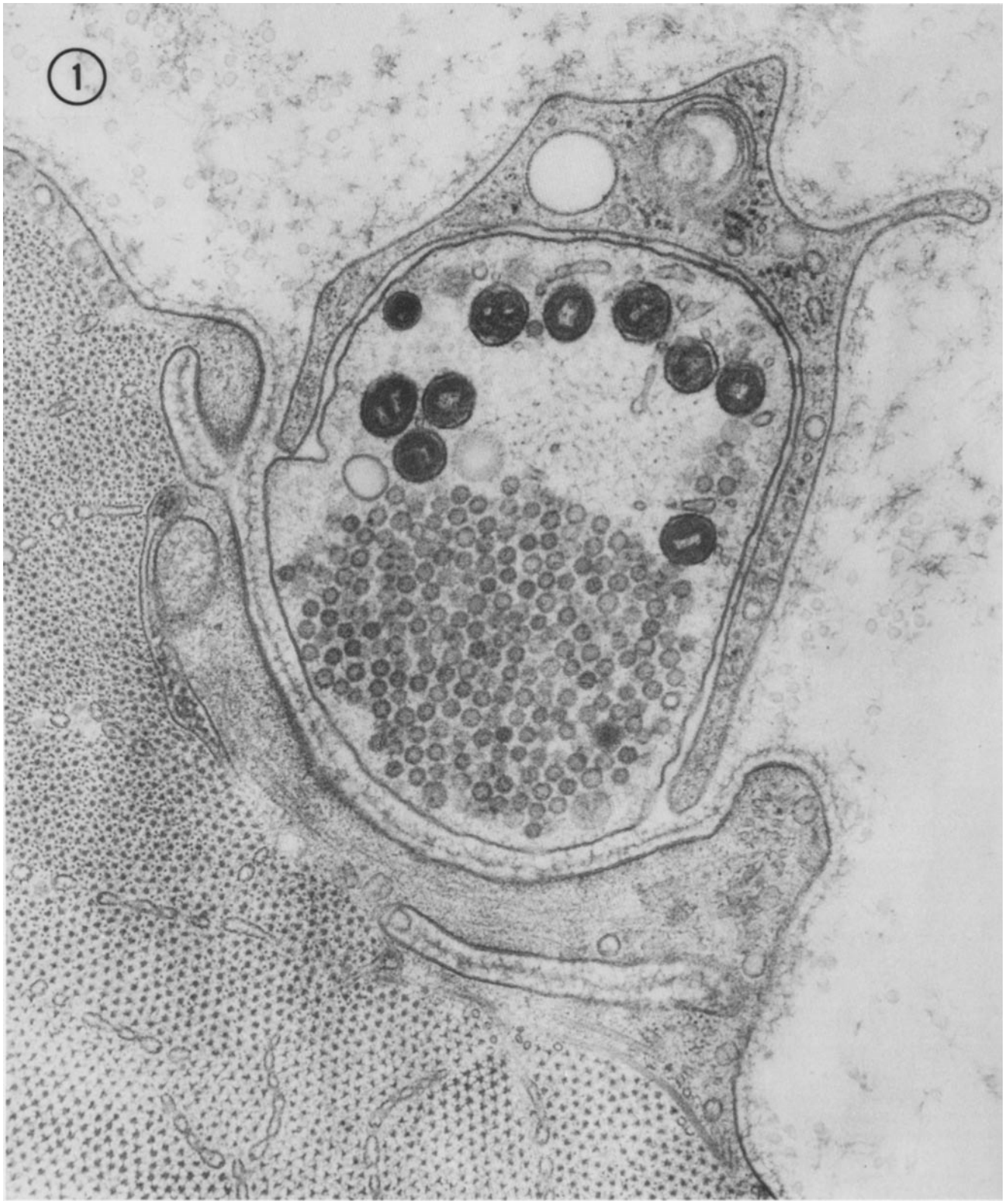


FIGURE 1 Thin cross-sectional view of a nonintoxicated nerve terminal soaked in Ca^{++} Ringer's with no stimulation. The nerve terminal is filled with numerous round synaptic vesicles and has a central core of neurofilaments, microtubules, and mitochondria. The matrix of the mitochondria is dark and no electron-dense granules are observed in the matrix. × 65,000.

10 mM calcium than in those stimulated in 2 mM calcium. (They were completely absent from nerves stimulated in magnesium alone.) Thus, 10 mM calcium was chosen for all subsequent experiments on the assumption that it would maximize the inward calcium currents into the nerve. Such a fivefold increase in extracellular calcium is known to increase transmitter output about twofold (8), but it is not such a high level of calcium that one need worry about it's blocking the conduction of action potentials into the nerve terminal during prolonged repetitive stimulation (25). Nevertheless, to be sure to avoid any problem with conduction block, we chose to deliver stimuli in intermittent bursts of ~40 stimuli delivered over 1.5 s (i.e., ~25 Hz), alternating with 1.5-s periods of rest, for the entire 5 min of stimulation (10).

With this paradigm chosen (5 min of interrupted 25 Hz stimulation in 10 mM calcium), several normal muscles were stimulated and prepared for examination by freeze substitution. The swelling of their mitochondria was easily visible and was

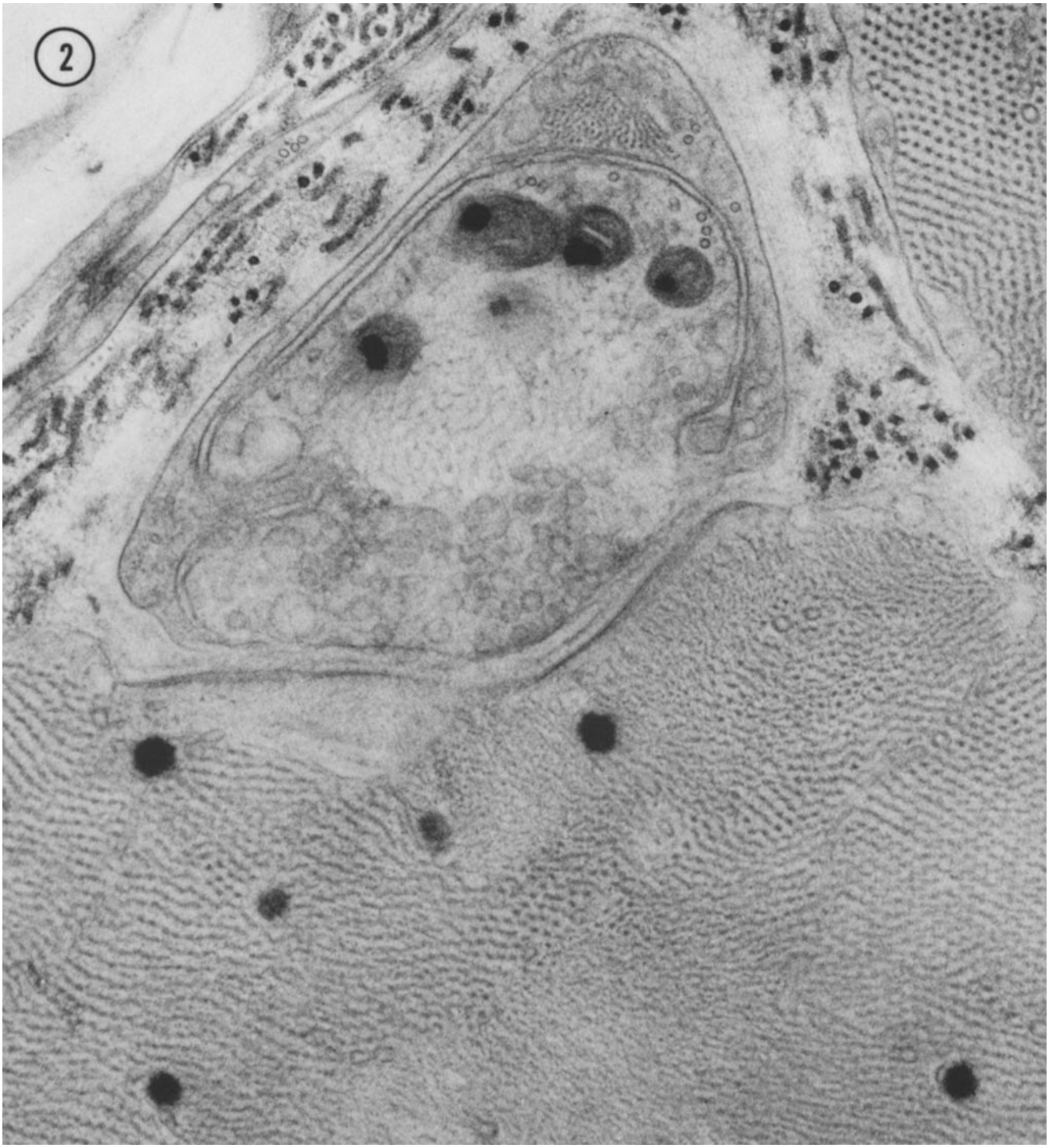


FIGURE 2 Thick sections of a nonintoxicated nerve terminal soaked in Ca^{++} Ringer's with electrical stimulation to show appearance of pronounced electron-dense granules in the mitochondrial matrix. Similar dense granules are localized in cisternae of muscle cells. × 74,000.

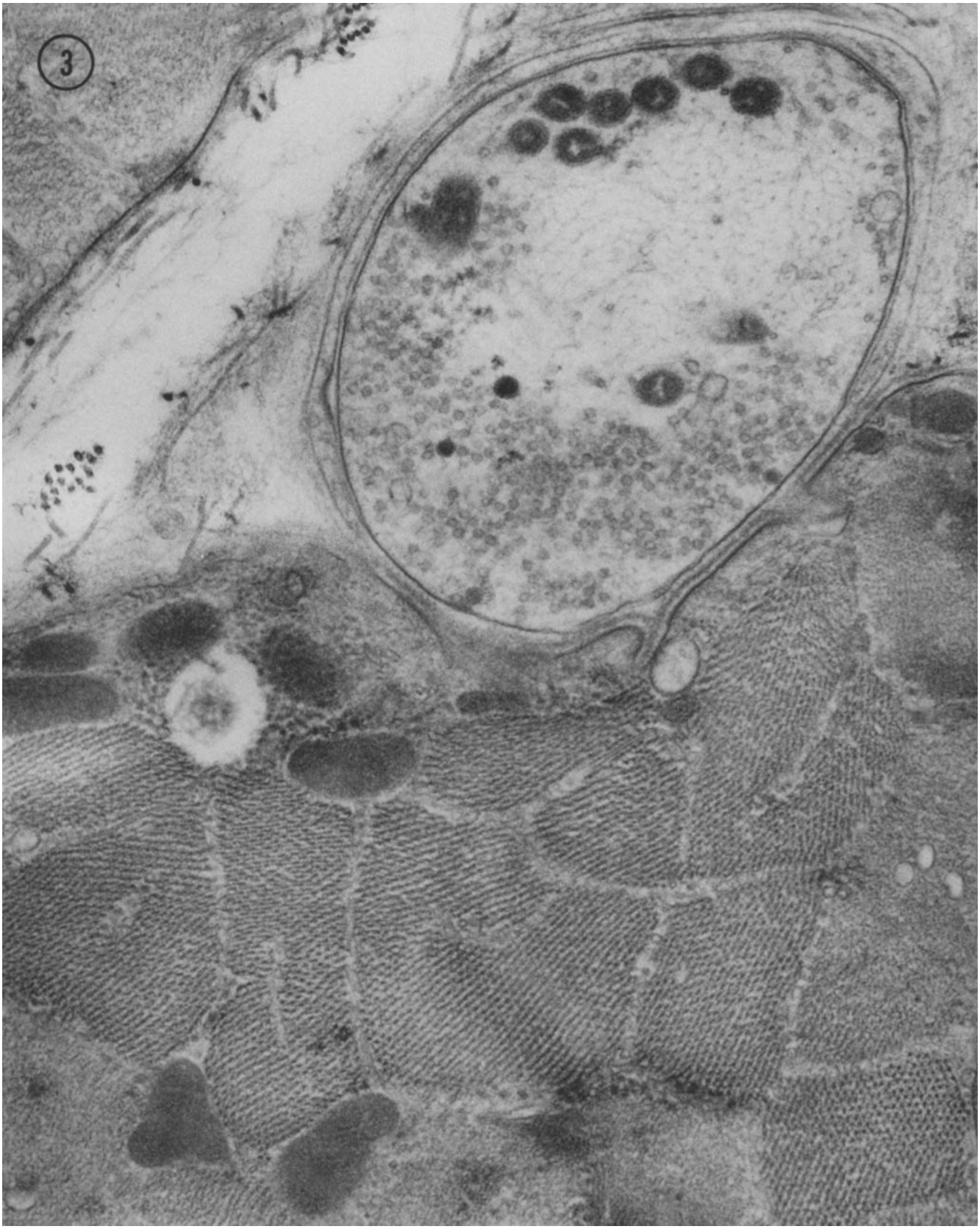


FIGURE 3 Thick section of an intoxicated nerve terminal soaked in Ca^{++} Ringer's with electrical stimulation. The structures in the terminals are not different from those in nonintoxicated muscle without stimulation. Mitochondria do not show swelling, and electron-dense granules are not found in the matrix. Note that electron-dense granules are not observed in mitochondria or other structures in the muscle cell. × 53,000.

documented by measuring their narrowest diameters and comparing them with normal mitochondria (Table I). Also visible was an obvious decrease in the density of the internal matrices of stimulated mitochondria and the appearance of prominent electron-dense granules within them (Figs. 2, 5, and 6). It is worth mentioning that after such stimulation, electron-dense deposits were also found in small cisterns in the muscle (32) and in muscle mitochondria (Fig. 2).

Botulinum-intoxicated Neuromuscular Junctions

Consistent with previous reports (13, 43), there were no obvious structural abnormalities in botulinum-intoxicated nerves. Like normal unstimulated nerves, toxin-treated ones contained small mitochondria with dense internal matrices which lacked electron-dense granules (Fig. 7).

The most important result, however, was that when the above paradigm of electrical stimulation was applied to botulinum-intoxicated nerves, there was no visible sign of muscle twitch, and after freeze-substitution there were none of the mitochondrial changes that were seen in normal nerves (Table I). That is, at the end of 5 min of 25 Hz stimulation, the mitochondria in intoxicated nerves remained small and electron dense and displayed no visible electron-dense granules (Figs. 3 and 8 and Table I).

However, when such botulinum-intoxicated nerves were soaked in 1 mM 4-aminopyridine (4-AP) for 15 min and then stimulated, they partially recovered their ability to make the muscle twitch, and when stimulation was continued for 5 min their mitochondria became dramatically swollen (Table I) and loaded with large electron-dense granules (Figs. 9–11). Again, similar electron-dense granules were also found in small cisterns and in the matrix of muscle mitochondria (Figs. 10 and 11).

In a subsequent series of experiments, normal and intoxicated muscles were stimulated in frog Ringer's that contained 10 mM strontium instead of 10 mM calcium, before freeze-substitution. These muscles showed all the same morphological changes described above, but throughout these experiments the muscles twitched less vigorously (presumably because strontium is a less potent trigger of quantal transmitter release [9]).

BWSV

As has been seen before (5), we observed that normal nerves exposed to BWSV for 1 h became swollen and severely depleted of synaptic vesicles, and their mitochondria became greatly swollen (Figs. 12 and 13). We found, in addition, that after either quick-freezing and freeze-substitution or more standard fixation in high calcium solutions, these swollen mitochondria contained a very large number of extraordinarily prominent electron-dense granules (Figs. 12 and 13). (Also, large calcium deposits were found in the muscle mitochondria and elsewhere in the muscle cells, presumably because of the prolonged action of acetylcholine discharging from the venom-treated nerves [Fig. 12].)

Botulinum toxin did not inhibit this change, as it did the changes resulting from electrical stimulation. Even in fully intoxicated nerves, mitochondria became considerably swollen after exposure to BWSV (Fig. 14). These mitochondria also developed prominent electron-dense granules, just as did the mitochondria in normal nerves exposed to BWSV (Fig. 13). (So, also, did the muscle mitochondria.)

X-Ray Microanalysis

X-ray microanalysis detected a prominent calcium K-α emission between 3.6 and 3.8 keV whenever the beam was applied to the electron-dense granules in nerve mitochondria or in muscle mitochondria and when it was applied to the small electron-dense deposits that appeared elsewhere in the muscles after the treatments described above (Fig. 11).

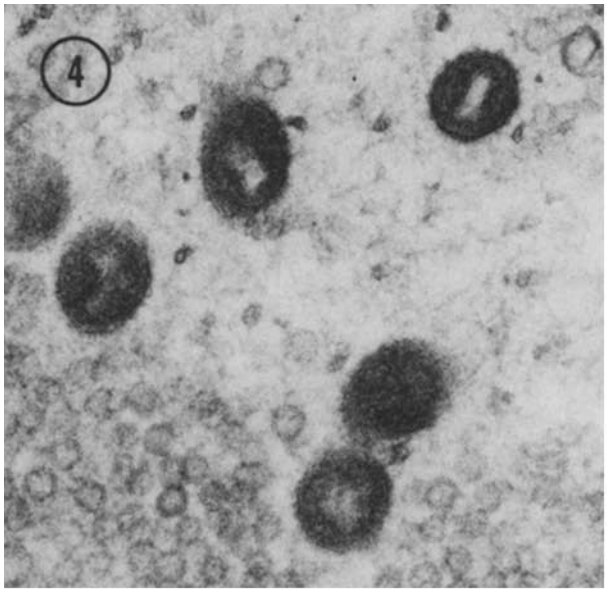
Chemical Fixation

Using conventional aldehyde fixatives with high calcium or high strontium, we observed basically the same mitochondrial changes as seen by freeze-substitution described above; but the preservation of the electron-dense mitochondrial granules was much poorer. Granules were dramatic only after stimulation in strontium Ringer's, presumably because strontium is of a higher atomic number than calcium. Fig. 15 shows a nerve terminal in a normal muscle soaked in strontium Ringer's, electrically stimulated, and fixed with high strontium fixative. Its mitochondria became swollen and dense granules could be seen in their interiors. Fig. 16 is a nerve terminal from a botulinum-intoxicated muscle soaked in strontium Ringer's, electrically stimulated, and fixed with high strontium fixative. Again, the important result was that in such intoxicated nerves, mitochondria remained small and dense, and electron-dense granules did not appear in their interiors.

DISCUSSION

In the present study, whenever nerve stimulation led to grossly visible muscle twitch (indicating that depolarization-induced calcium influx had occurred and that transmitter had been released) we found that nerve terminal mitochondria became swollen and developed prominent electron-dense granules in their interiors. X-ray microanalysis clearly indicated that these granules contained abundant calcium. These results fit well with previous works (14, 16, 17, 31, 32, 34, 35), all of which suggest that mitochondrial swelling and calcium sequestration is a natural consequence of the calcium influx into nerve terminals. The critical new observation in the present study was that such mitochondrial changes did not occur in botulinum-poisoned nerves. This clearly was not a result of some indirect effect of the toxin on mitochondria, because when calcium was driven into such intoxicated nerves by other means—for example, by stimulating them in 4-AP or by exposing them to BWSV—their mitochondria were still fully capable of swelling and accumulating calcium. Thus, the toxin effect seems to be caused by a direct block of the calcium entry that normally accompanies nerve depolarization.

Blockage of calcium entry into the nerve terminal could explain how botulinum toxin paralyzes animals. Without the calcium entry that normally accompanies nerve depolarization, quanta of acetylcholine could not be released in response to motor nerve activity and neuromuscular transmission would fail. This is exactly what is seen physiologically. During the onset of the paralysis of botulism, the number of quanta discharged in response to each nerve impulse declines progressively (13). Clinically, the developing paralysis can be reversed by applying high extracellular calcium or by guanidine, both of which temporarily increase the number of quanta discharged. Recently, it has been shown that agents that prolong the action potential—tetraethylammonium or 4-AP—also partially reverse this paralysis (7, 26, 29, 41, 45), and again it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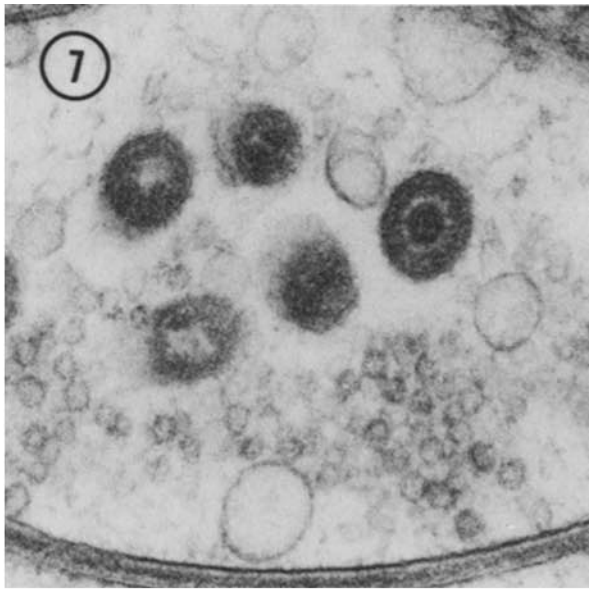
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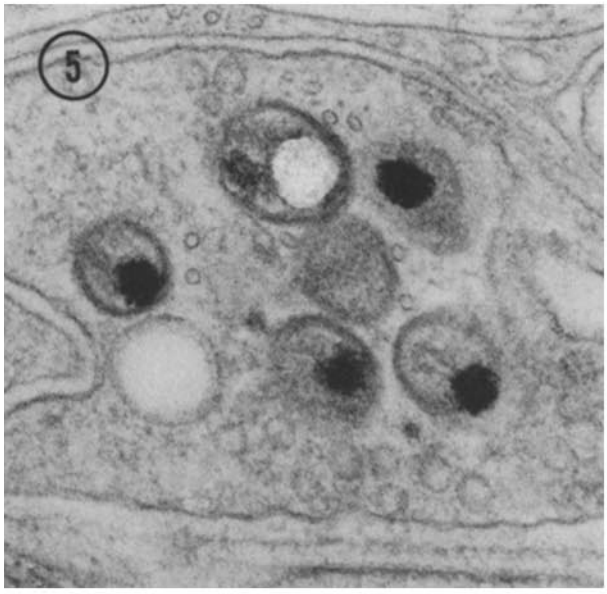
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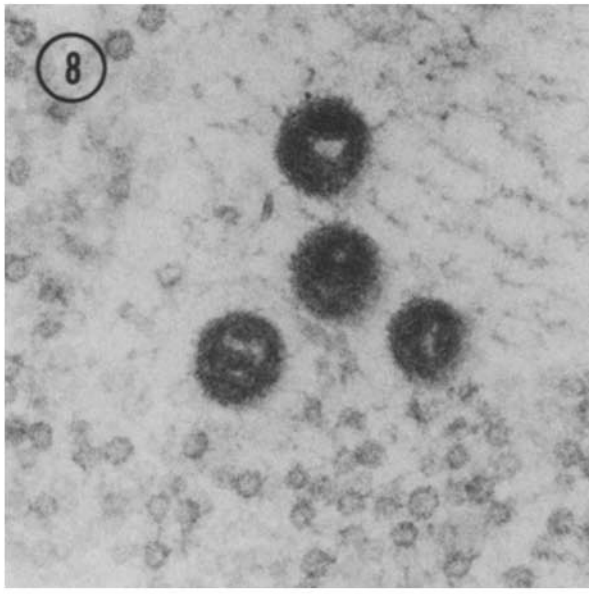
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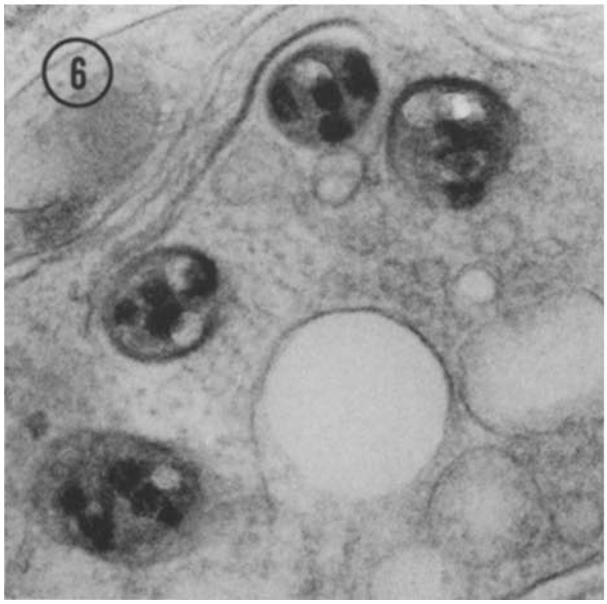
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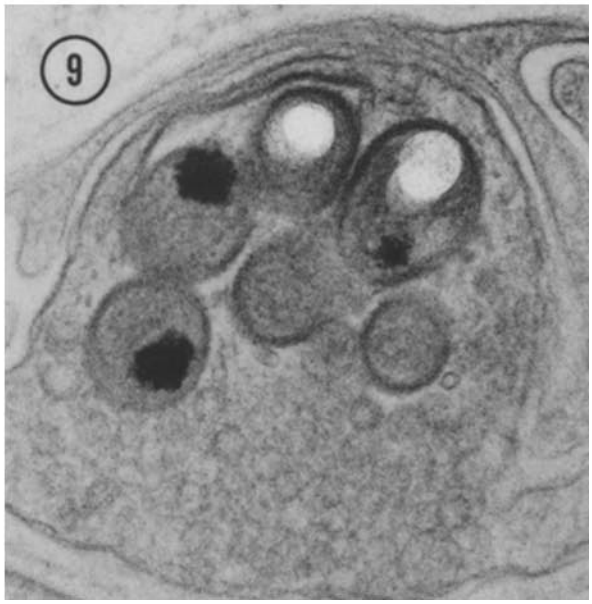
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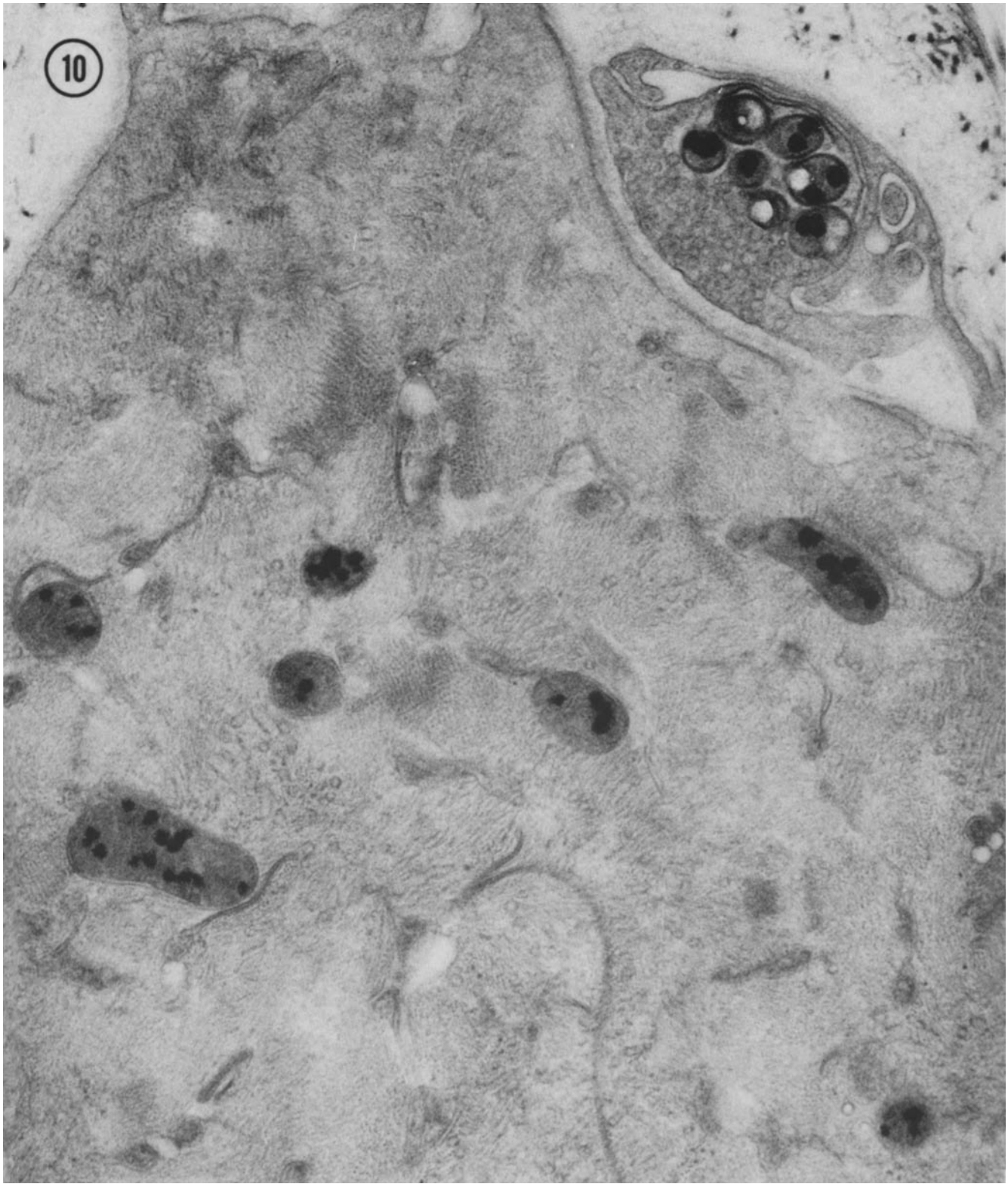


FIGURE 10 A thick section of an intoxicated nerve terminal and a muscle cell stimulated after incubation with 4-AP to show swollen mitochondria in the nerve terminal and prominent electron-dense granules in the mitochondrial matrix of both the nerve terminal and the muscle cell. × 46,000.

FIGURES 4–9 Thick sections of nerve terminals soaked in Ca^{++} Ringer's to show structural changes in the mitochondria. × 88,000. Fig. 4, no toxin, no stimulation. Mitochondrial matrix is dark and electron-dense granules are not observed in the matrix. Fig. 5, no toxin, stimulation. Mitochondria become swollen. Electron-dense granules appear in the pale matrix. Some granules fall out and leave a hole during sectioning. Fig. 6, no toxin, stimulation after incubation with 4-AP. Mitochondria are swollen and numerous electron-dense granules do exist in the matrix. Fig. 7, toxin, no stimulation. Mitochondrial matrix is dark and no electron-dense granules are found. Fig. 8, toxin, stimulation. The appearance of the mitochondria is similar to that in the intoxicated terminal with no stimulation. Mitochondria do not show swelling. Fig. 9, toxin, stimulation after incubation with 4-AP. Mitochondria become swollen, and prominent electron-dense granules appear in the pale matrix. Some granules have fallen out.

is known that these agents also increase the number of quanta discharged after each action potential, apparently by prolonging the action potential and the calcium entry that follows it (7, 19).

Eventually, the paralyzing effect of botulinum toxin progresses until no quanta are released at all in response to normal nerve stimulation. Yet, quanta must remain present inside the nerve because they can be forced out by such abnormal treatments as gross nerve damage, BWSV, etc. (7, 37). Consistent with this, all observers agree that there is no loss of synaptic vesicles at this time (13). Presumably, all these abnormal ways of forcing quanta to discharge result from some alternate avenue of calcium entry into the nerves, which bypasses the normal depolarization-triggered calcium channels. For example, it is well known that BWSV is itself a potent calcium ionophore (6, 11, 37, 42), and the massive amounts of calcium which it lets into the nerve can be seen quite clearly in the normal neuromuscular preparations we freeze-substituted in the course of the above experiments (Figs. 12 and 13).

The only observations which our hypothesis of the channel-blocking effect of botulinum toxin cannot readily explain are the following. First, Cull-Candy et al. (7) and Simpson (41) have reported that calcium ionophore A23187 and batrachotoxin produce less quantal release in toxin-poisoned muscles than they do in normal muscles, and have argued on this basis that the toxin reduces not calcium entry but the subsequent calcium sensitivity of the release mechanism. This conclusion presumes that the amount of calcium that these ionophores let in is the same in normal and intoxicated nerves, which may well be so, but it does not consider the possibility that the final intracellular concentration of calcium might end up to be quite different in normal versus intoxicated nerves. For example, the

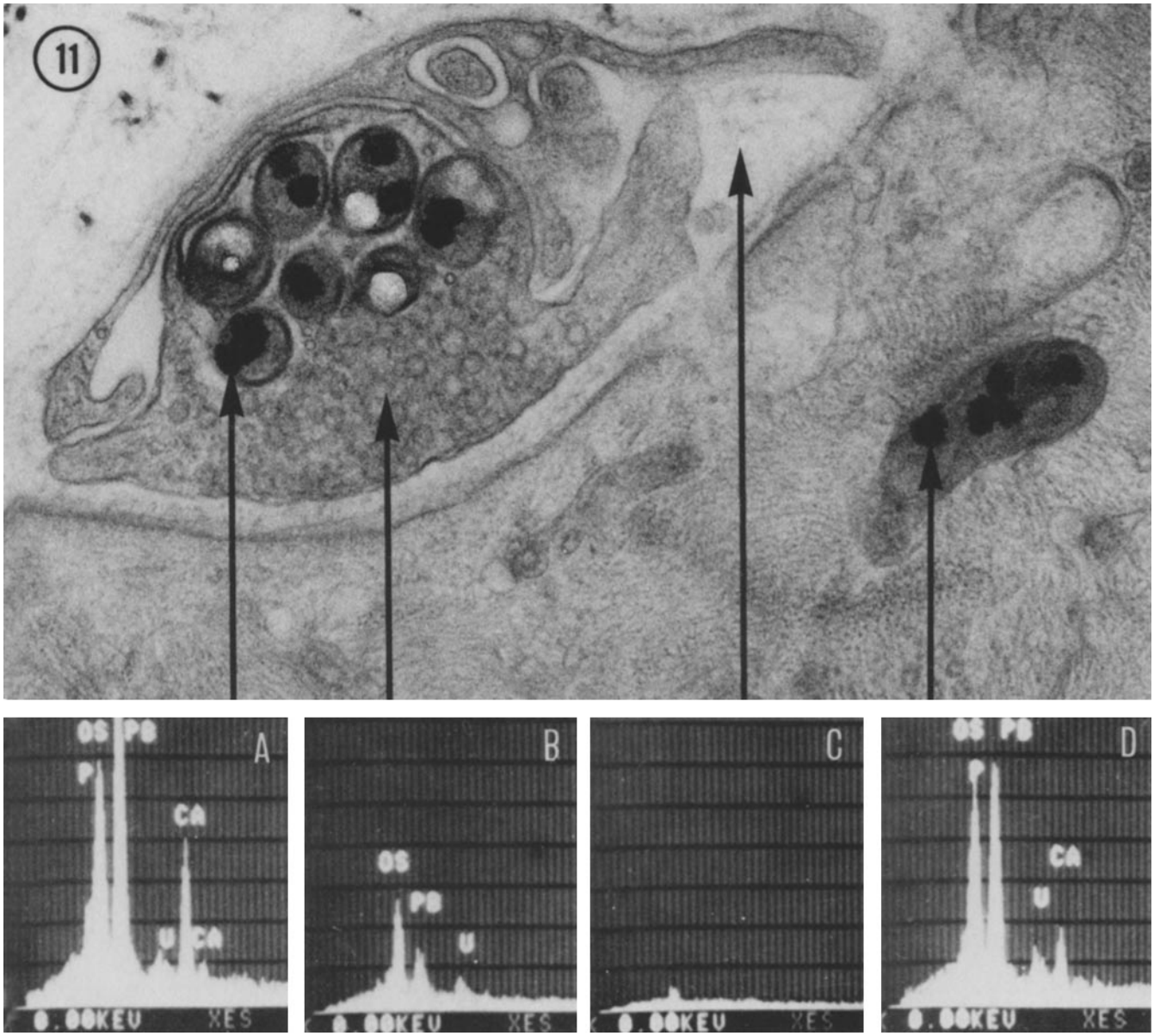
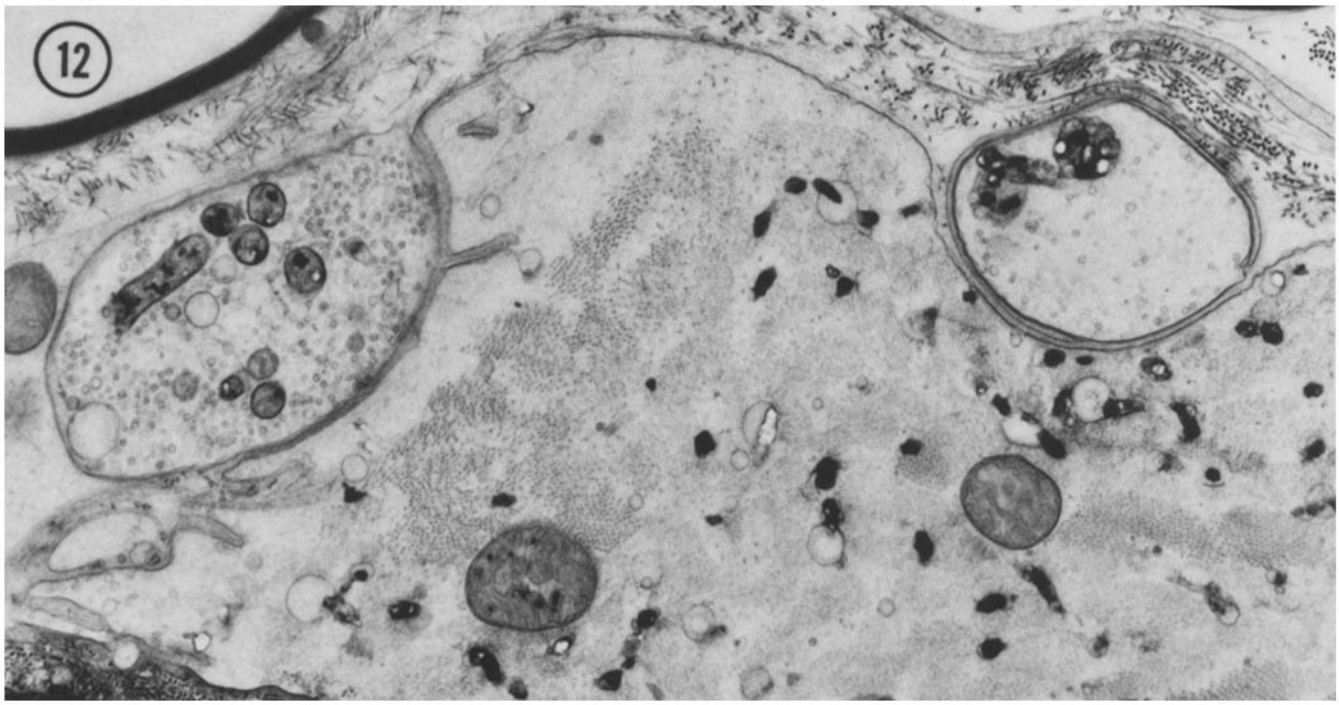
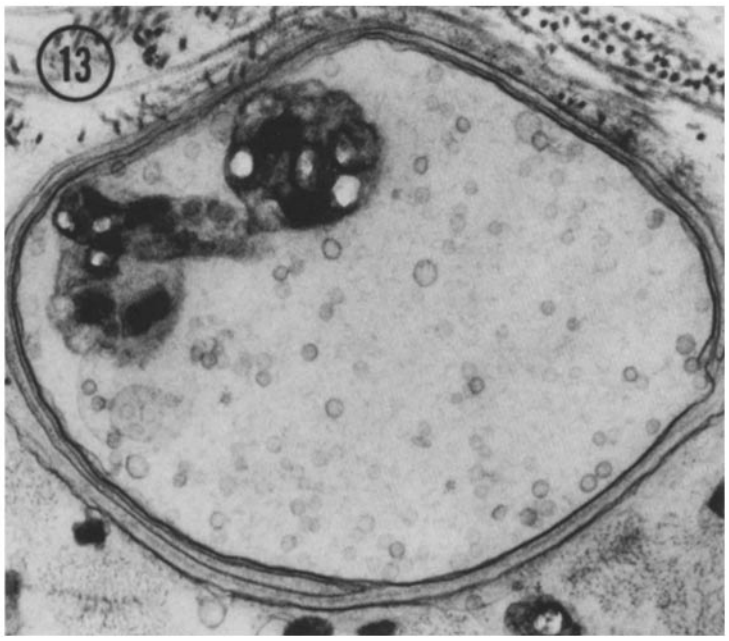


FIGURE 11 Thick section of the intoxicated neuromuscular junction stimulated after incubation with 4-AP to show typical x-ray spectrum from probing the areas pointed by arrows. (*A*) Electron-dense granules in the terminal mitochondria. (*B*) synaptic vesicles in the terminal. (*C*) Extracellular space. (*D*) Electron-dense granules in the muscle mitochondria. Prominent peaks at the energy characteristic of calcium (3.6–3.8 keV) are distinguishable at electron-dense granules in *A* and *D*. × 73,000.

present results would argue that intracellular calcium stores are drastically depleted in botulinum-intoxicated nerves because the prolonged period of less than normal calcium entry. If this is so, then whatever calcium entered these nerves would be more strongly buffered than normal and whatever sodium entered them via ionophores would displace less calcium from their internal stores. Both of these consequences would depress the ability of an ionophore to raise intracellular calcium, but this would not mean that calcium was less effective in eliciting transmitter release. Of course, even if this is so, our data do not rule out the possibility that the toxin depresses sensitivity to calcium as well as blocking calcium entry. The second phenomenon which the calcium channel-blocking idea cannot explain is the following. Ever since the original electrophysiological studies of botulinum toxin action, investigators have marvelled at the fact that the amplitude of spontaneously discharged





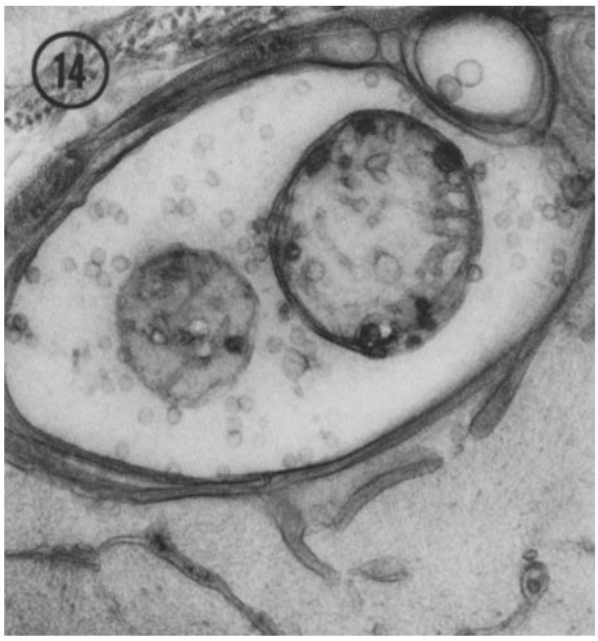
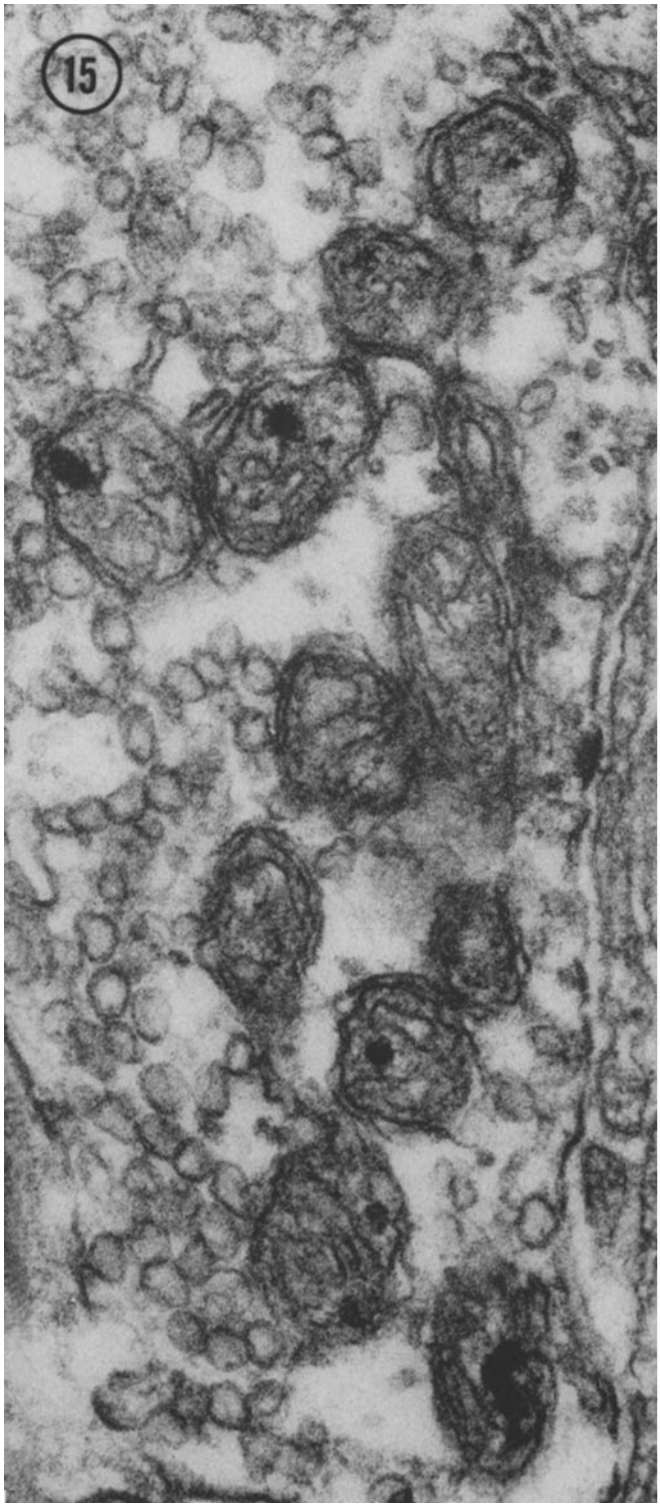


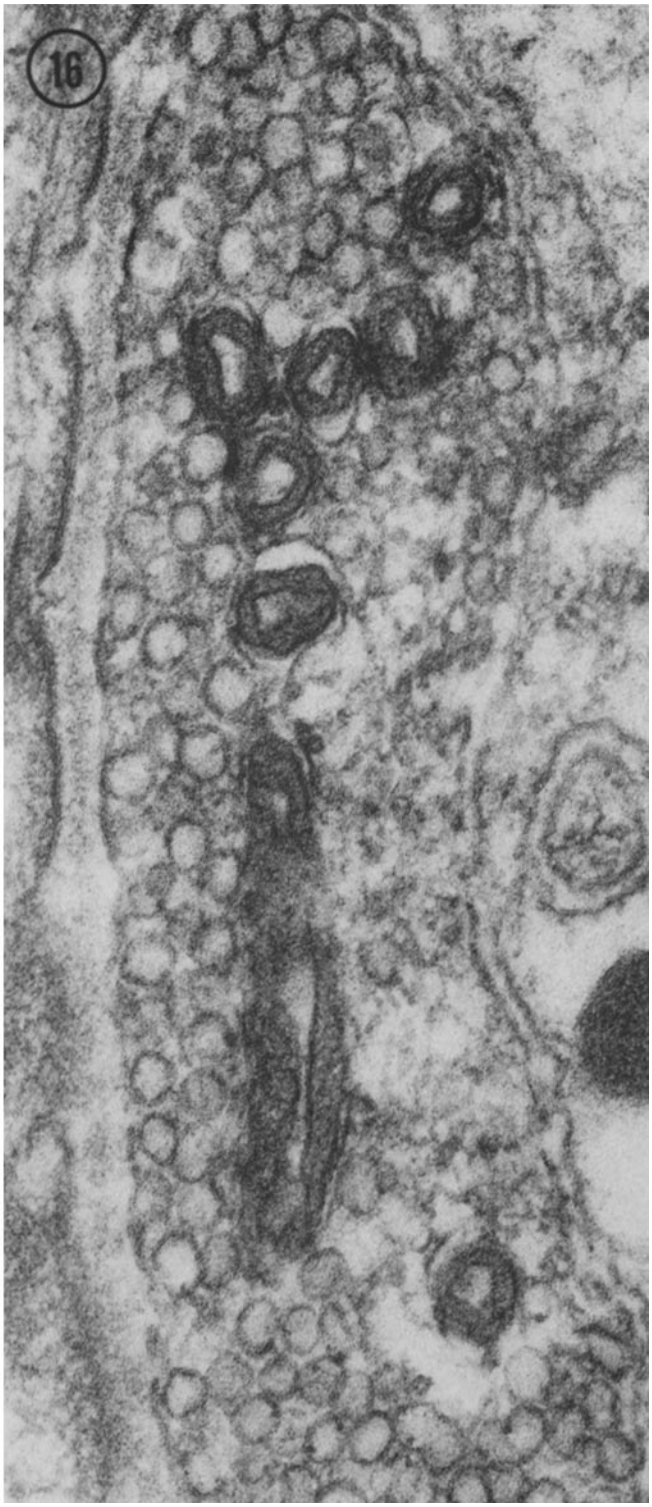
FIGURE 12 Thick section of a nonintoxicated neuromuscular junction soaked with BWSV. The mitochondria show swelling and develop prominent electron-dense granules in the nerve terminals. Vesicles are depleted much more and mitochondrial changes are more remarkable in the right terminal than in the left one. × 16,000.

FIGURE 13 High magnification of the nerve terminal in Fig. 12. × 48,000.

FIGURE 14 Thick sections of an intoxicated terminal soaked with BWSV. Vesicles are depleted. Mitochondrial swelling and electron-dense granules are clearly observed. × 48,000.

miniature endplate potentials (mepp's) declines progressively during the onset of paralysis (13). This has been shown to occur without decline in postsynaptic sensitivity, and has in general been thought to result from a reduction in the number of acetylcholine molecules in each quantum. But the puzzling aspect of this phenomenon is that it happens only to spontaneously released quanta; those released in response to nerve stimulation apparently remain of normal amplitude and simply become less abundant as the toxin takes effect. Nothing in our idea of the channel-blocking effect of botulinum toxin could explain the small spontaneous mepp's. They remain a total enigma.





FIGURES 15 and 16 Thin sections of nerve terminals soaked in Sr^{++} salines with electrical stimulation at 25 Hz for 5 min followed by fixation with 1% glutaraldehyde and 2% formaldehyde fixative containing 50 mM $SrCl_2$. Fig. 15, nonintoxicated nerve terminal. Mitochondria become swollen and electron-dense granules develop in the mitochondrial matrix. Fig. 16, intoxicated nerve terminal. We cannot observe mitochondrial swelling and electron-dense granules in the matrix.

The first question that emerges from the present observations and the interpretation that we offer for them is, of course, how could the toxin block calcium channels? We have no idea but would like to recall a basic fact about the toxin's action; namely, the rate of onset of paralysis is activity-dependent (2). That is, in the absence of nerve stimulation (as, for example, in an isolated nerve-muscle preparation resting in an experimental chamber) the rate of onset of paralysis is very slow and requires at least several hours. Nerve stimulation at any time during this period will make paralysis develop almost immediately. Thus, our working hypothesis is that the toxin is taken up by nerves in the course of their normal membrane recycling that accompanies transmitter secretion (16), and that the toxin can only alter calcium channels by acting from the inside. (This would be somewhat analogous to the uptake and intracellular action of other protein toxins and colicins [30].) If this were so, it would explain why we have been unable to demonstrate a localized binding of botulinum toxin to the active zones of nerves, where the calcium channels are supposed to be located. Binding before endocytic uptake could occur anywhere. Moreover, an "action from the inside" could well be enzymatic in nature, which would explain the extreme potency of this toxin, which has been called the "most poisonous poison" (27).

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REFERENCES

1. Alnaes, E., and R. Rahamimoff. 1975. On the role of mitochondria in transmitter release from motor nerve terminals. *J. Physiol. (Lond.)*. 248:285–306.
2. Boroff, D. A., J. del Castillo, W. H. Evoy, and R. A. Steinhardt. 1974. Observations on the action of type A botulinum toxin on frog neuromuscular junctions. *J. Physiol. (Lond.)*. 240:227–253.
3. Brooks, V. B. 1956. An intracellular study of the action of repetitive nerve volleys and botulinum toxin on miniature endplate potentials. *J. Physiol. (Lond.)*. 134:264–277.
4. Burgen, A. S. V., F. Dickens, and L. J. Zatman. 1949. The action of botulinum toxins on the neuro-muscular junction. *J. Physiol. (Lond.)*. 109:10–24.
5. Ceccarelli, B., F. Gorohovaz, and W. P. Hurlbut. 1979. Freeze-fracture studies of the neuromuscular junctions during intense release of neurotransmitter. I. Effects of black widow spider venom and Ca^{+++}-free solution on the structure of the active zone. *J. Cell Biol.* 81:163–177.
6. Clark, A. W., W. P. Hurlbut, and A. Mauro. 1972. Changes in the fine structure of the neuromuscular junction of the frog caused by black widow spider venom. *J. Cell Biol.* 52: 1–14.
7. Cull-Candy, S. G., H. Lundh, and S. Thesleff. 1976. Effects of botulinum toxin on neuromuscular transmission in the rat. *J. Physiol. (Lond.)*. 260:177–203.
8. Del Castillo, J., and L. Stark. 1952. The effect of calcium ions on the motor endplate potentials. *J. Physiol. (Lond.)*. 116:507–515.
9. Dodge, F. A., R. Miledi, and R. Rahamimoff. 1969. Strontium and quantal release of transmitter at the neuromuscular junction. *J. Physiol. (Lond.)*. 200:267–283.
10. Dunn, F. T. 1955. The blockage of motor impulses in an asynchronized volley at the neuromuscular junction. *J. Cell Comp. Physiol.* 46:348–370.
11. Finkelstein, A., L. L. Rubin, and M.-C. Tzeng. 1976. Black widow spider venom: effect of purified toxin on lipid bilayer membranes. *Sciences (N. Y.)*. 193:1009–1011.
12. Greenawalt, J. W., C. S. Rossi, and A. L. Lehninger. 1964. Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria. *J. Cell Biol.* 23: 21–38.
13. Harris, A. J., and R. Miledi. 1971. The effect of type D botulinum toxin on frog neuromuscular junction. *J. Physiol. (Lond.)*. 217:497–515.
14. Heuser, J. E. 1976. Morphology of synaptic vesicle discharge and reformation at the frog neuromuscular junction. *In* Motor Innervation of Muscle. S. Thesleff, editor. Academic Press, Inc., London. 51–115.
15. Heuser, J. E., B. Katz, and R. Miledi. 1971. Structural and functional changes of frog neuromuscular junctions in high calcium solutions. *Proc. R. Soc. Lond. B Biol. Sci.* 178: 407–415.
16. 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.* 57:315–344.
17. Heuser, J. E., and T. S. Reese. 1976. Freeze-substitution applied to the study of quick-frozen synapses. *J. Cell Biol.* 70(2, Pt. 2):357 *a* (Abstr.).
18. Heuser, J. E., T. S. Reese, and D. M. D. Landis. 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* 3:109–131.
19. Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell. Biol.* 81:275–300.
20. Hirokawa, N., and T. Kirino. 1980. An ultrastructural study of nerve and glial cells by freeze-substitution. *J. Neurocytol.* 9:243–254.
21. Hirokawa, N., and M. Kitamura. 1975. Localization of radioactive ^{125}I-labelled botulinum toxin at the neuromuscular junction of mouse diaphragm. *Naunyn-Schmiedebergs Arch. Pharmakol.* 287:107–110.
22. Hirokawa, N., and M. Kitamura. 1979. Binding of *Clostridium botulinum* neurotoxin to the presynaptic membrane in the central nervous system. *J. Cell Biol.* 81:43–49.
23. Katz, B., and R. Miledi. 1965. The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. Lond. B Biol. Sci.* 161:469–503.
24. Katz, B., and R. Miledi. 1965. The timing of calcium action during neuromuscular transmission. *J. Physiol. (Lond.)*. 189:535–544.
25. Krnjevic, K., and R. Miledi. 1958. Failure of neuromuscular propagation in rats. *J. Physiol. (Lond.)*. 140:440–461.
26. Kusano, K., R. Livengood, and R. Werman. 1967. Correlation of transmitter release with membrane properties of the presynaptic fiber of the squid giant synapse. *J. Gen. Physiol.* 50:2579–2601.
27. Lamanna, C. 1959. The most poisonous poison. *Science (Wash. D. C.)*. 130:763–772.
28. Leninger, A. L. 1970. Mitochondria and calcium ion transport. *Biochem. J.* 119:129–138.
29. Llinas, R., K. Walton, and V. Bohr. 1976. Synaptic transmission in squid giant synapse after potassium conductance blockage with external 3- and 4-aminopyridine. *Biophys. J.* 16:83–86.
30. Neville, D. M., and T. Chang. 1978. Receptor-mediated protein transport into cells. Entry mechanisms for toxins, hormones, antibodies, viruses, lysosomal hydrolases, Asialoglycoproteins, and carrier proteins. *Curr. Top. Membr. Transp.* 10:65–150.
31. Ornberg, R. L., and T. S. Reese. 1978. A method for localizing divalent cations in freeze-substituted thin-sectioned tissue. *J. Cell Biol.* 79(2, Pt. 2):257 *a* (Abstr.).
32. Ornberg, R. L., and T. S. Reese. 1980. A freeze-substitution method for localizing divalent cations: examples from secretory systems. *Fed. Proc.* In press.
33. Oschman, J. L., and B. J. Wall. 1972. Calcium-binding to intestinal membranes. *J. Cell Biol.* 55:58–73.
34. Pappas, G. D., and S. Rose. 1976. Localization of calcium deposits in the frog neuromuscular junction at rest and following stimulation. *Brain Res.* 103:362–365.
35. Parducz, A., and F. Joo. 1976. Visualization of stimulated nerve endings by preferential calcium accumulation of mitochondria. *J. Cell Biol.* 69:513–517.
36. Peachey, L. D. 1964. Electron microscopic observations on the accumulation of divalent cation in intramitochondrial granules. *J. Cell Biol.* 20:95–111.
37. Pumplin, D. W., and T. S. Reese. 1977. Action of brown widow spider venom and botulinum toxin on the frog neuromuscular junction examined with the freeze-fracture technique. *J. Physiol. (Lond.)*. 273:443–457.
38. Rahamimoff, R. 1976. The role of calcium in transmitter release at the neuromuscular junction. *In* Motor Innervation of Muscle, S. Thesleff, editor. Academic Press, Inc., New York. 117–149.
39. Sampson, H. W., R. E. Dill, J. L. Matthews and J. H. Martin. 1970. An ultrastructural investigation of calcium-dependent granules in the rat neurophil. *Brain Res.* 22:157–162.
40. Simpson, L. L. 1971. Botulinum Toxin in Neuropoisons—Their Pathophysiological Actions. L. L. Simpson, editor. Plenum Press, New York. 1:303–323.
41. Simpson, L. L. 1978. Pharmacological studies on the subcellular site of action of botulinum toxin type A. *J. Pharmacol. Exp. Ther.* 206:661–669.
42. Smith, J. E., A. W. Clark, and T. A. Kuster. 1977. Suppression by elevated calcium of black widow spider venom activity at frog neuromuscular junctions. *J. Neurocytol.* 6:519–539.
43. Thesleff, S. 1960. Supersensitivity of skeletal muscle produced by botulinum toxin. *J. Physiol. (Lond.)*. 151:598–607.
44. Yates, R. D., and J. C. Yates. 1968. The occurrence of intramitochondrial granules in nerve cells. *Z. Zellforsch. Mikrosk. Anat.* 92:388–393.
45. Yeh, J. Z., G. S. Oxford, C. H. Wu, and T. Narahashi. 1976. Interactions of aminopyridines with potassium channels of squid axon membranes. *Biophys. J.* 16:77–81.