Reversibility and Mode of Action of Black Widow Spider Venom on the Vertebrate Neuromuscular Junction

A. GORIO and A. MAURO

From The Rockefeller University, New York 10021. Dr. Gorio's present address is Fidia Research Laboratories, Abano Terme, Padova, Italy.

ABSTRACT Black widow spider venom (BWSV) stimulates transmitter release and depletes synaptic vesicles from muscles bathed in a sodium free medium containing 1 mM EGTA. However, frog neuromuscular junctions treated with BWSV in glucosamine Ringer's and post-treated with antivenin recover normal function. This suggests that probably the permanent block of neuromuscular transmission is due to changes in permeability of the nerve ending plasma membrane to cations such as Na^+. When BWSV is applied in a medium lacking divalent cations and containing 1 mM EGTA, in most of the cases no effect is observed. We found that this inhibition can be overcome in three ways: (*a*) by adding divalent cations to the medium; (*b*) by increasing the tonicity of the medium with sucrose; (*c*) by raising the temperature of the medium. These results suggest that the lack of divalent cations influences the membrane fluidity. Moreover, in view of the report by Yahara and Kakimoto-Sameshima (1977. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4511–4515) that hypertonic media induce capping of surface receptors in lymphocytes and thymocytes, we think that these data further support the hypothesis that BWSV stimulates release by a dual mode of action; namely, it increases the nerve ending permeability to cations and also stimulates release directly via a process of redistribution of membrane components, a process which may also inhibit vesicle recycling.

INTRODUCTION

When a crude homogenate of black widow spider venom (BWSV) glands is applied to a frog neuromuscular preparation, the miniature endplate potential (mepp) frequency rapidly increases several hundredfold. This rate of release is sustained for several minutes, after which it subsides and mepp's become rare, endplate potentials (epp's) cannot be evoked, and nerve endings are found to be depleted of synaptic vesicles (Clark et al.; 1970; Longenecker et al., 1970). While the mepp frequency is high, there is a corresponding increase in acetylcholine (ACh) release. Furthermore, after 1 h when mepp's are rare, neuromuscular transmission is blocked and vesicles are depleted; subsequent stimulation with a K+-rich solution does not affect the rate of mepp and of ACh release (Gorio et al., 1978 *a*). A protein, α-latrotoxin, of 130,000 mol wt, an isoelectric point ranging from pH 5.2 to 5.5 and without apparent lipolytic or proteolytic activity is responsible for all of these actions at the vertebrate neuromuscular junction

(Frontali et al., 1976). The fact that a single protein was able to stimulate ACh release and block vesicle recycling was surprising and stimulated work to understand its mode of action.

Finkelstein et al. (1976) applied α-latrotoxin to artificial black lipid membranes and observed a large increase in permeability to cations. Therefore they suggested that the toxin, after binding to the nerve terminal plasma membrane, increases the permeability to cations such as Ca^{++} or Na^{+}, leading to transmitter release. In addition, a provocative suggestion concerning how α-latrotoxin stimulates release was made based on experiments which indicated that during venom action, a redistribution of the nerve terminal plasma membrane components occur, which is similar to the patching and capping of the surface receptors in lymphocytes when they are incubated with bivalent antibodies (Rubin et al., 1978). Later, it was shown that BWSV stimulates release and depletes synaptic vesicles in the absence of both Na^{+} and Ca^{++}, but it was also shown that the enormous swelling of the nerve endings, observed when large doses of BWSV are applied, was absent if the medium contained glucosamine as a sodium substitute. This led to the suggestion that venom has a double mode of action: it increases the permeability of the nerve terminal plasma membrane to cations, and it stimulates ACh release by a mechanism which may not be dependent on these permeability changes (Gorio et al., 1978 *b*). Using electrophysiological and morphological means, we have continued these studies to test the hypothesis of BWSV dual mode of action, and we believe that the experiments reported here reinforce this hypothesis.

MATERIALS AND METHODS

Preparations

All the experiments using pectoris nerve muscle preparations from small frogs, *Rana pipiens*, were performed at room temperature. Muscles were mounted in a lucite chamber, stretched over a lens and pinned down (Hurlbut and Ceccarelli, 1974). The nerve, dissected to the spinal cord, was drawn into two wells insulated with vaseline and stimulated with square pulses of 0.5 ms duration at an intensity three or four times the threshold for evoking muscle twitch. Muscles were usually mounted in Ringer's, and only preparations which twitched in response to nerve stimulation were used. Diaphragm neuromuscular preparations were quickly dissected from mice of 20 g weight, and mounted as described for the frog preparation. The solutions were changed by flushing the bath with the fluid contained in a suspended syringe.

Solutions

The frog Ringer's solution consisted of 116 mM NaCl, 2.1 mM KCl, 1.8 mM $CaCl_2$, and 6 mM Tris-HCl; the low-Ca^{++} Ringer's consisted of 112 mM NaCl, 2.1 mM KCl, 0.6 mM $CaCl_2$, 4 mM $MgCl_2$, and 6 mM Tris-HCl, both at pH 7.2. D-Glucosamine was used as a sodium substitute in Na^{+}-free Ringer's. Glucosamine was used as a sodium substitute in Na^{+}-free Ringer's, inasmuch as glucosamine does not pass through venom-induced channels in artificial lipid membranes (Gorio et al., 1978 *b*). Approximately 7 mM KOH was added to the glucosamine solutions to obtain pH 6.5. At this pH glucosamine is about 90% ionized. For the recovery experiments, the Na^{+}-free solutions consisted of 112 mM D-glucosamine-HCl, 4 mM $MgCl_2$, and 6 mM Tris-HCl; the low-Ca^{++} Ringer's was supplemented with 10 mM glucose and 100 μM choline. BWSV antivenin was purchased

from Merck Sharp & Dohme, West Point, Pa. The Ca^{++}-free Ringer's and the Ca^{++}-free glucosamine Ringer's contained 1 mM EGTA and Mg^{++} concentrations ranging from 10^{-5} to 4×10^{-3} M. EGTA was titrated at pH 7 with Tris-base. When K^+ was used as a Na substitute, the solutions consisted of 116 mM K-propionate, 4 mM $MgCl_2$, 1 mM EGTA, and 6 mM Tris-HCl at pH 7.2. The Kreb's solutions for mouse diaphragms contained 142 mM NaCl, 8 mM $NaHCO_3$, 4 mM $KHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 1 mM Mg Cl_2, and 2 mM $CaCl_2$. The divalent cations-free solution contained 1 mM EGTA. The glucosamine divalent cations-free Krebs' consisted of 150 mM glucosamine-HCl, 3 mM Tris-HCl, 10 mM glucose, and 1 mM EGTA at pH 6.5.

Electrophysiology

Electrophysiological studies were carried out using standard intracellular recording techniques with glass microelectrodes filled with 3 M KCl and with a resistance ranging from 10 to 30 MΩ. Records were displayed on a dual beam oscilloscope; one beam was AC-coupled at high gain to record mepp's and epp's and the other one was DC-coupled at low gain to record resting potentials.

Electron Microscopy

Muscles were fixed in the recording chamber by draining the bath and filling it with fixative. Fixations were carried out with solutions consisting of 2% gluteraldehyde and 0.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer pH 7.2 for 45 min. Then small pieces of muscle, suspected to be rich in neuromuscular junctions, were cut and put again into fresh fixative for 45 min. Successively the muscle bits were washed twice for 10 min each in phosphate buffer, and post-fixed in cold 1% osmium tetroxide in 0.1 M phosphate buffer for 90 min. Then the specimens were dehydrated in a graded series of alcohols and flat embedded in Epon 812 (Shell Chemical Co., New York). The pieces of muscles were oriented so that longitudinal or cross sections of nerve endings could be obtained. Semi-thin sections were cut with a glass knife, stained with toluidine blue and examined with a light microscope. When sections contained numerous suspected neuromuscular junctions, serial thin sections were cut with a diamond knife, double-stained with uranyl acetate and lead citrate and examined in an electron microscope. Direct fixation with cold 2% osmium tetroxide, avoiding prefixation with aldehyde, was used in the experiments where K^+ was used as a Na substitute.

Black Widow Spider Venom

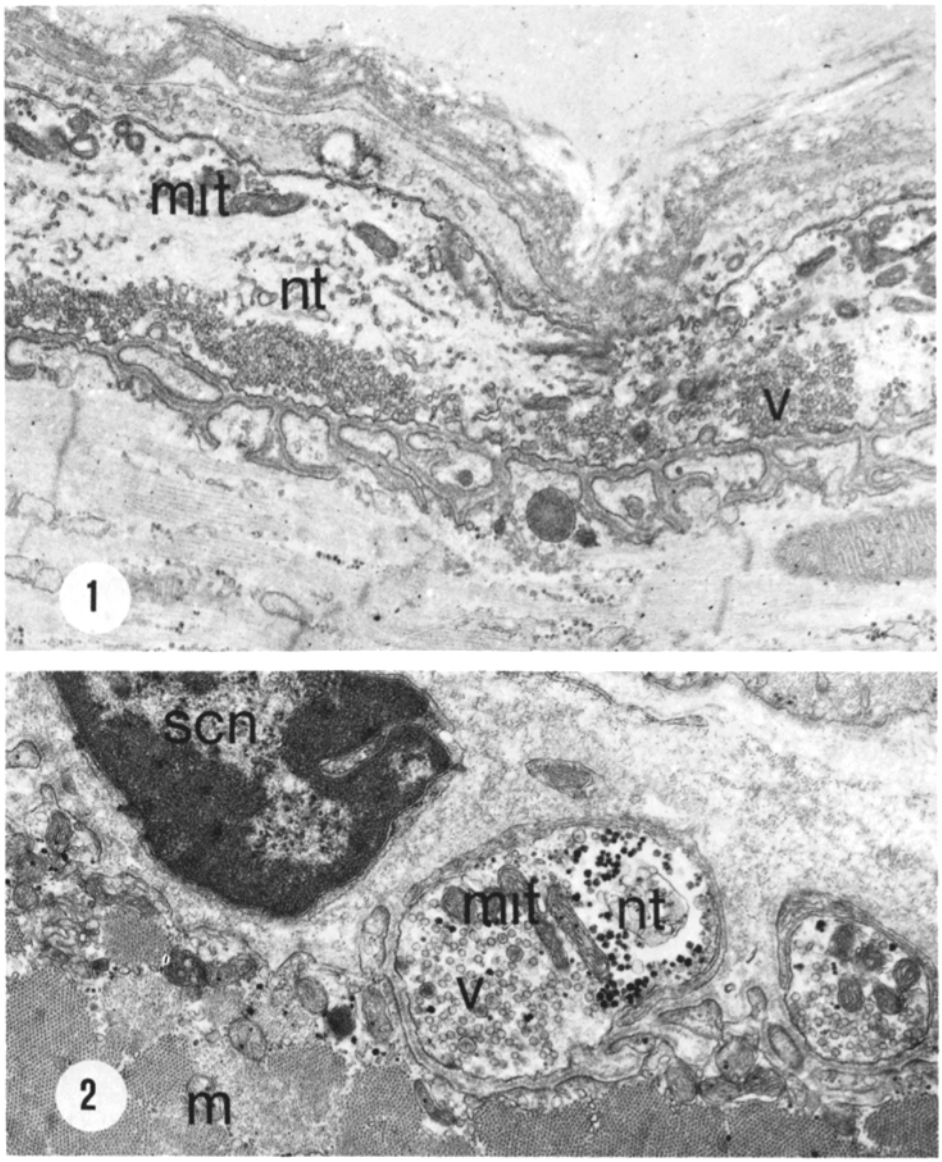
Italian black widow spiders, *Latrodectus mactans tredecimguttatus,* collected outside of Rome, were decapitated and the cephalothoraxes kept frozen at −20°C. When needed four cephalothoraxes were thawed, and the two glands from each of them were pulled out and homogenized in 1 ml of 120 mM Tris-HCl. The homogenate was stored in a cold room at 3°C. Either 50 or 150 μl of the crude homogenate was the standard dose used. When 150 μl was used, it will be indicated in the text. Since each venom gland contains about 2.5 μg of α-latrotoxin, the toxin concentration in the bath was 0.5 or 1.5 μg/ml. Venom activity was routinely tested as described by Frontali et al. (1976).

RESULTS

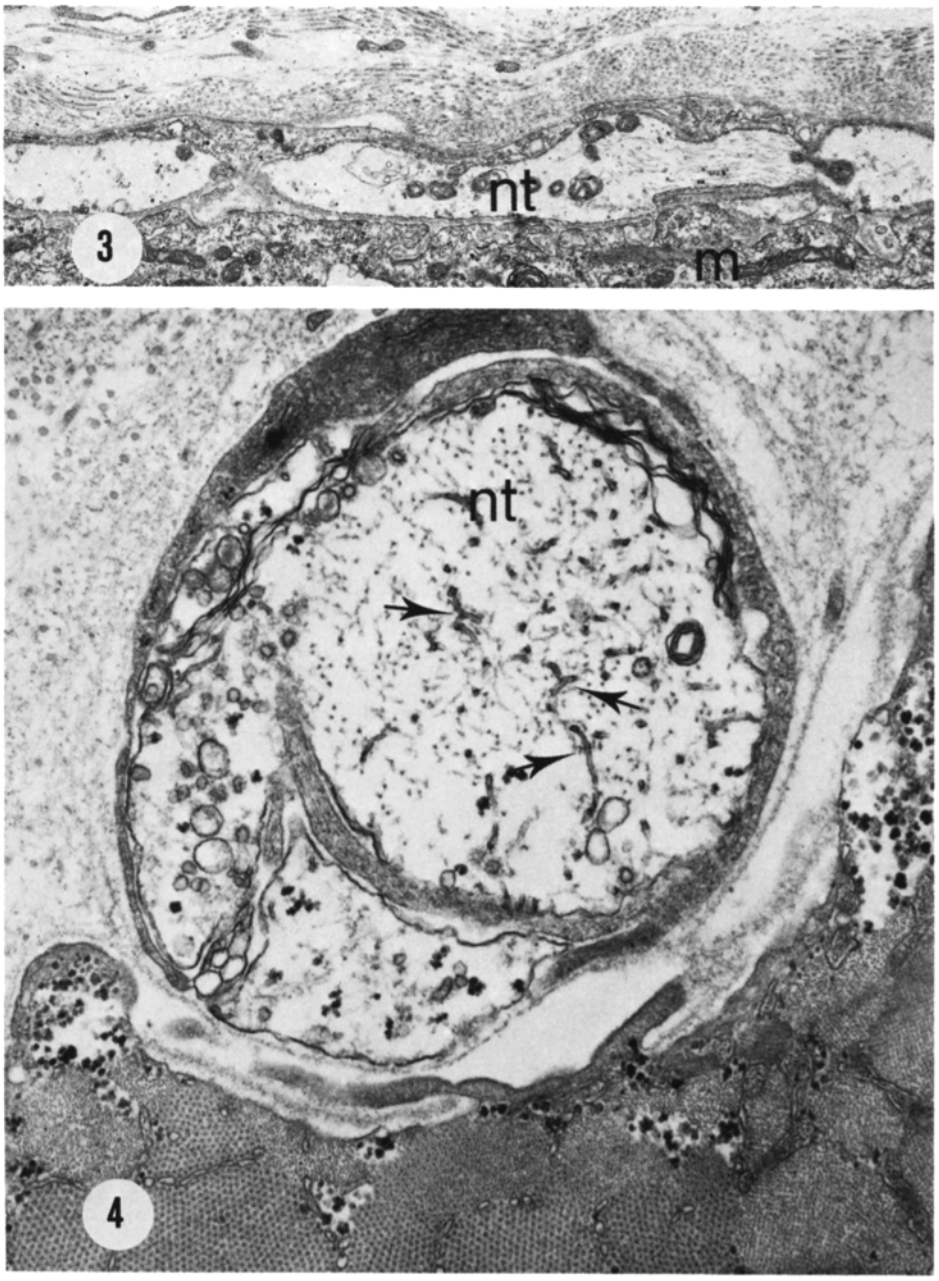
Effects of BWSV in Na^+-Free, Ca^{++}-Free Medium

In the past we have reported that nerve terminals of neuromuscular preparations soaked in a Na^+-free, Ca^{++}-free medium for 75 min are well preserved and the vesicle population is normal (Gorio et al., 1978 *b*). The addition of 1 mM

EGTA to this medium did not alter the result. Figs. 1 and 2 are electron micrographs of sections of two nerve endings from specimens which were bathed for 75 min in a glucosamine Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. The terminals appear normal and contain a full complement of synaptic



FIGURES 1 and 2. Electron micrographs of frog nerve endings (nt). The endings show a normal vesicle (v) distribution and clusters of mitochondria (mit). Where vesicles are absent cytoplasm is filled with glycogen. The preparation was fixed after a 75-min incubation in a glucosamine-Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. (scn) Schwann cell nucleus; (m) myofibrils. Fig. 1: × 12,000. Fig. 2: × 16,500.



FIGURES 3 and 4. Electron micrographs of neuromuscular junctions from a preparation fixed after 60 minutes treatment with BWSV in glucosamine Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. The endings (nt) are depleted of synaptic vesicles. Many coated vesicles are present in Fig. 4. Arrows indicate smooth endoplasmic reticulum; (m) myofibrils. Fig. 3: × 8,000. Fig. 4: × 30,000.

vesicles and mitochondria. Thus incubation in Na^+-free, Ca^{++}-free medium containing 1 mM EGTA did not alter the structure of the nerve endings. This is a sharp contrast to the observations made by Pumplin and Reese (1977) on preparations soaked for 120 min in the same medium. They observed a great loss of synaptic vesicles, which was never apparent in our experiments. Inasmuch as they used glucosamine Ringer's buffered at pH 7.2, and inasmuch as at this pH a large amount of glucosamine is not ionized, we favor the hypothesis that the uncharged glucosamine could diffuse freely through the membrane and create an osmotic imbalance which might have led to the observed loss of vesicles. Our solutions were at pH 6.5 (see Materials and Methods).

Figs. 3 and 4 show sections of nerve endings which were treated with BWSV when bathed in a glucosamine-Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. Preparations were perfused for 15–20 min with this medium to eliminate most of the Na^+ and then treated with BWSV. The nerve terminal is depleted of synaptic vesicles, the mitochondria are fairly well preserved, and the swelling of the ending is limited, all these effects being characteristic of the preparations treated with BWSV in Na^+-free, Ca^{++}-free medium (Gorio et al., 1978 *b*). This result is again in contrast with the observations made by Pumplin and Reese (1977), because when they applied brown widow spider venom in Na^+-free, Ca^{++}-free medium, no depletion of synaptic vesicles was observed. These authors claim that brown widow and black widow spider venoms have a common mode of action, but perhaps this assumption deserves reexamination. The results were even more striking when we looked at cross sections of nerve endings from control and treated preparations. We found that after soaking in glucosamine the nerve endings contained 94 ± 31 vesicles per random cross section, whereas venom treated nerve endings contained only 4 ± 3 vesicles.

On the Reversibility of BWSV Effects

(*a*) In Fig. 5 we have a series of electrophysiological records showing that a preparation treated with BWSV is able to recover and show normal neuromuscular transmission. A muscle fiber is impaled in low-Ca^{++} Ringer's and epp's, evoked by indirect stimulation, are recorded (trace A). Then the preparation is perfused with a Na^+-free, Ca^{++}-free medium and no electrical activity can be recorded since glucosamine is impermeant to the post-synaptic membrane of the frog neuromuscular junction (trace B). After soaking for 20 min in this medium, BWSV is added and left for 1 h. The venom is then washed out with the same medium and the preparation is treated with 50 μl of antivenin for 15 min to inactivate the venom molecules bound to the plasma membrane of the nerve endings. After this treatment the preparation is returned to low-Ca^{++} Ringer's, but no mepp's could be recorded nor epp's evoked by nerve stimulation. The resting potential during the soaking in glucosamine Ringer's was approximately constant around −70 mV, because the medium contained ≈7 mM K (see Materials and Methods); upon returning to Na^+ Ringer's the resting potential recovered to −85 mV. At this stage of the experiment neuromuscular transmission is blocked; however, since we treated the preparation with antivenin there should be no venom molecules actively attached to the nerve endings. Trace C of Fig. 5 shows two small mepp's recorded 30 min after we returned the

muscle to Na^+ Ringer's. With time, mepp's became much more frequent. 1 h after the reappearance of small mepp's, epp's could be evoked by stimulation of the nerve (trace D). The long soaking period in a Na^+-free medium cannot account for the late appearance of the epp's, because, in control experiments after a return to Na^+ Ringer's, mepp's and epp's are recorded in 2–3 min after 75 min in a Na^+-free Ringer's (Rubin et al., 1978). The morphological and electrophysiological aspect of this recovery process is now under detailed examination.

To obtain a reproducible time for recovery to take place, it is very important to follow strictly the described procedure. If antivenin is added after the preparation has been returned to Na^+ Ringer's, the recovery time is very erratic and it may be as long as 6 h. Furthermore, muscles when bathed in regular Na^+ Ringer's and treated with BWSV very rarely show any sign of recovery when

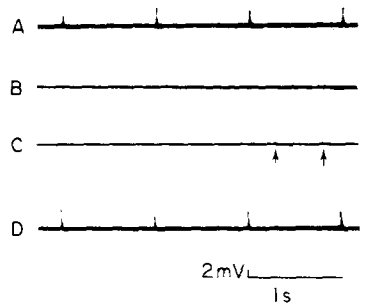


FIGURE 5. Oscilloscope traces from electrophysiological recordings of experiments on the reversibility of BWSV effects. (A) The frog muscle was impaled in low-Ca^{++} Ringer's and epp's were recorded. (B) When glucosamine Ringer's is the bathing fluid no electrical activity can be recorded; see text for details. BWSV is applied for 60 min in this medium, then the preparation is treated with antivenin and successively returned to low-Ca^{++} Ringer's. (C) After 30 min few mepp's are recorded, and (D) only after 90 minutes epp's could be evoked. Arrows indicate miniature endplate potentials. Recording was done with AC coupling.

treated with antivenin. Therefore, it seems that the presence or the absence of Na^+ in the bathing medium during the application of BWSV is related to the reversibility of the toxin effect. Since, in the past we had reported evidence that an increased permeability to Na^+ induced by the toxin was probably responsible for the swelling of the nerve endings (Gorio et al., 1978 *b*), it seems reasonable to postulate that perhaps the same mechanism leads to swelling and to permanent block of the neuromuscular transmission.

In one single case a preparation recovered spontaneously from the venom treatment, namely, after a recovery time of 6 h high frequency bursts of mepp's and epp's could be recorded. This muscle had the peculiarity that the nerve endings were resistant to new applications of BWSV. Even very large doses could not increase the mepp frequency and block neuromuscular transmission. In contrast, the nerve endings of preparations which recovered after the

procedure described previously utilizing antivenin, were again sensitive to BWSV. In this case it is likely that antivenin pulls away venom molecules from a receptor which is now again available, whereas in the spontaneously recovering nerve endings either an inactive venom molecule is still bound to the receptor or the receptor may no longer be present.

(*b*) We have recently reported that the addition of venom to preparations bathed in isotonic K_2SO_4 gave results which were difficult to interpret (Rubin et al., 1978). Because the preparation was totally depolarized during the exposure to K^+, the experiment could not be monitored electrophysiologically while the toxin was applied and only when the muscle was returned to Na^+ Ringer's could electrical activity be recorded. Control preparations bathed for 60 min in K^+ Ringer's recover epp's after a 30–60-min flushing with normal Ringer's. Extensive washing is necessary perhaps because K^+ is trapped inside the perineurium and it takes a long time to wash out. On the other hand, muscles bathed in this medium and treated with BWSV for 60 min show, during the recovery time, high frequency bursts of mepp's and after ~ 1 h some of the muscle fibers show epp's (Rubin et al., 1978). We confirmed these data also using K-propionate instead of sulfate, and adding 1 mM EGTA to the medium (see Materials and Methods). Only electron microscopic analysis of these experiments could have revealed whether venom was able to deplete the nerve endings of synaptic vesicles during the 1 h of exposure to the toxin.

Fig. 6 is an electron micrograph of a nerve ending from a preparation which was soaked for 75 min in K^+ isotonic Ringer's containing 4 mM Mg^{++} and 1 mM EGTA. Synaptic vesicles are clustered near the active zones, but the rest of the cytoplasm is almost devoid ot them. This is the general feature of the end-plates examined.

Figs. 7 and 8 are micrographs of two sections of the same nerve ending from a preparation that, after a 15-min soaking in isotonic K^+ Ringer's containing 4 mM Mg^{++} and 1 mM EGTA, was treated for 60 min with BWSV. The entire nerve terminal including the areas near the active zones is completely devoid of synaptic vesicles, which is as expected if BWSV was able to act in spite of the substitution of Na^+ with K^+.

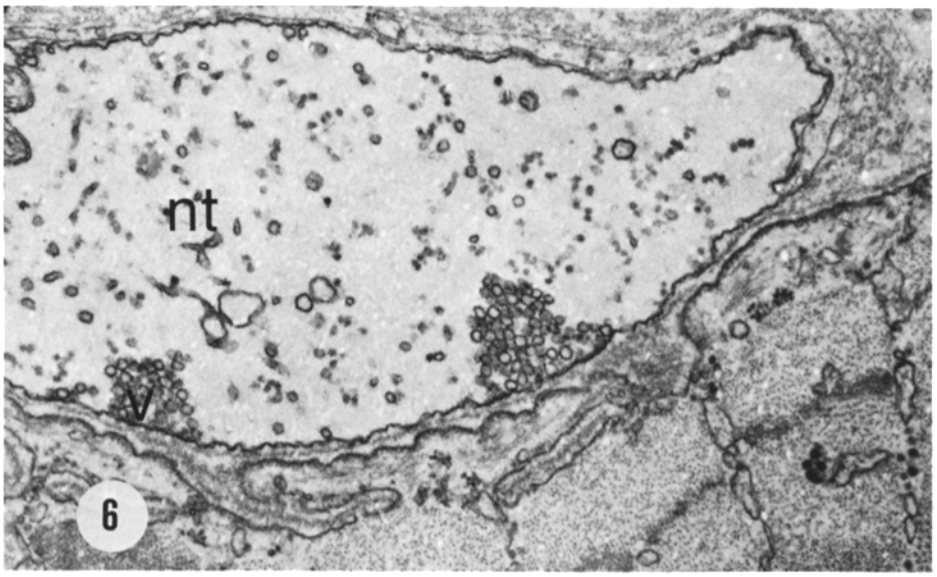
Interestingly, in the control experiments, high-K^+ solutions were able to stimulate vesicle release and to obtain a partial depletion in spite of the presence of 1 mM EGTA.

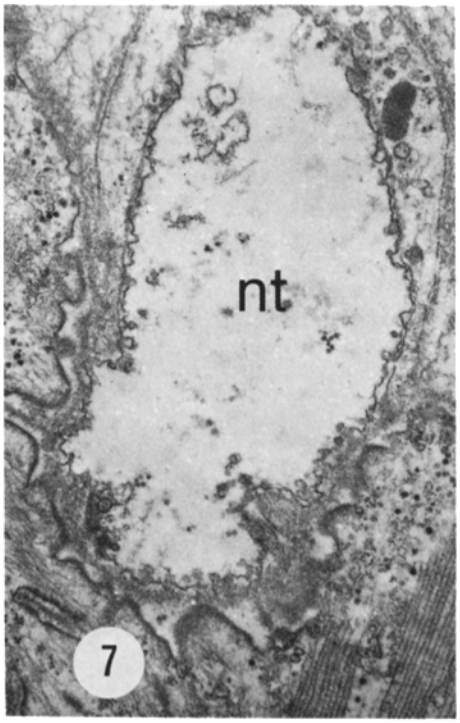
FIGURE 6 (*opposite*). Neuromuscular junctions from a frog preparation fixed after a 75-min incubation in isotonic K^+-Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. The synaptic vesicle (v) population is very reduced and only near the active zones of the nerve terminal (nt) there are two large clusters of vesicles. × 30,000.

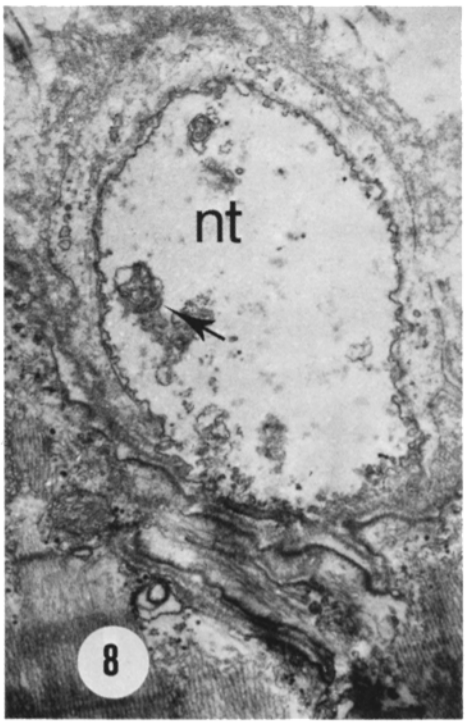
FIGURES 7 and 8 (*opposite*). Electron micrographs of two sections of the same neuromuscular junction from a frog muscle fixed after a 60-min treatment with BWSV in isotonic K^+ Ringer's containing 1 mM EGTA and 4 mM Mg^{++}. The nerve ending (nt) is depleted of synaptic vesicles. The arrow points to a mitochondrion. Fig. 7: × 20,000. Fig. 8: × 16,000.

Divalent Cation Dependency of BWSV Action

We have shown before that BWSV depletes synaptic vesicles in the presence of 1 mM EGTA even in a sodium-free medium, however a small amount of divalent cation (e.g., Mg^{++}) must be present.







If BWSV is applied to a preparation soaked in a medium containing 1 mM EGTA, and if no Mg^{++} is added, in most instances no obvious changes in mepp frequency is observed (see also Ornberg, 1977). Unfortunately, when a muscle is bathed in this medium, spontaneous fibrillations occur and it is very difficult to keep a stable impalement. To overcome this problem the muscles were tightly stretched over the lens situated in the center of the recording chamber, and this greatly improved the impalements. Other investigators have used tetrodotoxin at a concentration of 2×10^{-6} g/ml (Ornberg, 1977) to block excitability. After a 15–20 min soaking in this medium, we incubated the preparation with 150 μl of BWSV for another 15 min. We used this higher dose of venom to optimize the response at low concentration of Mg^{++}. Then Ringer's containing 1 mM EGTA and different concentrations of Mg^{++} were perfused into the chamber. Fig. 9

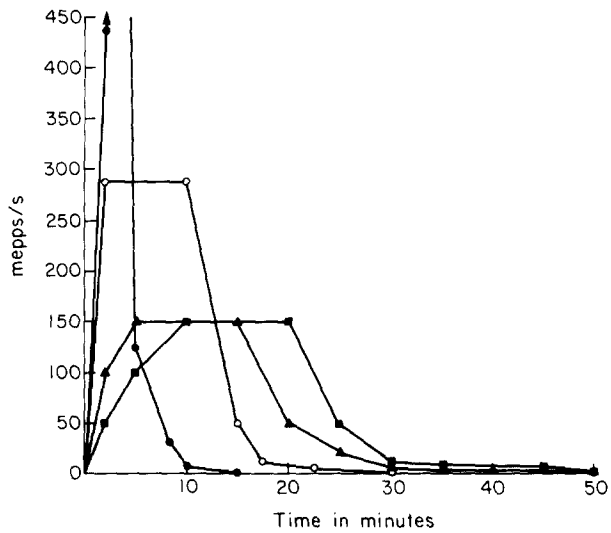


FIGURE 9. Series of curves showing the effects of different Mg^{++} concentrations on the increase of mepp frequency induced by BWSV. Frog preparations were incubated for 15–20 min with BWSV in a Ringer's lacking divalent cations and containing 1 mM EGTA. Then solutions containing Mg^{++} and 1 mM EGTA were perfused into the recording chamber; the observed increase of mepp frequency was dependent on Mg^{++} concentration. With 10^{-5}M (■) and 10^{-4}M (▲) Mg^{++}, the mepp frequency increased up to 150/s in a few minutes but when Mg^{++} was at concentrations of 1 mM (○) or 4 mM (●) the increase was very abrupt. With 4 mM Mg^{++} the frequency increased abruptly several hundredfold and lasted only 4–5 min.

shows the effect of Mg^{++} concentrations ranging from 10^{-5} to 4×10^{-3} M on the venom-induced mepp discharge. With 4 mM Mg^{++} the mepp frequency increased abruptly several hundredfold but subsided within a few minutes. With lower concentrations of Mg^{++}, the increase of mepp frequency was slower but lasted longer. In all cases, after 1 h of treatment with BWSV, the mepp rate was very low, but in the case of 0.1 mM Mg^{++}, if Mg^{++} was raised to 4 mM, a burst of mepp's lasting 20–30 s was always observed. Each point of Fig. 9 is an average of two experiments which were performed with the same venom gland

homogenate. With different homogenates we obtained series of curves with a slightly slower increase in the frequency of mepp's but with the same characteristics. (A single experiment was performed at the 10^{-5} M Mg^{++} concentration and it is shown in Fig. 9.) Probably this difference is due to the different amount of active α-latrotoxin contained in each gland.

In one experiment after addition of BWSV to a preparation bathed in Ringer's with EGTA and no Mg^{++}, the mepp frequency increased to about 150/s. To check if this increase was caused by some calcium contained in the crude homogenate we perfused the chamber with a solution containing 10^{-5} M Mg^{++} and 1 mM EGTA. The mepp frequency continued unchanged for 10 min then slowly subsided to low levels.

In a few experiments we have even used cobalt as a divalent cation. The preparation was incubated with 150 μl of venom in the absence of divalent cations, and was then flushed with solutions containing 1 or 4 mM Co^{++} and 1 mM EGTA. With 1 mM Co^{++} the mepp frequency in 10 min gradually reached the frequency of about 150/s, which was sustained for 12 min then gradually subsided to 5–6/s. If solutions containing 4 mM Co^{++} and 1 mM EGTA were used, the mepp sharply increased to 400/s, similar to the effect observed when 4 mM Mg^{++} and 1 mM EGTA were used.

Osmolarity and BWSV

When a neuromuscular preparation is soaked in a hypertonic medium the mepp frequency increases by several fold and is sustained for several minutes, then it subsides and the nerve endings are depleted of vesicles (Clark et al., 1970). In Table I we show the effects of Con A and colchicine on the mepp

TABLE I

EFFECTS OF CON A AND COLCHICINE ON THE MEPP FREQUENCY INDUCED BY 50% HYPERTONIC MEDIUM

	After application of Con A		
Control	Dissolved in Ringer's	Dissolved in 1 M NaCl*	After application of colchicine and Con A
	miniature endplate potentials per second		
69±39 (14)	78±20 (5)	5±4 (5)	54±30 (4)

Number in parentheses is number of trials.

*The inhibitory action occurs only after Con A has first been dissolved in 1 M NaCl, a solution of relatively high ionic strength as compared with Ringer's. Undoubtedly, high ionic strength serves to enhance the solubility of Con A and, presumably, to minimize the tendency for Con A to aggregate.

frequency induced by hypertonic Ringer's. In these experiments sucrose was added to low-Ca^{++} medium to increase the osmolarity by 50%. When a stable impalement was obtained and mepp's recorded, the hypertonic Ringer's was then perfused into the recording chamber and 5 min later the mepp's were recorded. In 14 experiments the rate was 69 ± 39 mepp's/s. The strategy of the experiments was to reapply the hypertonic medium after the preparation had

been soaked for 1 h with 300 μg/ml of Con A. When Con A was dissolved in 1 M NaCl and applied directly to the bath, the next increase in osmolarity failed to raise mepp frequency to the previous level. However if Con A was dissolved in Ringer's and applied, no protection was observed (Table I). In five experiments the mepp frequency recorded was 78 ± 20 per second. The application of Con A, even if dissolved in 1 M NaCl, does not affect the epp amplitude and the K^+-induced release, as previously reported (see Rubin et al., 1978). When preparations were treated, before application of Con A dissolved in 1 M NaCl, with 10^{-4} M colchicine the response to the second application of the hyperosmotic medium was almost normal, namely 54 ± 30 mepp's/s. It must be noticed also that BWSV action was prevented by Con A and that colchicine reversed the effect of Con A (Rubin et al., 1978). Neither Con A nor colchicine had any striking effect on the resting mepp frequency which was identical before and after treatment.

In the previous sections we have described that when BWSV is applied to a preparation bathed in divalent cation-free Ringer's containing 1 mM EGTA in most of the cases no increase in mepp frequency is observed. In addition, the rate of mepps induced by hypertonic medium lacking divalent cations decreased from 69 ± 39 to 18 ± 8/s (n = 4). However, if BWSV and the hypertonic medium are applied together, a synergistic effect is observed (Misler and Hurlbut, 1978). In Fig. 10 we show three curves from experiments performed

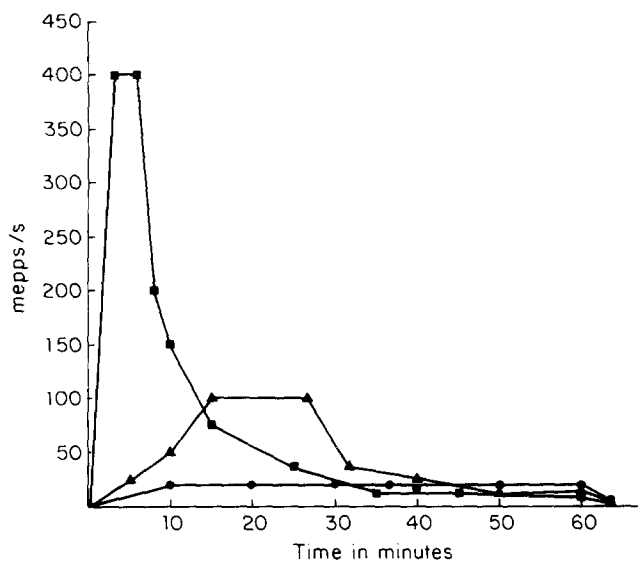


FIGURE 10. In this figure we show two examples of the effects of hypertonic medium on BWSV action. Frog preparations were bathed in Na^+-Ringer's lacking divalent cations and containing 1 mM EGTA. When the same medium with the tonicity increased 50% (●) was flushed into the chamber, the mepp frequency rose to 18 ± 8 and was sustained for all the time of exposure. However, if BWSV was added to the soaking medium the perfusion with the hypertonic solutions gave a wide spectrum of mepp frequency increase, ranging from a slow rise to 100/s (▲) to a very abrupt increase to ~ 400/s (■). See text for more details.

with BWSV and hypertonic medium. In these experiments preparations were bathed for 15–20 min in a medium lacking divalent cations containing 1 mM EGTA and then BWSV was added. After 15 min the same Ringer's with osmolarity raised by 50% was flushed into the recording chamber. We obtained a wide spectrum of increase from fiber to fiber extending from a slow rise in mepp frequency to 100/s which was sustained for ~ 10 min, to a very abrupt increase to ~ 400/s which lasted a few minutes before subsiding.

Temperature and BWSV

The mouse diaphragm was used to study the effect of temperature on the action of BWSV in the range of 20–30° C. Treatment of mouse diaphragms with BWSV leads to the same series of events already described for frog neuromuscular junctions, namely, the mepp frequency transiently increases and after 1 h the neuromuscular transmission is blocked and the nerve endings are depleted of vesicles (Gorio et al., 1978 *a*). The absence of divalent cations from the bathing medium inhibits venom action at room temperature 20–22° C; however, at 36° C normal venom effect is observed. To determine the critical temperature, at which BWSV can trigger release in spite of the absence of divalent cations, we constantly perfused the diaphragm for 20 min with a Krebs' lacking divalent cations and containing 1 mM EGTA. Then the flow was stopped and 50 μl of BWSV added into the recording chamber. After 15 min incubation the temperature of the bath was raised at a rate of 0.33° C per minute. In four of our four experiments it was found that the critical temperature for the onset of activity was between 30 and 31° C. The increase in mepp frequency could be relatively slow reaching a peak in 3–4 min or fast as shown in Fig. 11, where in 30 s the mepp frequency rose from <1/s to >400/s. In one experiment the temperature

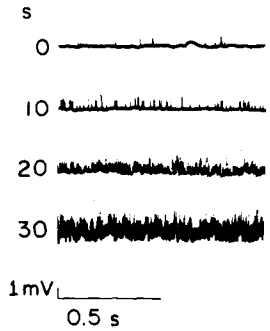


FIGURE 11. Oscilloscope traces from electrophysiological recordings of experiments on the effects of temperature and BWSV. Mouse diaphragm neuromuscular preparations were bathed at room temperature in a medium lacking divalent cations and containing 1 mM EGTA for 20 min. Then 50 μl of BWSV were added and after 15 min the temperature rose. At 30.5° C the mepp frequency increased in 30 s from a few seconds to >400/s. The slow fluctuation shown at time 0 s was probably due to muscle contractions induced by the lack of divalent cations. Recording was done with AC coupling.

was raised at the rate of 1° C per minute until 32° C was reached, whereupon the mepp frequency jumped from a few to several hundreds per second without an intermediate increase. This behavior suggested that all the release sites were activated simultaneously.

To exclude the possibility that an increase in permeability to Na^+ could be responsible for the venom activity at higher temperature, some experiments were performed using glucosamine as a sodium substitute (see Materials and Methods). We perfused the preparation for 20 min at 36° C with the glucosamine-containing and divalent cation-free Krebs', then we added venom and allowed the preparation to cool down to room temperature over a period of 45 min. (In this procedure, venom is in contact with the preparation at temperatures above 30° C for 25–30 min.) Then cold divalent cation-free, Na^+ Krebs' was perfused into the chamber, whereupon only few mepps were recorded in three experiments; moreover, addition of Ca^{++} failed to increase mepp frequency in three experiments. In the control experiments BWSV was not added. Immediately after the reappearance of mepp's in the divalent cation-free, Na^+ Krebs', normal Krebs' was reintroduced into the recording chamber and a few minutes later the BWSV was applied. A normal venom effect was observed. These experiments showed that BWSV can stimulate release even in the absence of Na^+ and divalent ions at higher temperatures.

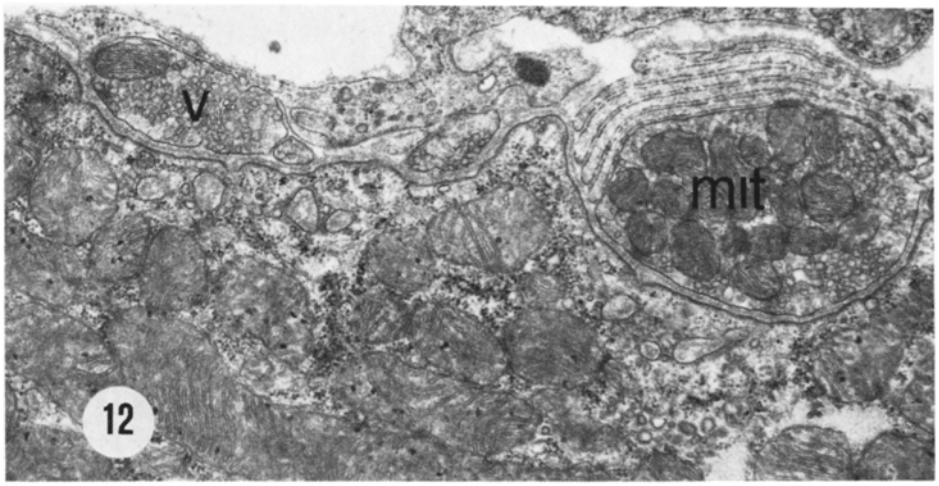
These observations were corroborated by a morphological examination of the control and venom-treated preparations, which, after attaining room temperature were fixed and processed for electron microscopy. Fig. 12 is an electron micrograph of a cross section of a nerve ending from a control muscle which contains a good complement of vesicles, whereas the venom preparation shown in Fig. 13 is devoid of them.

DISCUSSION

Further confirmation has been obtained that α-latrotoxin, the component of BWSV active at the vertebrate neuromuscular junction, depletes synaptic vesicles and blocks neuromuscular transmission by a mechanism which may not involve induced permeability changes. Moreover, there are strong suggestions that venom may act by inducing a redistribution of the membrane components, which results in a change of the relationship between plasma membrane and cytoplasmic structures leading to vesicle fusion.

BWSV can stimulate release even in Na^+- and Ca^{++}-free medium. As a Na^+ substitute we used D-glucosamine, which is impermeant to the postsynaptic membrane and to the venom-induced channels in black lipid membranes. The reduction in size and disappearance of any electrical activity during the introduction of glucosamine Ringer's into the recording chamber can be used as a parameter to monitor the time-course of the substitution of Na^+ with glucosamine. If Na^+ Ringer's is substituted with one which consists of 50% NaCl and 50% glucosamine-HCl, the amplitude of mepp's decreases to ~ 50% (Misler, 1976). In our experiments electrical activity is abolished from superficial fibers in < 1 min of perfusion with Na^+-free solution, indicating rapid elimination of Na^+ from the synaptic cleft. Therefore, it seems clear that perfusion for 15–20 min is sufficient to eliminate most of the Na^+ from the

extracellular space or at least to reduce it to a concentration comparable to that within the cytoplasm. Because of the presence of EGTA and the very reduced concentration of Na^+, we can conclude that in this condition venom does not stimulate release by increasing Ca^{++} or Na^+ entry.



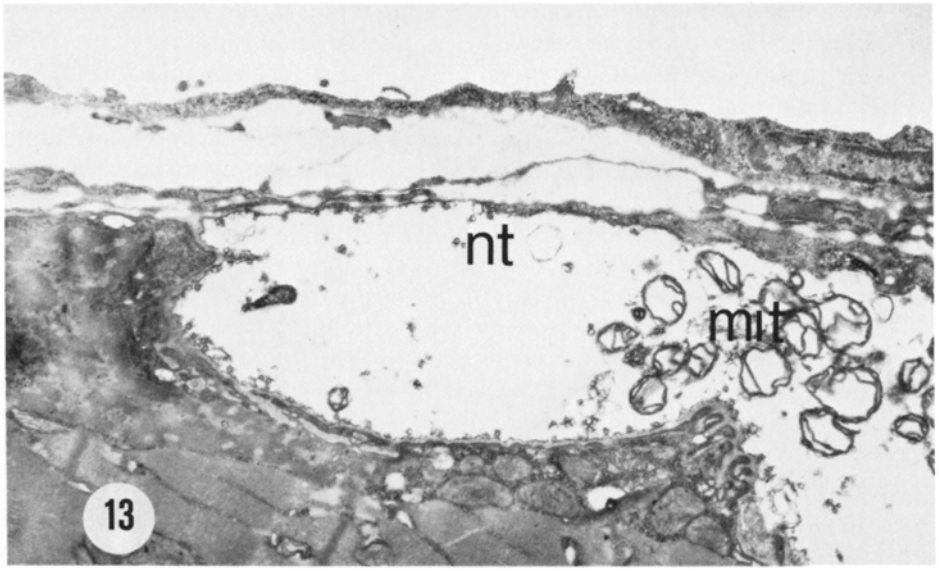


FIGURE 12. Section of nerve ending from mouse diaphragms fixed after having been incubated in glucosamine Krebs' lacking divalent cations. (See Results for details). The nerve terminal is well preserved and contains a good number of vesicles (v) and mitochondria (mit). × 17,500.

FIGURE 13. Section of endplate from mouse diaphragm fixed after treatment with BWSV while incubated in glucosamine Krebs' lacking divalent cations (see Results for details). The nerve ending (nt) is depleted of vesicles and contains mitochondria (mit). × 10,000.

It has been suggested that an increase in cytoplasmic Na^+ concentration can displace Ca^{++} from intracellular stores leading to increased vesicle release (Lowe et al., 1976; Alnaes and Rahamimoff, 1975). Another suggested way to displace intracellular Ca^{++} is to decrease intracellular K^+ (Baker and Crawford, 1975), which could be induced by toxin channels in the presence of Na substitutes. But we have shown that BWSV is able to deplete the endings of synaptic vesicles even when K^+ is substituting for Na^+ therefore, even this hypothesis should be discarded.

On the other hand, our evidence indicates that indeed an increase in nerve terminal permeability does occur during BWSV treatment. Nerve endings treated with large doses of venom swell enormously, but if Na^+ was substituted with glucosamine no swelling was observed (Gorio et al., 1978 *b*). Furthermore, it seems that the presence of Na^+ in the bathing medium is strongly related to the irreversibility of the venom effect. All these data suggest that the swelling and irreversible block of the neuromuscular transmission are due to the same mechanism, namely, an increase of the Na^+ permeability induced by the toxin.

The fact that mitochondria of nerve endings from preparations bathed in a Ca^{++}-containing medium and treated with BWSV are enormously swollen also suggests that venom increases the terminal Ca^{++} permeability (Smith et al., 1977). Soaking in Ca^{++}-containing Ringer's without treatment with the toxin does not change mitochondrial shape (Gorio et al., 1978 *b*), and mitochondria are known to swell as a consequence of Ca^{++} uptake.

Another interesting aspect of BWSV's mode of action is the requirement for divalent cations in the bathing medium. If venom is added to a preparation bathed in a medium lacking divalent cations and containing 1 mM EGTA, only on a few occasions was an effect observed. However, the addition to this medium of 0.1 mM Mg^{++}, which corresponds to a concentration of about 5×10^{-7} M free Mg^{++} (Schmid and Reilly, 1957), consistently led to increased mepp frequencies. If the Mg^{++} concentration in the bath is increased there is a parallel increase in mepp frequency; with 4 mM Mg^{++} the discharge is very vigorous and lasts only a few minutes. This much stronger stimulation obtained with 4 mM Mg^{++} might be explained by an influx of Mg^{++} into the nerve ending, which was suggested by Hurlbut et al. (1971) to explain the Mg^{++} dependence of the increase in mepp frequency induced by tetanic stimulation of the nerve in EGTA-containing medium. In a study recently completed, Misler and Hurlbut[1] obtained convincing evidence for the correlation of mepp frequency with the electrochemical potential difference of Mg^{++} across the frog nerve terminal membrane in preparations treated with BWSV. Thus, the results presented here and those of Ornberg (1977) and of Misler and Hurlbut[1] support the hypothesis that one of the modes of action of BWSV is to increase permeability of the nerve terminal to Mg^{++} and also to other divalent cations.

In a previous report we showed that if preparations are bathed in a medium containing low concentrations of concanavalin A (Con A) The BWSV action is

[1] Misler, S., and W. P. Hurlbut. Action of black widow spider venom on quantitized release of acetylcholine at the frog neuromuscular junction: dependence upon external Mg^{2+}. *Proc. Natl. Acad. Sci. U. S. A.* In press.

inhibited; however, if the preparation was preincubated with colchicine the full effect was observed (Rubin et al., 1978). This led to the suggestion that perhaps venom stimulates release and inhibits recycling of vesicles by a mechanism involving a redistribution of the membrane components similar to patching and capping of lymphocyte surface receptors exposed to multivalent antibodies. Yahara and Kakimoto-Sameshima (1977) have reported that surface receptors of mouse lymphocytes or thymocytes redistribute into caps if exposed to a hypertonic medium of ~ 600 mM. This effect is strongly inhibited by Con A, and the inhibition is reversed by colchicine. In Fig. 10 we show that BWSV inhibition caused by the absence of divalent cations can be reversed if the osmolarity of the medium is raised by 50%. These observations further suggest the involvement of a rearrangement of surface molecules in BWSV action. We feel that this correlation is significant. In this regard, we note that Con A is able to inhibit not only BWSV but also the increase of mepp frequency induced by a hyperosmotic medium which is also reversed by colchicine. If divalent ions are absent both stimulatory effects are reduced, particularly, that due to BWSV. However, if applied together they have a synergistic effect. This suggests that perhaps in both cases the same basic mechanism is involved in stimulating release.

A strong indication that the lack of divalent cations may interfere with the mobility of the membrane components comes from the experiments performed with mouse diaphragms. In these experiments a higher temperature (30–31° C) overcomes the block of venom action induced by the lack of divalent ions at room temperature perhaps by increasing the membrane fluidity. The possibility, that the observed effect may be due to an increase of Na^+ permeability is excluded by the electrophysiological and morphological experiments with glucosamine. The lack of Na^+ and divalent cations again suggest that the venom stimulatory effects may be mediated by a rearrangement of membrane components. Singer (1976) has suggested that the clustering of receptor molecules induces a stress in the membrane which, through a critical series of changes, may activate an actomyosin-like system attached to the cytoplasmic face of the membrane. Following this contractile process an endocytosis will occur. Indeed it was reported that in lymphocytes the formation of patches and of caps always leads to the formation of subpatches or subcaps containing actin and myosin (Burguignon and Singer, 1977). Moreover, it was shown that during BWSV action vesicles fuse near the active zone (Ceccarelli et al., 1976) and that, from our preliminary autoradiographic studies, α-latrotoxin binds to the nerve endings at the active zones.[2] Therefore we would propose that because of the nature of the active zones, which appear to be in a fixed position, contraction of actomyosin filaments could pull vesicles towards the plasma membrane, leading to exocytosis.

CONCLUSION

Venom from Italian black widow spiders, *Latrodectus mactans tredecigmuttatus*, is able to stimulate release, to inhibit vesicle recycling and to block permanently

[2] Gorio, A., M. C. Tzeng, and P. Siekevitz. Unpublished observations.

the transmission at frog neuromuscular junctions by a dual mechanism. BWSV increases the permeability of the nerve ending plasma membrane to cations (e.g., Na^+, Ca^{++}), but also may induce a redistribution of the membrane components, which results in a change of the relationship between plasma membrane and cytoplasmic structures leading to vesicle fusion. However, these two phenomena occur simultaneously and therefore in standard conditions (namely, if Ca^{++} is present), stimulation of release may be evoked by both mechanisms. If a single venom molecule is acting simultaneously in both ways or if there are two binding sites, one near the active zone to directly stimulate release and the other one in lipid patches of the ending plasma membrane acting as an ionophore, must still be determined. It is hoped that autoradiographic experiments in progress will give an answer to these questions.

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