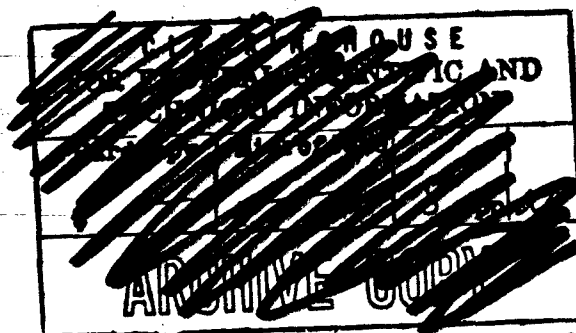


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PARSONS, RODNEY L., W. W. HOFMANN, AND GEORGE A. FEIGEN. *Mode of action of tetanus toxin on the neuromuscular junction.* *Am. J. Physiol.* 210(1): 84-90. 1966.—Studies on the source of the activated spontaneous discharge of miniature end-plate potentials (MEPP's) by tetanus toxin indicate that the MEPP's are those whose release mechanism is both calcium- and voltage-dependent. The potentiation of spontaneous release by toxin did not occur in the absence of calcium or magnesium; it was enhanced by increasing the calcium concentration. These results, together with the observation that depolarization by potassium blocked or reversed the peripheral effect of tetanus toxin, suggest that the toxin acts by lowering the presynaptic resting membrane potential. Studies of the direct effect of toxin on neuromuscular transmission in the rat phrenic nerve-diaphragm preparation indicate that the toxin decreases the probability of transmitter release by a nerve spike, the rate of initial transmitter depletion, and the level of sustained transmitter output.

miniature end-plate potentials; spontaneous neuromuscular activity; neurotoxin; neuromuscular transmission; bacterial toxins; transmitter release; transmitter depletion; intracellular potentials

BROOKS, CURTIS, AND ECCLES (1) have suggested that the site of the central paralytic action of crystalline tetanus toxin is at the synaptic junctions between the specific interneurons of the inhibitory pathway and the motoneuron, and that the characteristic paralysis results from blockade of spinal inhibition. The mode of chemical action is not known, but studies by van Heyningen (26, 27) on the nature of the chemical receptor substance have shown it to be a water-soluble ganglioside which exists in nervous tissue as water-insoluble complexes with cerebrosides and sphingomyelin. It is also significant that the centrally acting "tetanospasmin" can be inactivated by adsorption with these cerebroside-ganglioside complexes.

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Recent studies in this laboratory (10) have shown that impure tetanus toxin has, in addition to the classical central effect, a direct action on the skeletal-neuromuscular junction. The peripheral effect was recognized by an increase in the frequency of spontaneous miniature end-plate potentials (MEPP's) as recorded intracellularly from the intercostal muscles of the mouse. It was also shown from a comparison of the effect of toxin at various temperatures that an additional activation energy of 7 kcal is required for the MEPP-potentiating action of toxin. Since adsorption of the centrally acting tetanospasmin on protagon removed 98% of the central paralytic power but left the peripheral action unaltered, it was evident that the peripheral effect was caused by a principle distinct from tetanospasmin, although the effects of both components could be blocked by curare and by specific equine antitoxin. The change in MEPP frequency at 37 C caused by toxin thus far has appeared to be limited to an increase of approximately 50%.

The object of the present studies was to determine whether the MEPP's affected by toxin are of the kind whose release is dependent on the presynaptic membrane potential (9), and to assess the effects of toxin on impulse transmission when the probability of transmitter release is varied.

MATERIALS AND METHODS

Tissue Preparation

The electrophysiological assay techniques on the mouse intercostal preparation have been described previously (10). In the present study male Swiss-Webster mice, averaging 20 g in weight, were killed by decapitation; the thoracic cage was removed quickly and one-half of it mounted in a Perspex clamp, which was then positioned in a muscle bath (24) containing test solution. After an equilibration period of 20 min, MEPP's were recorded from 10 to 15 cells. Toxin was then introduced and after a lapse of 10 min the recording procedure was repeated.

Studies of the direct effect of tetanus toxin on neuromuscular transmission were performed in vitro on the rat

diaphragm-phrenic nerve preparation (2). For these experiments rats of the Wistar strain, unselected as to sex, weighing between 200 and 300 g were used. The left hemidiaphragm with the phrenic nerve intact was removed under profound ether narcosis and the animal sacrificed. The muscle strip and attached nerve were immediately immersed in well-oxygenated physiological solution and pinned under slight stretch on a paraffin holder which was then transferred to the muscle bath. Recordings of EPP's were made when neuromuscular transmission was partially blocked, either by 10 mM magnesium and 1.5 mM calcium or by curare in concentrations ranging from 1 to 5×10^{-7} g/ml. The quantum content of the EPP's was determined in one of two ways according to the conditions of the experiment: in the presence of high concentrations of magnesium, the quantum content (m) was determined by the ratio of the mean EPP amplitude to the mean MEPP amplitude (5, 18); in curare it was determined from the ratio of the variance to the mean EPP amplitude in trains of impulses (23).

Toxin

A partially purified preparation of tetanus toxin (TD594B) was obtained as a lyophilized powder from Dr. R. O. Thomson of the Wellcome Research Laboratories, Beckenham. Preparation TD594B contained 4×10^6 mouse LD₅₀/mg; it was 42% protein and had 278 Lf U/mg protein. Ultracentrifugal analysis showed three distinct components in the crude preparation, the major component having a sedimentation constant of 7S (Svedberg units) and two minor components having sedimentation constants of 4S and 13S. For electrophysiological testing the toxin was dissolved in 0.15 M phosphate buffer (pH 7.0) containing 0.1% (w/v) gelatin. The concentration of toxin used in these studies, between 10^{-4} and 10^{-3} mg/ml, was sufficient to produce a maximal stimulation of MEPP frequency.

TABLE 1. Influence of potassium on peripheral action of tetanus toxin at 37°C

mmoles [K] ⁺	Approx. Membrane Depolarization,* mv	MEPP Freq.† Before Toxin, sec ⁻¹	MEPP Freq.‡ After Toxin, sec ⁻¹	%Δ	N
3.0	3.5	6.72 ± 0.90	9.89 ± 0.78	+47.2	9
5.0	6	9.13 ± 0.64	13.66 ± 1.21	+49.6	13
10.0	11	22.26 ± 1.00	23.58 ± 2.39	+5.9	9
15.0	22	248.28 ± 15.58	181.96 ± 11.36	-26.7	6
20.0	30	742.55 ± 114.97	500.10 ± 59.68	-32.7	4

* Values taken from Croonian Lecture by Katz (16).

† Mean frequency $\pm$ SE computed from average frequencies of N preparations. ‡ In the presence of 1.19×10^{-3} mg/ml tetanus toxin (TD594B) final bath concentration.

Apparatus

Resting membrane potentials and transients were recorded with KCl-filled micropipettes connected by an Ag-AgCl bridge to the input of a cathode follower circuit (24). The potentials were displayed on a Tektronix (Tektronix Inc., Portland 7, Ore.) 502 twin-beam oscilloscope and photographed with a Grass (Grass Instrument Co., Quincy, Mass.) oscilloscope camera. When required, EPP's were elicited by stimulation of the phrenic nerve with repeated supramaximal square-wave stimuli delivered either by a Grass S4 stimulator or by an American Electronic Laboratories (Colmar, Penna.) 104-2 stimulator, at frequencies of either 25 or 30 cycles/sec for 1-2 sec.

Solutions

The tissues were maintained in a continually renewed physiological solution as described by Liley (17). The perfusion fluid was constantly circulated and was saturated with 95% O₂ + 5% CO₂ by means of a lift-pump system similar to that described by Szekeres and Vaughan Williams (25). The temperature was controlled by means of a Techne (Techne (Princeton) Ltd., Princeton, N. J.) Tempunit TU8, and unless otherwise indicated all experiments were carried out at 37.0 ± 0.1 °C.

The addition of MgCl₂, CaCl₂, and KCl was compensated for by omitting an osmotically equivalent amount of NaCl from the medium (12). Substitution of NaNO₃ and of NH₄Cl for NaCl was made on an equimolar basis.

RESULTS

Source of Activated Spontaneous Discharge

Specificity of activation. The possibility that the increased MEPP frequency observed in the presence of toxin was due to contaminating cations such as K⁺ or Ca⁺⁺ was eliminated in the previous study (10). In the present study we have ruled out the nonspecific influence of proteins by testing bovine gamma globulin and bovine

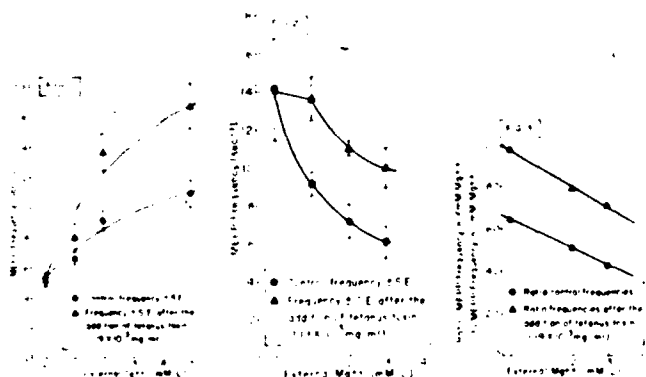


FIG. 1. Potentiation of the peripheral action of tetanus toxin by calcium.

FIG. 2. Influence of magnesium on the frequency of the spontaneous discharge.

FIG. 3. Depressant effect of magnesium on the peripheral action of tetanus toxin. The ratio of the MEPP frequency in x mM Mg⁺⁺ to the MEPP frequency in 0 mM Mg⁺⁺ is expressed as a function of the external magnesium concentration.

serum albumin in concentrations ranging from 10^{-4} to 10^{-1} mg/ml, and tetanus toxoid in a concentration range of 10^{-3} to 10^{-2} mg/ml. None of these substances had any measurable effect on the random discharge of MEPP's.

Influence of calcium and magnesium ions. Calcium and magnesium ions act antagonistically on the frequency of spontaneous discharge at the mammalian myoneural junction. Increasing the calcium ion concentration augments the frequency of MEPP's, whereas raising the magnesium ion concentration depresses the spontaneous rate of discharge (13). Magnesium acts solely by reducing the potentiating effect of calcium, but in itself it is without influence on MEPP frequency (13). The same antagonism is clearly seen in relation to any agent which increases the MEPP frequency by depolarizing the presynaptic membrane. Consequently depolarization-coupled potentiation of MEPP frequency becomes lost in the absence of calcium, augmented by increasing the calcium concentration above normal levels, and depressed drastically by high concentrations of magnesium (6, 19). Considering the importance of these two cations, the potentiating effect of toxin was studied while the calcium and magnesium ion concentrations were varied independently in the range 0–5 mM for calcium and 0–3 mM for magnesium.

The mean frequency of MEPP's increased as the calcium concentration was increased in the range 0–5 mM: the average frequencies were $5.31 \pm 0.39 \text{ sec}^{-1}$ (5 preparations—ca. 60 fibers), $9.13 \pm 0.64 \text{ sec}^{-1}$ (13 preparations), and $10.90 \pm 0.89 \text{ sec}^{-1}$ (5 preparations) at calcium concentrations of 0, 2, and 5 mM, respectively. The 42% reduction of MEPP frequency when the normal 2 mM calcium solution was replaced by calcium-free medium is similar in magnitude to that previously observed (13). From an examination of Fig. 1 in which the MEPP frequency is expressed as a function of the calcium concentration, it is seen that the toxin exerted

TABLE 2. Interaction between NH_4^+ and tetanus toxin on mammalian miniature end-plate potentials at 37°C

	Mean RP, mv	MEPP Freq. $\pm$ SE, sec^{-1}	MEPP Ampl. $\pm$ SE, mv	No. of Fibers
Control	60.6	8.33 ± 0.80	0.58 ± 0.04	23
10% NH_4^+	58.7	10.27 ± 1.02	0.40 ± 0.02	27
10% NH_4^+ + toxin†	58.1	11.61 ± 1.33	0.32 ± 0.02	29
Control	62.3	10.08 ± 0.71	0.42 ± 0.03	24
25% NH_4^+	44.1	123.07 ± 18.04	0.20 ± 0.02	17
25% NH_4^+ + toxin†	41.6	55.89 ± 12.54	0.19 ± 0.02	15

* Control measurements in Liley's solution; test measurements in Liley's solution in which either 10% or 25% of NaCl is replaced by NH_4Cl . † Toxin (TD594B) added to give final bath concentration of 1.19×10^{-3} mg/ml.

no effect on MEPP frequency when calcium ions were omitted from the perfusion fluids. However, when calcium was introduced and its concentration increased, the effectiveness of the toxin was potentiated. For a given maximal dose of toxin, the increase in MEPP frequency was 10% in 1 mM calcium, 50% in 2 mM calcium, and 53% in the solution containing 5 mM calcium.

An examination of the control MEPP frequencies recorded in various concentrations of magnesium as shown in Fig. 2 shows an inverse relationship between frequency of discharge and external magnesium concentration (calcium concn 2 mM/liter). By progressively increasing the magnesium concentration from 0 to 1, 2, and 3 mM, the mean rate of spontaneous activity was reduced from $14.14 \pm 2.67 \text{ sec}^{-1}$ (8 preparations) to 9.13 ± 0.64 , 7.20 ± 0.91 , and $6.11 \pm 0.85 \text{ sec}^{-1}$, respectively. This magnesium-induced depression of the MEPP frequency is greater than that described by Hubbard in 1961 (13); his results, however, were obtained from the rat phrenic nerve-diaphragm preparation, and the average reference MEPP frequency observed in the absence of magnesium (4.2 sec^{-1}) was considerably less than that in the mouse intercostal preparation (14.1 sec^{-1}) used in the present study.

From Fig. 2 it is evident that magnesium is essential to the initiation of the toxin-activated MEPP discharge; there was no acceleration of MEPP discharge by toxin when magnesium was omitted from the external solution. In Fig. 3 the ratio of MEPP frequency in x mM magnesium to MEPP frequency in 0 mM magnesium is plotted as a function of the external magnesium concentration (x) for control and toxin-treated tissues. If the action of toxin had been potentiated by an increase in the magnesium concentration, the control and toxin lines should have diverged. Although the two lines appear to converge, the standard errors of the frequencies as shown in Fig. 2 are such that no definite conclusion regarding the relationship between magnesium concentration and toxin potentiation of MEPP discharge can be drawn.

Effect of presynaptic depolarization. The purpose of this series of experiments was to determine the effectiveness of a constant dose of toxin after the nerve terminals had been depolarized either by increasing the external

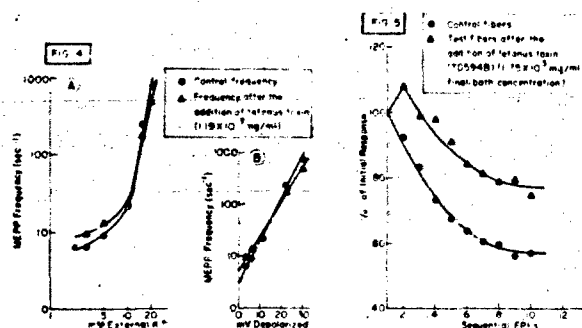


FIG. 4. Interaction between potassium and tetanus toxin on the rate of spontaneous discharge. A: MEPP frequency plotted as a function of the external potassium concentration (logarithmic coordinates). B: spontaneous discharge rate expressed as a logarithmic function of the depolarization produced at the various concentrations of potassium.

FIG. 5. Influence of toxin on the rate of initial transmitter depletion. Each point represents average of several fibers plotted as percent of mean initial end-plate potential amplitude; stimulation rate 50 cycles/sec.

TABLE 3. *Effect of nitrate on MEPP frequency in tetanus toxin at 37 C*

% NO ₃	Control Freq., sec ⁻¹	Test Freq.,* sec ⁻¹	%Δ	No. of Prep.
0	9.13 ± 0.64	13.66 ± 1.21	49.6	13
25	10.53 ± 1.37	16.01 ± 1.94	52.0	4
50	11.01 ± 0.98	16.33 ± 1.88	48.1	5
100	11.24 ± 1.35	17.28 ± 2.11	53.7	4

* In the presence of 1.194×10^{-2} mg/ml tetanus toxin (TD₅₀4B).

potassium concentration, in the range of 5–20 mM, or by substituting NH₄Cl for NaCl.

The relationship between MEPP frequency and external potassium was similar to that reported for other mammalian tissues (19). The logarithmic plot of control frequency as a function of external potassium concentration was linear between 10 and 20 mM, as is shown in Fig. 4A. It is apparent from Table 1 and Fig. 4A that, as the potassium concentration was increased, the MEPP-stimulating effect of toxin diminished. No significant toxin potentiation occurred at a potassium concentration of 10 mM, and with concentrations above 15 mM toxin depressed the frequency of random discharge. From the plot of the logarithm of the MEPP frequency as a linear function of the amount of depolarization produced by the respective external potassium concentrations (Fig. 4B), it appears that when the nerve terminals are depolarized by 10–20 mV there is no activation of MEPP frequency. Above 20 mV of depolarization the effect of toxin is reversed from a MEPP-stimulating to a MEPP-depressing action.

In order to exclude the possibility that the MEPP reduction which occurred in the potassium solutions after adding toxin was the result of depletion of transmitter stores, the depolarization experiments were repeated after prolonged exposure of the tissues to the high-potassium solutions. Tissues maintained in high-potassium solutions for as long as 60 min before testing continued to liberate quanta at frequencies not significantly different from those of the preparations tested immediately.

Because the amplitude of the MEPP is determined partially by the level of polarization of the postsynaptic muscle fiber, the size of the miniature potentials was diminished in those solutions containing high concentrations of potassium. However, the decrease in amplitude was not great enough to interfere with accurate measurement of the frequency of the spontaneous discharge. The noise level was approximately 50 μ V and the mean MEPP between 400 and 500 μ V; thus, a MEPP amplitude reduced by 50% would still be four to six times greater than the noise level.

When the nerve terminal was depolarized by ammonium ions (11) a decrease in the effect of toxin was seen similar to that obtained with potassium. It will be recalled that after a depolarization of more than 20 mV (as estimated from the relationship given in Fig. 4B) the toxin depressed rather than potentiated the MEPP

frequency. When 10% of the NaCl was replaced by NH₄Cl, the mean resting discharge rate at 37 C in two experiments increased from 8.33 ± 0.80 to 10.27 ± 1.02 sec⁻¹ (Table 2). After the subsequent addition of a maximum effective dose of toxin, the frequency was further increased by only 13% to 11.61 ± 1.33 sec⁻¹. Concurrently, there was a small drop in the muscle resting potential and a 30% decrease in the mean MEPP amplitude. In two separate preparations a 25% replacement with NH₄Cl increased the MEPP frequency 12-fold over the control: from 10.08 ± 0.71 to 123.07 ± 18.04 sec⁻¹. The subsequent addition of the same dose of toxin depressed the MEPP frequency 55%: from 123.07 ± 18.04 to 55.89 ± 12.54 sec⁻¹. At this magnitude of replacement the resting potential of the muscle cells dropped by approximately 20 mV, and the average MEPP amplitude was only 50% of that recorded in the normal solutions (Table 2).

Furukawa et al. (11), in 1957, reported that in the frog fibrillation of the muscle fibers occurred when NH₄Cl was substituted for NaCl. The fibrillation apparently occurred because the muscle cells were not depolarized as rapidly as the nerve terminals; consequently, the outburst of MEPP's caused fibrillations of the muscle fibers. In those studies carried out at 37 C we did not observe fibrillation, probably because the muscle cells were rapidly depolarized. In one preparation tested at 25.5 C, however, in which 25% of NaCl was replaced by NH₄Cl, the mean frequency increased from 1.16 (16 fibers) to 128.40 sec⁻¹ (7 fibers). In this preparation there was some evidence of fibrillation, and the amplitude was reduced only 18% from 0.42 to 0.35 mV. Whereas, at 37 C, the muscle cells were completely depolarized within 10 min after the addition of NH₄Cl, the depolarization at 25.5 C appeared to be slower, requiring some 20–30 min. At both temperatures the maximal increase in frequency was reached within 10 min after the NH₄Cl solution was added.

Importance of chloride ions. The reversal of the toxin effect at a given level of depolarization suggested that the activation might have been related to a conductance change involving some ion such as chloride, whose equilibrium potential had been reached. Substitution of another anion with different conductance properties for chloride might then be expected to alter the response to toxin. It is apparent from Table 3, however, that substitution of nitrate for chloride neither potentiated nor depressed the action of a maximal effective dose of toxin.

Summary. From the studies concerning the source of the activated MEPP discharge it is apparent that the action of toxin is dependent on the level of presynaptic polarization and on the character of the external ionic composition. The MEPP's activated were found to be of the calcium-dependent, voltage-dependent type (9). The activation of random discharge by toxin required magnesium and was potentiated by calcium, suggesting that the increased spontaneous discharge is linked to a depolarization of the presynaptic terminals (13, 19).

Direct Effect on Neuromuscular Transmission

In order to determine the effects of toxin on certain aspects of stimulus-linked neuromuscular transmission, more specifically, on transmitter release and replenishment, a series of experiments was performed in the rat phrenic nerve-diaphragm preparation.

In a train of impulses evoked by repetitive stimulation, it is possible to determine whether a given agent has altered the depolarization-coupled release, and whether it does so by increasing the probability of release or the available store of readily releasable units. The effect of toxin on the probability of release was first determined when the quantum content was reduced to the level of one quantal unit by elevating the external magnesium and reducing the external calcium. The effect of toxin on transmitter depletion and mobilization was then studied after blocking neuromuscular transmission by curare.

Effect on quantum content. When neuromuscular transmission is partially blocked by elevating the magnesium and lowering the calcium ion concentrations, the probability of transmitter release initiated by a nerve spike becomes very low (3, 4, 7, 8); under these conditions, the quantum content can be calculated simply by the expression

$$m = \frac{\text{mean EPP amplitude}}{\text{mean MEPP amplitude}}$$

When neuromuscular transmission was depressed by magnesium (3), tetanus toxin had an effect on both the spontaneous release and the probability of release by the presynaptic nerve impulse. In the presence of toxin (Table 4) the spontaneous activity increased 26%, from a control level of 14.44 ± 1.43 to 18.33 ± 2.20 sec⁻¹. In consideration of the fact that any depolarization-coupled MEPP activation would be reduced considerably by the elevated magnesium concentration, this change was assumed to be maximal and similar in magnitude to that observed in the mouse intercostal preparations. Despite the potentiation of the spontaneous release, there occurred a definite reduction in the amount of transmitter liberated by the nerve spike: the quantum content

TABLE 4. Effect of tetanus toxin on MEPP frequency and quantal content in rat diaphragm at 37 C

	Control Fibers*	Test Fibers†	%Δ
MEPP freq. ± SE, sec ⁻¹	14.44 ± 1.43	18.33 ± 2.20	+26.9
No. of fibers	(30)	(22)	
m ± SE	1.39 ± 0.06	1.07 ± 0.02	-23.0
No. of fibers	(26)	(18)	

* Neuromuscular transmission partially blocked with 10.0 mM Mg⁺⁺ and 1.5 mM Ca⁺⁺; m calculated from ratio of the mean EPP/mean MEPP during repetitive stimulation at 25 cycles/sec. † In the presence of 8.759×10^{-4} mg/ml crude tetanus toxin (TD594B) final bath concentration.

was reduced by 23%, from 1.39 ± 0.06 to 1.07 ± 0.02 after the addition of toxin.

Effect on transmitter depletion and mobilization. The effects of toxin on transmitter depletion and mobilization can be studied when neuromuscular transmission is blocked with curare (19). In a train of EPP's elicited by repetitive stimulation of a curarized preparation, the first EPP is usually the largest, and sequential responses show a brief decay of amplitude followed by a steady "plateau."

The rate of depletion of the store of readily releasable transmitter was determined by plotting the sequential responses as ratios of the original response (19-22). The rate of transmitter depletion was decreased in the presence of toxin, as is shown in Fig. 5. In the control fibers at 37 C, the second response was reduced to 95% of the first, whereas, after the addition of toxin, the average second response was potentiated to 107% of the first.

This sparing effect on the rate of transmitter depletion in the presence of toxin could result either from a reduction in the amount of transmitter liberated or from a more rapid replenishment of vacated membrane sites. There did occur a reduction in the amount of transmitter liberated; at 37 C the mean content of the first EPP in a train was 165 quanta, while in the presence of toxin the transmitter content of the initial EPP was reduced by 50% to 83 quanta. To test the second possibility, the effect of toxin on the level of sustained transmitter release was determined. The data in Table 5 show that the mean level of sustained output at 37 C was reduced from a control value of 108.7 ± 13.5 to 79.2 ± 4.5 quanta/impulse after the addition of toxin. This observation indicated that toxin had no stimulating effect on the rate of transmitter replenishment.

DISCUSSION

The first series of experiments concerning the source of MEPP activation suggest that the toxin-potentiated spontaneous activity was the result of depolarization of the presynaptic nerve terminals.

Previously, del Castillo and Katz (6) and Liley (19) have shown that depolarization by presynaptically applied cathodic currents increases the MEPP frequency but does not affect the amplitude. Depolarization by increasing the external potassium ion concentration also has been shown to accelerate the frequency of miniature discharge, the logarithm of the MEPP frequency being linearly related to the amount of depolarization produced at the respective potassium concentrations (16). Such potentiation of MEPP discharge by depolarization (produced either by presynaptically applied cathodic currents or elevated potassium concentrations) has been shown to require calcium; it is enhanced by raising the calcium concentration and depressed by raising the magnesium concentration (6, 13, 19).

The acceleration of MEPP frequency by tetanus toxin is consistent with presynaptic depolarization. Acceleration did not occur when calcium was absent and was potentiated as the calcium concentration was elevated.

TABLE 5. Effect of tetanus toxin on sustained transmitter release at 37 C

	Control Fibers*	Test Fibers†
Avg RP	71.3	72.1
Avg $m \pm SE$	108.7 ± 13.5	79.2 ± 4.5
No. of fibers	(39)	(40)

* Neuromuscular conduction partially blocked by *d*-tubocurarine 2.5×10^{-7} g/ml. *m* Calculated from ratio of the variance to the mean EPP amplitude during repetitive stimulation at 50 cycles/sec. † In the presence of 1.75×10^{-3} mg/ml tetanus toxin (TD594B) final bath concentration.

In the case of high magnesium no conclusion could be drawn. Depolarization of the nerve terminals by 10–20 mv, brought about by increasing the external potassium, abolished the effect of toxin. In addition, these findings demonstrate that the MEPP's activated by toxin are those calcium-associated, voltage-dependent quanta which are released by voltage changes generated across the nerve terminal membrane.

The observation that toxin exerted no effect when magnesium ions were absent from the external medium was an unexpected result (Fig. 2). However, the previous finding (10) that 7 kcal is required for the MEPP-potentiating action of toxin suggested that this process might be enzymatic. If that is the case, it is not unlikely that magnesium ions are essential to, or at least are cofactors in, such an enzymatic process.

The means by which toxin acts to depolarize the nerve terminal is not clear. It would appear that there occurs a change in the conductance properties of the nerve terminal to one, or to a combination, of ionic species. From the present data neither sodium nor potassium alone seems responsible for the potential changes. From the fact that 100% substitution of NaNO_3 for NaCl had

no influence on the MEPP activation by toxin, it is also unlikely that chloride is the ionic species involved.

The second series of experiments demonstrated that toxin reduces the quantum content in both initial and sequential stimulus-evoked EPP's, but does not alter the rate of transmitter replenishment. The similarities between the effects of cathodic depolarization and tetanus toxin on spontaneous transmitter release raises the question as to whether presynaptic depolarization may also account for the observed reduction of end-plate potential quantum content. It is generally believed that at the synapse the magnitude of the postsynaptic activity is partly a function of the presynaptic spike height. Hubbard and Schmidt (14) showed that at the mammalian neuromuscular junction the logarithm of the amplitude of the EPP varies linearly with the amplitude of the presynaptic spike potential. In other studies Hubbard and Willis (15) demonstrated that at both the curarized and the magnesium-poisoned neuromuscular junction, during the passage of depolarizing currents on the presynaptic terminals, the EPP's elicited by stimulation of the phrenic nerve were reduced in amplitude. The reduction of the EPP amplitude, expressed as a fraction of the control amplitude, was, in their experiments, linearly related to the strength of the depolarizing current. As in the present toxin experiments, there occurred no change in the amplitude of MEPP's recorded in the magnesium-poisoned preparations, showing that the change in EPP size was not a postsynaptic effect. The evidence from both Hubbard and Willis' experiments and the toxin experiments presented here is therefore taken to mean that the peripheral neuromuscular properties of tetanus toxin result from depolarization of the motor nerve terminals.

REFERENCES

1. BROOKS, V. B., D. R. CURTIS, AND J. C. ECCLES. The action of tetanus toxin on the inhibition of motoneurons. *J. Physiol., London* 135: 655–672, 1957.
2. BÜLBING, E. Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.* 1: 38–61, 1946.
3. DEL CASTILLO, J., AND L. ENGBAER. The nature of the neuromuscular block produced by magnesium. *J. Physiol., London* 124: 370–384, 1954.
4. DEL CASTILLO, J., AND B. KATZ. The effect of magnesium on the activity of motor nerve endings. *J. Physiol., London* 124: 553–559, 1954.
5. DEL CASTILLO, J., AND B. KATZ. Quantal components of the end-plate potential. *J. Physiol., London* 124: 560–573, 1954.
6. DEL CASTILLO, J., AND B. KATZ. Changes in end-plate activity produced by pre-synaptic polarization. *J. Physiol., London* 124: 586–604, 1954.
7. DEL CASTILLO, J., AND B. KATZ. Biophysical aspects of neuromuscular transmission. *Proc. Biophys. Biophys. Chem.* 6: 121–170, 1956.
8. DEL CASTILLO, J., AND L. STARK. The effect of Ca^{++} ions on the motor end-plate potential. *J. Physiol., London* 116: 506–515, 1952.
9. ECCLES, J. C. *The Physiology of Synapses*. New York: Academic, 1954.
10. FEIGEN, G. A., N. S. PETERSON, W. W. HOFMANN, G. H. GENTHER, AND W. E. VAN HEYNINGEN. The effect of impure tetanus toxin on the frequency of miniature end-plate potentials. *J. Gen. Microbiol.* 33: 489–495, 1963.
11. FURUKAWA, T., A. FURUKAWA, AND T. TAKAGI. Fibrillation of muscle fibers produced by ammonium ions and its relation to the spontaneous activity at the neuromuscular junction. *Japan. J. Physiol.* 7: 252–263, 1957.
12. HEILBRUNN, L. V. *An Outline of General Physiology* (3rd ed.). Philadelphia: Saunders, 1952.
13. HUBBARD, J. I. The effect of calcium and magnesium in the spontaneous release of transmitter from mammalian motor nerve endings. *J. Physiol., London* 159: 507–517, 1961.
14. HUBBARD, J. I., AND R. F. SCHMIDT. An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol., London* 166: 145–167, 1963.
15. HUBBARD, J. I., AND W. A. WILLIS. Reduction of transmitter output by depolarization. *Nature* 193: 1294–1295, 1962.
16. KATZ, B. The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. *Proc. Roy. Soc., London, Ser. B* 155: 455–479, 1962.
17. LILEY, A. W. An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol., London* 132: 650–666, 1956.
18. LILEY, A. W. The quantal components of the mammalian end-plate potential. *J. Physiol., London* 133: 571–587, 1956.
19. LILEY, A. W. The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. *J. Physiol., London* 134: 427–443, 1956.

20. LILEY, A. W., AND K. A. K. NORTH. An electrical investigation of effects of repetitive stimulation on mammalian neuro-muscular junction. *J. Neurophysiol.* 16: 509-527, 1953.
21. LUNDBERG, A., AND H. QUILSCH. Presynaptic potentiation and depression of neuro-muscular transmission in frog and rat. *Acta Physiol. Scand.* 30: Suppl. 111, 111-120, 1953.
22. LUNDBERG, A., AND H. QUILSCH. On the effect of calcium on presynaptic potentiation and depression at the neuro-muscular junction. *Acta Physiol. Scand.* 30: Suppl. 111, 121-128, 1953.
23. MARTIN, A. R. A further study of the statistical composition of the end-plate potential. *J. Physiol., London* 130: 114-132, 1955.
24. PETERSON, N. S., AND G. A. FEIGEN. Effect of NO_2 on atrial action potentials and contraction as modified by Na and Ca. *Am. J. Physiol.* 202: 950-955, 1962.
25. SZEKERES, L., AND E. M. VAUGHAN WILLIAMS. Antifibrillatory action. *J. Physiol., London* 160: 470-482, 1962.
26. VAN HEYNINGEN, W. E. Tentative identification of the tetanus toxin receptor in nervous tissue. *J. Gen. Microbiol.* 20: 310-320, 1959.
27. VAN HEYNINGEN, W. E. The fixation of tetanus toxin, strychnine, serotonin, and other substances by ganglioside. *J. Gen. Microbiol.* 31: 375-387, 1963.

