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THE MODE OF ACTION AT THE MOUSE NEUROMUSCULAR JUNCTION OF THE PHOSPHOLIPASE A-CROTAPOTIN COMPLEX ISOLATED FROM VENOM OF THE SOUTH AMERICAN RATTLESNAKE

BARBARA J. HAWGOOD & J.W. SMITH[1]

Department of Physiology, Queen Elizabeth College, Campden Hill Road, London W8 7AH

1 Phospholipase A_2-crotapotin complex (P–C complex) isolated from the venom of *Crotalus durissus terrificus* induced an irreversible blockade of neuromuscular transmission when twitch tension was measured in the mouse phrenic nerve-hemidiaphragm preparation *in vitro* at 37°C.

2 A similar concentration of the phospholipase A_2 (10 μg/ml) alone did not affect neuromuscular transmission and no priming action was detected on later addition of crotapotin.

3 The rate of neuromuscular blockade induced by P–C complex (15 μg/ml) was not altered by raising the frequency of nerve stimulation. Lower temperatures markedly increased the time of onset and reduced the rate of blockade (Q_{10} (27–37°C) of 4.4) whilst replacement of Ca by Sr in the medium prevented this activity. These latter results suggest that enzymatic activity is important in the neurotoxicity of the complex.

4 A myotoxic action was shown by 30 μg/ml P–C complex and 30 μg/ml phospholipase A_2.

5 P–C complex (150 μg) was injected into the tail vein of mice and the intoxicated hemidiaphragm preparation removed for intracellular recording at 25°C.

6 In fully intoxicated hemidiaphragms, resting membrane potentials were unaltered and endplate potentials (e.p.ps) varied in average amplitude from zero to less than 3 mV.

7 Miniature endplate potential (m.e.p.p.) frequency was lower at fully poisoned endplates than at controls; the frequency rose during a 50 Hz tetanus but was unaffected by either raising external K or the application of the Ca-ionophore A23187.

8 E.p.ps were recorded in partially intoxicated hemidiaphragms with (+)-tubocurarine (0.5–1.0 μg/ml) added to prevent contraction. Evoked release was abnormal as 50 Hz tetanus elicited e.p.ps of very variable amplitude, no facilitation of response was shown to paired stimuli, and tetraethylammonium (0.5 mM) failed to increase e.p.p. amplitudes.

9 M.e.p.ps and e.p.ps were recorded at partially poisoned endplates in low Ca–high Mg solution. A reduction in the quantal content of evoked transmitter release was observed in comparison with controls.

10 M.e.p.ps recorded at partially and at fully intoxicated endplates showed an altered amplitude distribution with a higher proportion of large potentials.

11 It is concluded that P–C complex has a presynaptic site of action and may interfere with depolarization–secretion coupling at the motor nerve terminals.

Introduction

The venom of the South American rattlesnake (*Crotalus durissus terrificus*) is highly toxic to man and is unusual in producing flaccid paralysis and respiratory failure as well as the features of cardiovascular shock. The paralysis and respiratory failure are of peripheral origin (Brazil, 1966) and attributable to a neurotoxic fraction, crotoxin, which was first isolated from whole venom by Slotta & Fraenkel-Conrat (1938). Crotoxin is not a homogeneous protein but can be separated by chromatography on carboxymethyl cellulose into two basic phospholipases A which are isoenzymes of the A_2 type (mol. wt. 14,500) and an acidic protein, crotapotin (mol. wt. 8900) (Hendon & Fraenkel-Conrat, 1971; Breithaupt, Rübsamen & Habermann,

[1] Present address: Pharmacological Laboratories, Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET.

1974; Breithaupt, Omori-Satoh & Lang, 1975; Breithaupt, 1976a). The phospholipases A alone are much less toxic than crotoxin although their specific enzymatic activities are higher (Rübsamen, Breithaupt & Habermann, 1971; Breithaupt, 1976a). Crotapotin alone is non-toxic and devoid of enzymatic activity; when it is recombined with crotalus phospholipase A to form P–C complex, the toxicity is restored although the complex form lowers the enzymatic activity of phospholipase A (Rübsamen *et al.*, 1971).

Previous studies on the neurotoxicity of crotoxin have shown that natural crotoxin or crotoxin formed by reconstitution of phospholipase A and crotapotin (P–C complex) blocks the frog neuromuscular junction by a presynaptic action (Brazil & Excell, 1970; Brazil, Excell & Santana de Sa, 1973) but in the rat causes a decrease in postsynaptic sensitivity (Brazil, 1972) as well as myotoxicity (Breithaupt, 1976b).

In the present study, the mode of action of the P–C complex on mouse neuromuscular transmission was investigated as the complex is much more toxic to the mouse than to the rat (Rübsamen *et al.*, 1971). The aims of the investigation were two-fold: (1) to identify the site of action of the P–C complex at the mouse neuromuscular junction, (2) to investigate the role, if any, of enzyme activity in the neurotoxicity. A preliminary account of this work has been published (Hawgood & Smith, 1977).

Methods

Toxins

Basic phospholipase A_2 (PhA) and crotapotin each isolated from the venom of *Crotalus durissus terrificus* was the gift of Professor Habermann, University of Giessen. PhA was separated from fraction 6 of the whole venom and the intravenous LD_{50} in mice of the PhA and crotapotin were 4.0 mg/kg and >50 mg/kg respectively. By contrast the reconstituted P–C complex had an i.v. LD_{50} of 0.14 mg/kg when 2 parts of PhA were added to 1 part crotapotin on a weight basis (Habermann & Schaub, personal communication). This weight ratio which was used throughout the investigation of the P–C complex gives a molar ratio of 1:0.8. The stoichiometry of PhA and crotapotin in the natural crotoxin complex varies over a wide range but has a preference for a molar ratio of 1:1 (Breithaupt, 1976a). Both the PhA and crotapotin were dissolved in phosphate buffered saline to a concentration of 1 mg/ml and 1 ml samples were stored at −25°C until use.

Basic PhA_2 isolated from the venom of *Naja nigricollis* was kindly supplied by Dr D. Eaker, Institute of Biochemistry, University of Uppsala, Sweden.

Contractile response

All experiments were done on adult male TO mice of 20–25 g weight. The isolated phrenic nerve hemidiaphragm preparation (Bülbring, 1946) was mounted in an organ bath containing 10 ml of physiological saline of ionic composition (mM): NaCl 137, $NaHCO_3$ 15.0, NaH_2PO_4 1.0, KCl 2.5, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and glucose 11.0, which was aerated with 95% O_2 and 5% CO_2. The P–C complex (expressed as the combined weights of the components) was added to the bath. In some experiments $CaCl_2$ 2 mM was replaced by $SrCl_2$ 4 mM. The temperature was maintained at 37 ± 0.5°C unless stated otherwise.

Preparations were stimulated indirectly with rectangular voltage pulses of 0.05 ms or less usually at 1 V which was supramaximal and at a frequency of 0.05 Hz unless otherwise stated. Directly stimulated preparations were maintained in physiological saline containing (+)-tubocurarine, 3 μg/ml, and the stimuli were rectangular voltage pulses of 1 ms duration and supramaximal strength. Isometric tension which developed in response to electrical stimulation was recorded with a force displacement transducer and displayed on a Washington 400 pen recorder. The resting tension of the muscle was adjusted to give the maximum contractile response.

In vivo *intoxicated muscles*

The P–C complex (150 μg) was injected into the tail vein of the mouse which was killed either at the stage when the limbs became paralyzed or when respiration ceased. The isolated phrenic nerve – hemidiaphragm preparation was mounted in a bath containing 25 ml of physiological saline. The entry of gas was so arranged that a bubble lift maintained a flow of solution over the muscle. The nerve was stimulated through a suction electrode. In order to retard the further progression of neuromuscular blockade *in vitro*, the intoxicated preparations were kept at 25 ± 1°C. This temperature was chosen in view of the high Q_{10} of the neurotoxic action of the P–C complex (see Figure 3).

Intracellular recording

Resting membrane potentials, end plate potentials (e.p.ps) and spontaneous miniature endplate potentials (m.e.p.ps) were recorded with glass microelectrodes filled with 3 M KCl using standard techniques. Recordings were displayed on a Mingograf 34 pen recorder (time constant 0.15 s, frequency response 700 Hz). The effects of repetitive stimulation and of tetraethylammonium ions (TEA) on the e.p.ps were studied in partially paralyzed diaphragm preparations equilibrated with (+)-tubocurarine 0.5–1 μg/ml in

physiological saline. Control values were obtained from untreated diaphragm preparations in (+)-tubocurarine 2 μg/ml. In the experiments with TEA, e.p.ps were elicited at 0.5 Hz, and at each endplate the average of 16 e.p.ps was obtained using a signal averager (Datalab 102A).

M.e.p.ps were recorded for a 2-min period for the estimation of frequency and amplitude distribution. This involved 40–80 m.e.p.ps. To determine the quantal content of evoked transmitter release, the preparations were placed in physiological saline containing Ca 1 mM–Mg 6 mM and e.p.ps were elicited at 5 Hz for 30–90 s depending on the number of failures of response. As a standard procedure, e.p.ps were corrected for non-linear summation (Martin, 1955).

The Ca-ionophore A23187 (Eli Lilly Co.) was dissolved in absolute ethanol and added to the physiological saline to give a final concentration of 2×10^{-5} M of the ionophore and 0.5% ethanol. In these experiments 0.5% ethanol was present as control in the physiological solution before the addition of the ionophore. This concentration has been reported to have only a slight effect on m.e.p.p. frequency (Gage, 1965).

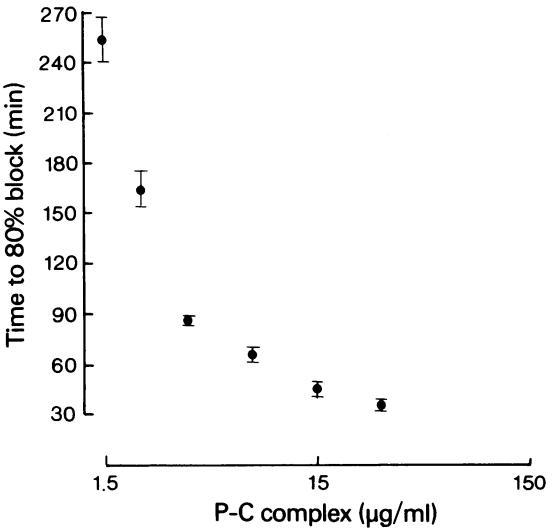

Figure 1 Effect of various concentrations of the phospholipase A_2-crotapotin (P–C) complex on the time to 80% block of the contractile response of mouse diaphragm muscle evoked by indirect stimulation at 37°C. Each point is the mean result from at least 5 muscles, vertical lines show s.e. means.

Results

Effect of phospholipase A_2-crotapotin complex on the development of twitch tension in indirectly and directly stimulated muscle

In the presence of the P–C complex (1.5–20 μg/ml), the twitch tension in response to indirect stimulation was progressively reduced until complete block developed. The mean times to 80% block are shown in Figure 1, and the mean time-course of blockade in response to 15 μg/ml of P–C complex is shown in Figure 2 (closed squares). When tested on the directly stimulated curarized muscle, 7.5 and 15 μg/ml of the complex were ineffective. However, raising the concentration to 30 μg/ml produced a reduction to 50% of the control tension within 90 min and was accompanied by a sustained contracture of the muscle. This indicates a myotoxic action which was also observed by Breithaupt (1976b) for P–C complex on the rat diaphragm. In all subsequent experiments in the present study, the concentration of the P–C complex did not exceed 15 μg/ml.

The degree of reversibility of the block of the response to indirect stimulation was determined by washing the preparation at various times after addition of 15 μg/ml of the P–C complex. After 80% block was reached, repeating washing produced no recovery over a period of 90 minutes. When the preparation was washed 25 min after the addition of the toxin, i.e. at an early stage of the block, there was a small but significant reduction in the subsequent rate of block. The mean time to 80% block in control experiments was 44.5 ± 2.8 min and in washed preparations was 68.2 ± 4.4 min (mean and s.e. mean of 5 muscles in each case). In muscles washed after 10 min contact with the complex, the rate of paralysis was considerably reduced, so that at 140 min, the mean twitch tension was still $45 \pm 5\%$ of control (s.e. mean of 5 muscles). These results suggest that the toxin is bound irreversibly to the preparation, and that fixation is almost complete within 25 min under these conditions.

The frequency of nerve stimulation markedly affects the rate of action of botulinum toxin (Hughes & Whaler, 1962) and taipoxin (Kamenskaya & Thesleff, 1974). The P–C complex showed no such dependency as increasing the rate of stimulation 40-fold from 0.05 Hz to 2 Hz had no significant effect on the rate of block produced by either 2.3 μg/ml or by 15 μg/ml P–C complex in 6 experiments.

Effect of phospholipase A_2 and crotapotin

In the rat diaphragm, PhA or crotapotin given alone are ineffective even at high concentrations (Breithaupt, 1976b). At the frog neuromuscular junction although crotapotin was ineffective, PhA had a weak blocking action (Brazil *et al.*, 1973). In the present experiments

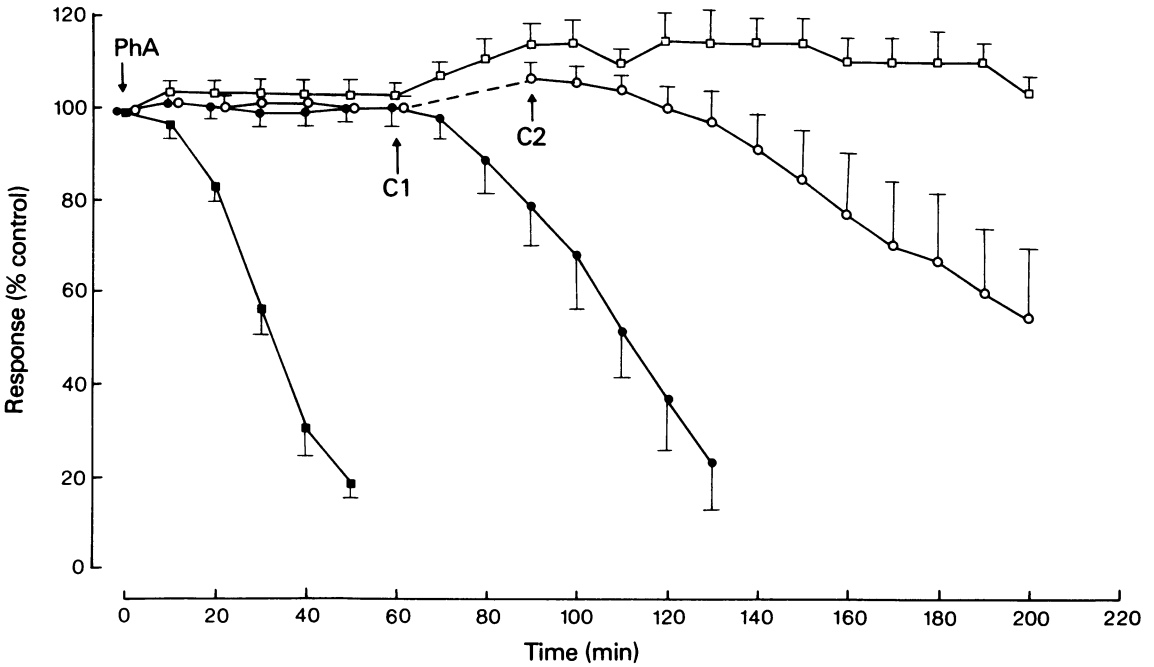

Figure 2 The effect on the indirectly evoked twitch tension in mouse diaphragm muscles of treatment with phospholipase A_2 (PhA, 10 μg/ml) for various times before the addition of crotapotin (5 μg/ml). Temperature 37°C. (□) PhA alone; (■) PhA added with crotapotin (P−C complex) at zero time; (●) PhA present for 60 min before addition of crotapotin at C1; (○) PhA present for 60 min followed by a 30 min wash, indicated by the broken line. Crotapotin then added at C2. Mean of at least 4 muscles; vertical lines show s.e. means.

on the mouse hemidiaphragm 10 μg/ml PhA (Figure 2) or 10 μg/ml crotapotin had no effect on twitch tension in the indirectly stimulated muscle. However, higher concentrations of PhA (30 and 50 μg/ml) had a myotoxic effect as shown by the reduction in response to direct stimulation of curarized muscle.

To determine if PhA were able to prepare the neuromuscular junction for the later action of crotapotin, PhA (10 μg/ml) was placed in contact with the preparation for 60 min before the addition of crotapotin (5 μg/ml). As shown in Figure 2, the rate of block of the response to indirect stimulation after the addition of crotapotin was similar to that of 15 μg/ml of the P−C complex given at zero time. This was interpreted as indicating that PhA had no priming action and that complex formation was rapid. In further experiments some muscles were washed extensively after 60 min exposure of the preparation to PhA. Crotapotin was then added and a slow rate of block of neuromuscular transmission ensued (Figure 2); this is possibly due to complex formation with residual PhA as the enzyme is known to be difficult to desorb from surfaces (Breithaupt, 1976a). These results suggest that the complex form is necessary for neurotoxic activity.

The specificity of crotapotin for *C.d. terrificus* PhA was tested by determining if crotapotin potentiated the action of the basic PhA isolated from the venom of *N. nigricollis*; 1 μg/ml of this enzyme with or without crotapotin (0.5 μg/ml) had no effect on the indirectly evoked twitch tension of the mouse diaphragm. Higher concentrations of *N. nigricollis* PhA (4 and 10 μg/ml) showed signs of a myotoxic action as the reduction of the contractile response was associated with sustained contracture of the muscle. Crotapotin had no effect on the rate of development of this action.

Effects of temperature and of strontium

Figure 3 shows the marked effect of temperature on the time of onset and on the rate of block of neuromuscular transmission produced by the P−C complex (15 μg/ml). The time to 50% block of contractile response had a Q_{10} (27–37°C) of 4.4; at 37°C, 32°C and 27°C the slopes changed in proportion to these times. This suggests that the enzymatic activity

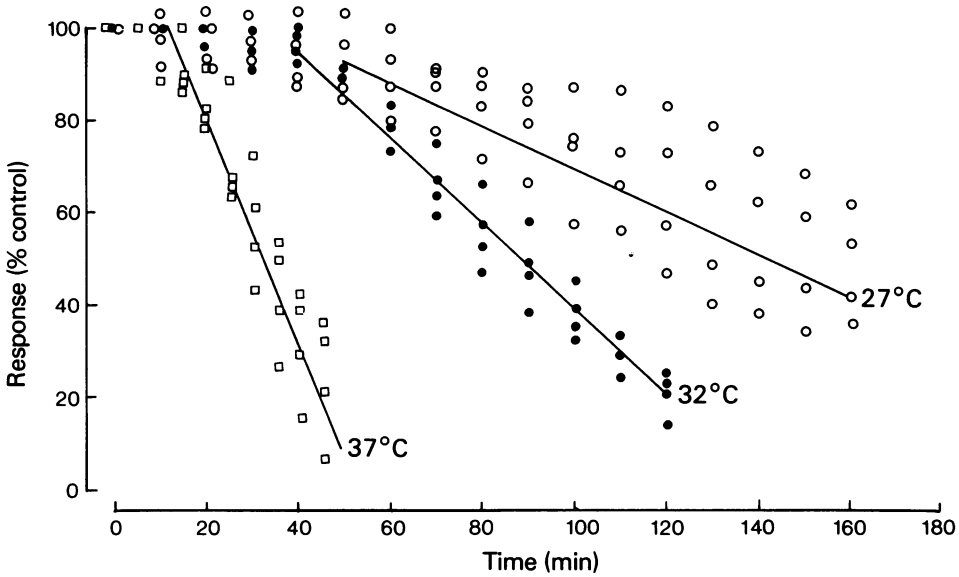

Figure 3 The effect of temperature on the rate of action of the phospholipase A_2-crotapotin (P–C) complex (1·5 μg/ml) as determined on the indirectly evoked twitch response of mouse diaphragm muscle. A straight line was fitted to the experimental points by the method of least squares and gave values for the slopes (± s.e. mean) of -2.30 ± 0.281, -0.95 ± 0.129 and -0.52 ± 0.085 min^{-1} at 37, 32 and 27 °C respectively.

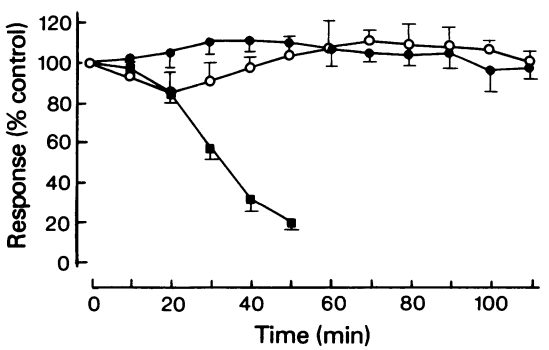

Figure 4 The effect of replacement of Ca 2 mM by Sr 4 mM on the blocking activity of 15 μg/ml phospholipase A_2-crotapotin (P–C) complex as measured by the development of twitch tension of the indirectly stimulated diaphragm at 37°C. (●) Control muscles in Sr; (○) muscles treated with the P–C complex in Sr; (■) muscles treated with the P–C complex in Ca. Each point is the mean of at least 4 muscles; vertical lines show s.e. means.

of the complex may play an important role in neurotoxicity.

The catalytic activity of *C.d. terrificus* PhA as tested on an egg yolk medium *in vitro* is strongly Ca-dependent (Breithaupt, 1976a) and Sr cannot substitute for Ca in this activation (Habermann & Schaub, personal communication). To determine if a reduction in catalytic activity would be accompanied by a reduction of neurotoxicity, we investigated the effect of the replacement of Ca by Sr on the blocking activity of the P–C complex. Sr can maintain neuromuscular transmission in the absence of Ca (Dodge, Miledi & Rahamimoff, 1969), and in the present experiments the contractile response of control preparations to indirect stimulation in Sr 4 mM was similar to that in Ca 2 mM. However, as Figure 4 shows, Sr abolished the blocking activity of the P–C complex thus providing further support for an enzymatic role in neurotoxicity.

Intracellular recording from endplates intoxicated in vivo

A detailed investigation of the site of action of the P–C complex was undertaken with intracellular recording techniques to determine the effect on spontaneous and neurally evoked release of transmitter from single endplates which have been

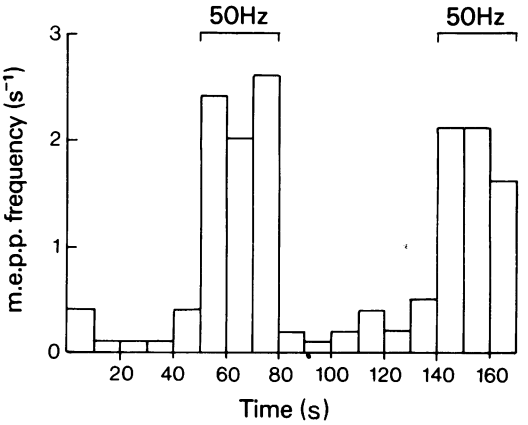

Figure 5 The time course of response of miniature endplate potential (m.e.p.p.) frequency (s^{-1}) to a repeated tetanus of 50 Hz for 30 s at endplates completely intoxicated with the phospholipase A_2-crotapotin complex (150 μg, i.v.). Recorded at 25°C.

intoxicated with 150 μg of P–C complex given intravenously to the mouse. Recordings were made at 25°C (see methods section).

Resting membrane potentials. When hemidiaphragms were removed from mice intoxicated to the point of complete respiratory paralysis, the mean resting membrane potential of the fibres was 71.6 ± 1.2 mV (s.e. mean of 53 fibres, 8 muscles) which is within the control range. As a check that the complex did not produce a progressive change in resting membrane potential with time, untreated muscles were exposed to the P–C complex *in vitro* at 37°C. At 60 min, the mean resting membrane potential of fibres exposed to 15 μg/ml of the complex was 69.1 ± 0.8 mV (s.e. mean of 38 fibres, 4 muscles) and in fibres exposed to 30 μg/ml of complex was 71.9 ± 0.8 mV (s.e. mean of 28 fibres, 3 muscles). These values do not differ significantly from the mean resting membrane potential of control fibres of 70.3 ± 0.7 mV (s.e. mean of 36 fibres, 3 muscles) at 60 minutes.

Presynaptic site of action. In hemidiaphragms removed from intoxicated mice at the stage of respiratory arrest, phrenic nerve stimulation either failed to evoke e.p.ps or elicited e.p.ps of up to 3 mV amplitude. As m.e.p.ps could be recorded this indicated a severe reduction of evoked release to a few quanta. The frequency of spontaneous release was significantly lower than in controls (Table 1). To determine if action potentials could invade the nerve terminals at the stage when e.p.ps were completely abolished, the effect on m.e.p.p frequency of a tetanus of 50 Hz for 30 s was determined. An approximately 14-fold increase in mean m.e.p.p. frequency resulted (Table 1) although the response did not outlast the duration of the stimulus (Figure 5). This is in marked contrast to the raised frequency seen after tetanus at normal mammalian endplates in low Ca, high Mg solution (Liley, 1956). These results suggest that action potentials were able to reach the terminals in intoxicated preparations which is in agreement with the finding of Breithaupt (1976b) that high doses of the P–C complex did not interfere with conduction in the phrenic nerve. As a further test of the ability of the nerve terminal to respond to depolarization, the K concentration of the medium was raised from 2.5 mM to 10 mM. This failed to increase the m.e.p.p. frequency of intoxicated endplates in contrast to the response of controls (Table 1). A similar finding was observed at the frog neuromuscular junction in the presence of crotoxin (Brazil *et al.*, 1973). These observations suggest that the P–C complex depresses

Table 1 Comparison of miniature endplate potential (m.e.p.p.) frequencies recorded at control and completely intoxicated endplates under various experimental conditions

	M.e.p.p. frequency (s^{-1})	
	Control endplate	*Intoxicated endplate**
Normal medium	0.53 ± 0.03 (50, 6)**	0.30 ± 0.03 (80, 8)
50 Hz tetanus	–	4.42 ± 0.38 (15, 3)
K 10 mM	10.60 ± 0.42 (20, 3)	0.30 ± 0.05 (30, 3)
Ca ionophore A23187	1.46 ± 0.13 (30, 3)	0.46 ± 0.08 (28, 3)

Values are mean ± s.e. mean.
* 150 μg P–C complex given intravenously; ** number of fibres and number of muscles, respectively.

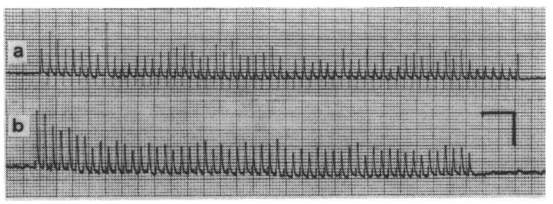

Figure 6 The effect of a train of stimuli at 40 Hz on endplate potential (e.p.p.) amplitudes recorded at (a) an endplate intoxicated with the phospholipase A_2-crotapotin complex (150 μg, i.v.) and (b) a control endplate; (+)-tubocurarine present at 1 μg/ml in (a) and 2 μg/ml in (b). Temperature 25°C. Time scale 100 ms, voltage scale 1 mV.

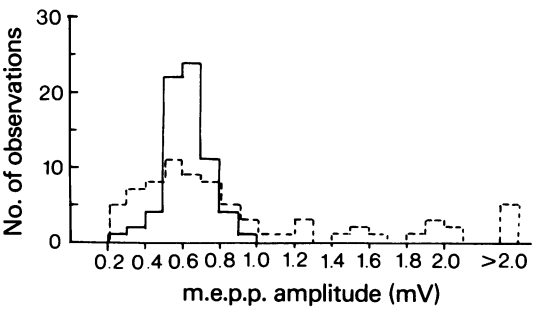

Figure 7 The amplitude distribution of miniature endplate potentials (m.e.p.ps) recorded at an intoxicated endplate (- - -, $n=76$) and at a control endplate, (——, $n=69$) in Ca 1 mM – Mg 6 mM physiological saline. n is the number of m.e.p.ps.

evoked quantal release of transmitter by interfering with depolarization-secretion coupling at the motor nerve terminal. As the antibiotic A23187 is believed to act as a Ca-ionophore allowing passage of divalent cations across biological membranes (Reed & Lardy, 1972), it was of interest to determine if spontaneous transmitter release could be increased from intoxicated endplates. Application of the ionophore (2×10^{-5} M) to control endplates increased m.e.p.p. frequency almost three-fold (Table 1) which was interpreted as the response to a small nett Ca influx. However, in intoxicated muscles a similar application of the ionophore failed to produce a statistically significant increase in the rate of spontaneous release (Table 1).

Neurally evoked release of transmitter

Effect of repetitive stimulation. Hemidiaphragm preparations were removed from partially intoxicated mice killed at the stage when limb paralysis was evident but before respiration had ceased. At this stage of intoxication, as phrenic nerve stimulation produced contraction of some of the fibres, the preparation was equilibrated with (+)-tubocurarine (0.5–1 μg/ml) for 30 minutes. An e.p.p. could be elicited by a single stimulus. A train of stimuli at 40 Hz evoked e.p.ps of very variable amplitude. This is in contrast to the initial decline in amplitude normally observed at control endplates (Figure 6). A similar response to tetanic stimulation has been reported for β-bungarotoxin (Chang, Chen & Lee, 1973). Facilitation of the evoked response was not observed at intoxicated endplates either during a 40 Hz tetanus or in response to pairs of stimuli separated by 10 ms. At control endplates partially blocked by (+)-tubocurarine 2 μg/ml, such paired stimuli produced an increased amplitude of the second e.p.p. with a facilitation ratio of 1 : 1.56 (20 endplates, 2 muscles). At intoxicated endplates the ratio was 1 : 1.04 (20 endplates, 2 muscles).

Effect of tetraethylammonium. TEA prolongs the spike and increases the amount of Ca which enters the nerve terminal during the action potential (Katz & Miledi, 1967). After the addition of TEA 0.5 mM, the mean amplitude of e.p.ps recorded in control muscles in the presence of (+)-tubocurarine 2 μg/ml increased from 1.69 ± 0.21 mV to 4.77 ± 0.36 mV (±s.e. mean of 10 endplates, 2 muscles). At intoxicated endplates in the presence of (+)-tubocurarine 0.5 μg/ml, there was no significant effect of TEA 0.5 mM. Mean e.p.p. amplitudes were 4.32 ± 1.15 mV before and 5.78 ± 2.02 mV (s.e. mean 10 endplates, 2 muscles) after addition of TEA. It is not known whether the action of the P–C complex interfered with the prolongation of the spike or with Ca entry into the terminal.

Experiments with low calcium, high magnesium. Hemidiaphragm preparations were removed from mice at the stage of limb paralysis following the intravenous injection of P–C complex (150 μg) as in the previous experiments. To record both spontaneous and neurally evoked transmitter release from these and from control preparations, the diaphragms were placed in physiological saline containing 1 mM Ca–6 mM Mg.

Spontaneous transmitter release. The mean amplitude of m.e.p.ps recorded at these partially intoxicated endplates was greatly increased in comparison with controls when group values were compared. Thus the mean of the mean amplitudes of m.e.p.ps recorded at 8 endplates in 3 control muscles was 0.41 mV with a mean of the s.d. of 0.15 mV; at 9 intoxicated endplates in 3 muscles the mean amplitude was 0.78 mV with a mean s.d. of 0.49 mV. The histogram of m.e.p.p. amplitude distribution showed that a higher proportion of large potentials was present at a treated endplate (Figure 7). This was

reflected in the high mean amplitude of the group and was also indicated by an elevated mean coefficient of variation (CV) of amplitudes at individual endplates (0.63 at intoxicated endplates in comparison with a control mean CV of 0.35). A similarly altered distribution of m.e.p.p. amplitudes was observed at fully intoxicated endplates in normal physiological saline. Thus at 6 such endplates in 3 muscles, the mean m.e.p.p. amplitude was 0.68 mV with a mean s.d. of 0.43 mV and mean CV of 0.62. The mean frequency of spontaneous release was reduced at partially intoxicated endplates in comparison with controls in this modified medium as in fully intoxicated endplates in normal physiological saline (Table 1).

Neurally evoked transmitter release. At partially intoxicated endplates in Ca 1 – Mg 6 mM solution there was a high failure rate of evoked response to nerve stimulation (31–91% of stimuli) indicating an increased block of transmitter release in comparison with controls (0–23% failures). Statistical analysis was used to determine if the evoked transmitter release during intoxication was quantal in nature and could be described by the Poisson theorem. It is possible to calculate the predicted number of responses containing 0, 1, 2, 3 quantal components, i.e. n_0, n_1, n_2, n_3 from the Poisson distribution if the mean quantal content (m) is known. As it was difficult to determine the quantum size (q) of the unitary event at many intoxicated endplates due to the altered distribution of m.e.p.p. amplitude (Figure 7), m was calculated from ln (number of impulses/number of failures) and q from mean e.p.p. amplitude/m (Katz, 1966). To analyze the observed distribution of quantal components at single endplates, histograms of e.p.p. amplitude were divided into classes with centres around q and its multiples and with class width $\pm q/2$. Errors due to variation in the overlap of classes were considered negligible (Wernig, 1975). Nine endplates in 3 muscles were studied and in general there was a satisfactory agreement between observed and predicted values of quantal components as illustrated in Table 2 for 5 intoxicated endplates.

This suggests that evoked transmitter release is quantal in nature at least at this stage of intoxication. For controls in Ca 1 mM – Mg 6 mM, mean quantal content was calculated from mean e.p.p. amplitude/mean m.e.p.p. amplitude. Comparison of intoxicated and control endplates showed that partial intoxication of the muscle significantly reduced the value of m from 3.78 ± 2.83 (mean $\pm$ s.d. of 8 endplates, 3 muscles) in controls to 0.61 ± 0.44 ($\pm$ s.d. of 9 endplates, 3 muscles; $P < 0.01$).

Discussion

The results of the present study show that the P–C complex acts presynaptically to interfere with the release of transmitter from motor nerve terminals. This is based on a number of observations including the finding that evoked transmitter release was severely reduced and the rate of spontaneous release was lowered. In addition the m.e.p.p. amplitude distribution was distorted with the appearance of a higher proportion of large potentials; mean m.e.p.p. amplitude consequently increased. Action potentials can apparently propagate into the nerve terminals of highly intoxicated preparations as occasional e.p.ps could be recorded and tetanic stimulation of the nerve increased the rate of spontaneous release in fully blocked preparations.

To investigate further the suggestion that the P–C complex interfered with depolarization-secretion coupling at the motor nerve terminal, intoxicated endplates were examined by various procedures considered to increase the entry of calcium ions into the nerve terminal and to promote transmitter release (Baker, 1974). In all cases the results were negative: K failed to increase spontaneous release; post-tetanic potentiation of m.e.p.p. frequency was not observed; TEA and double pulse stimulation failed to increase the evoked release of transmitter and the response to a

Table 2 Analysis of neurally evoked transmitter release at single endplates intoxicated with phospholipase A_2-crotapotin (P–C) complex *in vivo* and examined *in vitro* in Ca 1 mM – Mg 6 mM solution

		n *predicted/observed*		
Endplate	*m**	n_1	n_2	n_3
1	0.09	36/35	1/4	0/0
2	0.21	36/37	4/4	0/0
3	0.32	46/50	7/3	0/0
4	0.75	65/63	24/28	6/6
5	0.97	59/57	29/32	9/10

The number of e.p.ps predicted by the Poisson theorem to contain 1, 2, 3 quantal components (n_1, n_2, n_3) over the observed number of quantal components is shown. For details see text. Temperature 25°C.
* m = ln (number of stimuli/number of failures).

train of stimuli was abnormal. The increase in m.e.p.p. frequency which was observed during tetanus at the fully blocked endplate may have arisen from the release of internally bound Ca (Rahamimoff, Erulkar, Alnaes, Meiri, Rotshenker & Rahamimoff, 1976). The presence of a calcium-ionophore failed to increase the rate of spontaneous release, however. The present results provide indirect evidence that the P–C complex interferes with Ca entry and/or the subsequent release mechanisms at the motor nerve terminal although further work is required to elucidate the exact mechanism.

Three other protein neurotoxins isolated from snake venoms depress evoked release of transmitter, viz. β-bungarotoxin from the venom of *Bungarus multicinctus* (Chang *et al.*, 1973), notexin from the venom of *Notechis scutatus scutatus* (Harris, Karlsson & Thesleff, 1973) and taipoxin from the venom of *Oxyuranus scutellatus scutellatus* (Kamenskaya & Thesleff, 1974). In addition taipoxin severely reduced the frequency of m.e.p.ps and altered their amplitude distribution with the appearance of both small and very large potentials. These changes were accompanied by a depletion of vesicles from the motor nerve terminals (Cull-Candy, Fohlman, Gustavsson, Lüllmann-Rauch & Thesleff, 1976).

Enzyme activity in relation to neurotoxicity

In the present study *C.d. terrificus* PhA had no effect on neuromuscular transmission in the doses used. However at the frog neuromuscular junction the enzyme showed a presynaptic mode of action, although a much higher dose of enzyme than of P–C complex was required (Brazil *et al.*, 1973; Santana de Sa, 1975). The role of PhA activity in the neurotoxic action of the P–C complex is uncertain, particularly as the enzyme activity of the complex *in vitro* is about three times lower than that of its component PhA. However, this inhibition of enzyme activity is prevented by deoxycholate in the medium (Breithaupt, 1976a) and may only reflect a lack of specificity of the complex for the micellar state of the egg lecithin.

Weak phospholipase A activity is also shown by the presynaptic neurotoxins, β-bungarotoxin (Wernicke, Vanker & Howard, 1975), notexin (Halpert & Eaker, 1975) and taipoxin (Fohlman, Eaker, Karlsson & Thesleff, 1976). Although the role of catalytic activity in the neurotoxic action of notexin has been contested (Halpert, Eaker & Karlsson, 1976) it is strongly implicated in the action of β-bungarotoxin (Abe, Limbrick & Miledi, 1976). In a study of the ionic requirements for enzymatic and toxic activities of β-bungarotoxin, replacement of Ca by Sr was shown to reduce both these activities of the molecule in parallel (Strong, Goerke, Oberg & Kelly, 1976). The PhA activity was therefore considered a necessary but insufficient causal factor alone. Similarly the replacement of Ca by Sr prevented the P–C complex from blocking neuromuscular transmission which may correspond with the reduction in enzymatic activity of its component PhA by Sr. Crotapotin is required for full toxicity, and this combination may allow the PhA to gain direct access to its specific site of action. We suggest that PhA hydrolyses phospholipids within the pre-synaptic membrane to produce a local increase in lysophosphatides and thus changes the conformation and properties of the membrane. Lysolecithin has been shown to increase membrane fusion (Poole, Howell & Lucy, 1970) and the high proportion of large m.e.p.ps recorded from intoxicated endplates could result from an increase in the probability of fusion of vesicles. Kelly, Oberg, Strong & Wagner (1976) postulate also that the initial increase in m.e.p.p. frequency due to the action of β-bungarotoxin may be due to lysophosphatides producing an increased fusion of the vesicles with the terminal membrane.

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