Br. J. Pharmac. (1972), **46**, 329–343.

A myotoxin secreted by some piscivorous *Conus* species

SHIRLEY E. FREEMAN AND R. J. TURNER

Australian Defence Scientific Service, Defence Standards Laboratories, Melbourne 3032, Victoria, Australia

Summary

1. Toxins isolated from the venom apparatus of *Conus magus* and *Conus achatinus* have the same pharmacological properties, but differ from the toxins of several other piscivorous species of cone shells.

2. *C. magus* and *C. achatinus* toxins are heat labile at pH 8·5. A single lethal component with an approximate molecular weight of 10,000 was isolated from *C. achatinus* toxin by exclusion chromatography.

3. Animals died from a characteristic spastic paralysis after intravenous injection of the toxin.

4. Nerve transmission was unaffected by the toxin; skeletal muscle appeared to be the primary site of action. The toxin caused a persistent contracture of rat diaphragm muscle, and a dose-dependent decline in twitch tension. The contracture was potentiated by caffeine.

5. The decline in twitch tension was associated with depolarization of the cell membrane. Action potential height and the maximum rate of rise declined, and spike propagation failed when the resting potential had declined to approximately 60 mV. The muscle recovered very slowly on washout of the toxin. The depolarization could be reversed by exposure of the preparation to 5 mM Na^+ solution or tetrodotoxin or saxitoxin.

6. Miniature end-plate potential frequency in the rat diaphragm decreased, as did the quantal content of the end-plate potential. The acetylcholine-induced contraction and depolarization of the chronically denervated rat diaphragm were increased by low doses of toxin and reduced by higher, depolarizing toxin doses. The K^+-induced contraction and depolarization of innervated diaphragm were similarly affected by the toxin.

7. Cardiac and smooth muscle were relatively resistant to the toxin. The isotonic contraction of the isolated perfused guinea-pig heart was increased by the toxins from both Conidae. The heart rate decreased. Guinea-pig atrial cells showed a small decrease in action potential height and maximum rate of rise. Rabbit sino-atrial cells showed increases in action potential height, maximum diastolic potential and maximum rate of rise of the spike at low toxin levels, and no change in any of these parameters at high levels. There was a decrease in the rate of the spontaneously beating atrium. Atrio-ventricular nodal potentials showed no change other than a slight increase in the maximum rate of rise of the action potential.

8. It is postulated that the action of the toxin may be related to a change in the Ca^{++} permeability of the excitable membrane, which makes it unstable,

leading to a secondary, depolarizing entry of Na^+. The effects of the toxin are compared with those of batrachotoxin, which it somewhat resembles.

Introduction

The genus *Conus* comprises a family of predatory marine gastropods that have been reported to cause injuries and occasionally death to man (Cleland & Southcott, 1965 ; Halstead, 1965 ; Kohn, Saunders & Wiener, 1960). The symptoms described vary from a sharp stinging sensation which fades over a period of hours, to death from respiratory paralysis with no report of pain. This apparent conflict may be resolved by the recognition of three groups within the genus with different feeding habits. Vermivorous and molluscivorous cones are likely to secrete different toxins from those of piscivorous species. Human fatalities have all been ascribed to piscivorous species of Conidae, although the identification of the shell has not always been definite. Endean & Rudkin (1963), Whyte & Endean (1962) and Endean & Izatt (1965) have reported investigations of the toxins of a number of piscivorous species. Their results suggest that *C. magus* Linné toxin is qualitatively different from that of *C. geographus* Linné. We have noted differences between *C. striatus* Linné toxin and that of *C. magus* Linné ; *C. achatinus* Gmelin toxin closely resembles that of *C. magus*. Thus there may be a further division between the types of toxin secreted by different piscivorous Conidae.

The present communication describes an investigation of the pharmacology of myotoxins extracted from the venom ducts of *C. magus* and *C. achatinus*. An attempt has been made to test the toxins on as wide a spectrum of preparations as was possible having regard to the very limited supply of cone shells.

Methods

Toxin preparation

Specimens of *C. magus* and *C. achatinus* were collected and identified by Mr. A. G. Hinton in the Territory of Papua and New Guinea. Mr. Hinton suggested that *C. achatinus* may be a variant of *C. monachus* Linné ; however, we have adhered to the older nomenclature.

The shells, which averaged 4–6 cm in length, were frozen soon after collection, and were kept at −20° C. The venom apparatus was dissected out of the animals immediately after thawing, and an extract was made by homogenizing the venom ducts in 150 mM NaCl solution buffered to pH 8 with 5 mM Tris-HCl. The lethality of the extract was determined by injection of 0·1 ml of diluted samples into the lateral tail veins of albino mice, 25–30 g weight. Since individual cones varied greatly in toxicity, groups of 3–8 venom ducts were pooled to prepared extracts, which were stored at −20° C. A mouse unit (M.U.) was defined as the minimum lethal dose (MLD) for groups of 4–5 mice. Death within 30 min of intravenous injection was used arbitrarily to estimate the MLD.

C. achatinus toxin was partially purified by exclusion chromatography through a column of Sephadex G-50 superfine grade. Toxicity was eluted as a single discrete peak. The UV absorbance of the column fractions was monitored at 280 nm as a guide to protein concentration. Peak absorbance was obtained at the void volume, the lethal fractions contained relatively little UV absorbing material. The column was calibrated with trypsin, cytochrome C and insulin, and the position

of the lethal fraction relative to the marker proteins suggested an approximate molecular weight of 10,000. The pharmacology of the lethal column fractions was identical to that of the crude preparation and results obtained with both preparations have therefore been pooled.

C. magus and *C. achatinus* toxins were completely inactivated by heating to 100° C for 7 min at pH 8·5 ; *C. achatinus* toxin was not inactivated by heating at pH 6·0 or 7·2 for 10 minutes.

Pharmacological testing of toxins

The effects of the toxins on arterial pressure, right atrial pressure, rate and depth of respiration, electrocardiogram and heart rate were determined on male albino rats (220–300 g) and, in the case of *C. magus* toxin, on New Zealand rabbits (Freeman & Turner, 1969a). Rabbits were anaesthetized with urethane and maintained with chloralose, rats were anaesthetized with pentobarbitone, 40–50 mg/kg. In other experiments the phrenic nerve of the rabbit was exposed in the neck and platinum electrodes were placed on it. A myograph needle was placed in the diaphragm muscle, and the respiratory activity of nerve and muscle was displayed on a dual beam oscilloscope and photographed on moving film.

Isolated organ experiments

The effects of the toxins were determined on a number of isolated organ preparations. These were the isolated phrenic nerve-diaphragm preparation of the rat, the chronically denervated rat diaphragm (Freeman & Turner, 1969b), the isolated desheathed sciatic nerve of the toad, *Bufo marinus*, the isolated, perfused heart of the guinea-pig (Turner & Freeman, 1969) and the rat isolated ileum. The effect of the toxins on skeletal muscle resting and action potentials was determined by intracellular recording with glass microelectrodes (Freeman & Turner, 1970). Potentials were also recorded from sinoatrial and atrioventricular areas of isolated right atria from young (1·5 kg) New Zealand rabbits and atrial potentials were recorded from guinea-pig atria by a technique similar to that of Paes de Carvalho, Hoffman & Carvalho (1969). The action potential was differentiated to obtain the maximum rate of rise. This technique will be described in detail elsewhere.

Unless otherwise stated, experiments with the rat diaphragm preparation were carried out at 30° C. The toad sartorius muscle and sciatic nerve were maintained at 23° C, all isolated heart experiments were carried out at 37° C, as were the ileum experiments. The nutrient solutions used have been described (Freeman, 1968 ; Turner & Freeman, 1969). Because the action of the toxin was sensitive to changes in external Na^+, high K^+ solutions were made up without compensatory reduction in Na^+ level.

Results

Acute toxicity

The intravenous injection of either *C. magus* or *C. achatinus* toxin into unanaesthetized mice caused death after 3–30 minutes. The animals became ataxic and agitated; gasping respiration and convulsions preceded death. The heart continued to beat irregularly for some minutes after respiration ceased. The ani-

mals showed a characteristic extension of the hind limbs at death, and rigor set in rapidly. The bodies were quite rigid within 10 min of death.

Anaesthetized rabbits died within 10–40 min of the injection of 60–100 M.U. of *C. magus* toxin into the marginal ear vein (4 rabbits). Rats died 14–30 min after the injection of 5–10 M.U. of either *C. magus* or *C. achatinus* toxin into a lateral tail vein (4 rats). The cardiovascular changes were identical, whichever toxin was used. Figure 1 illustrates typical findings. After some minutes latency, rabbits showed an increase in systolic arterial pressure, with little change in diastolic pressure. The heart rate decreased, and there was a moderate increase in right atrial pressure. The respiratory rate decreased and respirations became shallow, largely intercostal, and finally ceased. The ECG showed no abnormality other than bradycardia until the animal became hypoxic, there was then intermittent atrioventricular blockade and gross T wave enlargement. The arterial pressure fell as the bradycardia became severe, but pulse pressure was maintained until

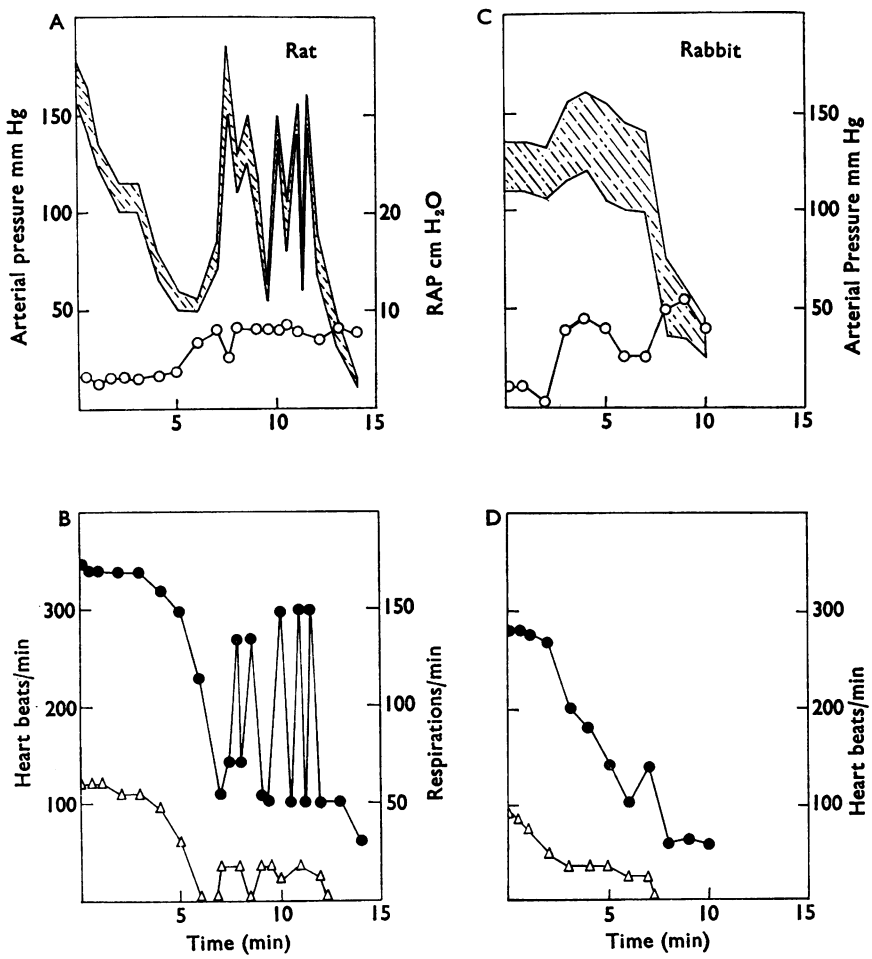

FIG. 1. The effect of *Conus magus* toxin on cardiovascular parameters in rat (A & B) and rabbit (C & D). The rat received 6 M.U. i.v. at time zero; the rabbit received 80 M.U. at time zero. Hatched areas show changes in systolic and diastolic pressure, right atrial pressure (R.A.P.) (○——○), heart rate (●——●) and respiratory rate (△——△). The amplitude of respiration fell markedly prior to apnoea.

close to death. It was possible to elicit a carotid occlusion reflex until the last few minutes of life; and the rabbits showed the usual arterial pressure increase in response to norepinephrine (2·5 μg/kg). Respiratory failure always preceded cardiovascular failure. The rabbits showed a moderate increase in salivation during the action of the toxin.

The response of rats to the toxins differed somewhat from the effects seen in rabbits. The rats showed no increase in systolic pressure such as was seen in the rabbit; there was a slow drop in arterial pressure, which preceded the onset of bradycardia. Coincident with this drop there was a moderate rise in right atrial pressure. Respiration became shallow and slowed, a period of apnoea of 60–90 s duration preceded several minutes of gasping, slow respiration before terminal apnoea. During this period of inadequate ventilation the arterial pressure showed marked oscillations. Figure 1 illustrates typical findings. The oscillations coincided with changes in heart rate, as the heart went through episodes of partial atrioventricular blockade.

The effect of Conus magus *toxin on phrenic nerve activity*

The respiratory depression induced by *C. magus* toxin in the rabbit was further examined by monitoring the impulses passing down the phrenic nerve, in conjunction with the output of a myograph needle placed in the diaphragm. The results of one such experiment are illustrated in Figure 2.

The anaesthetized animal had a respiratory rate of 36 per min, and an arterial pressure of 125/100 mmHg (1 mmHg≡1·333 mbar). Five min after a dose of 60 M.U. of *C. magus* toxin the arterial pressure fell to 115/90 mmHg, and myo-

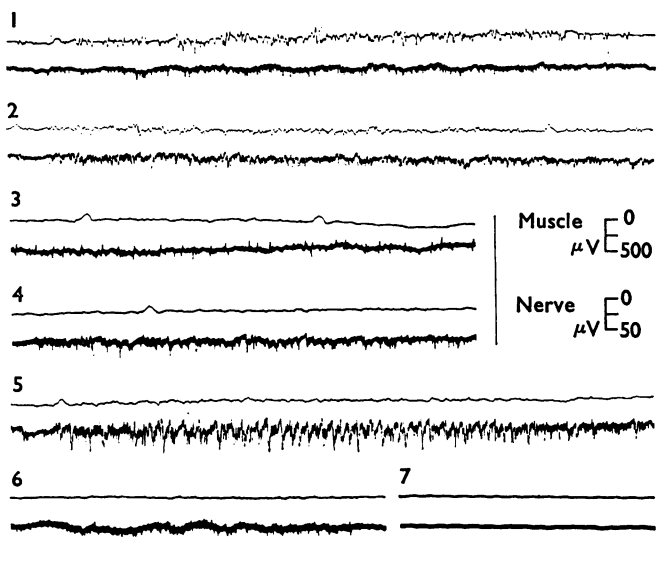

FIG. 2. The effect of *Conus magus* toxin on the electrical activity of the phrenic nerve (lower trace) and diaphragm (upper trace) of the rabbit. Panel 1, part of a control respiration; panel 2, 5 min after the i.v. injection of 60 M.U. toxin. Myograph activity has decreased, phrenic nerve activity has increased. Panel 3, 11 min after toxin. Panel 4, 14 min after toxin. Myograph and nerve records show reduced activity, respiration was intercostal. Panel 5, increased phrenic activity as the hypoxic animal made gasping attempts to breathe. Panel 6, 36 min after toxin, some phrenic activity is seen prior to death at 37 min (panel 7). Time marker: 20 ms.

graph activity was markedly reduced. At 11 min the myograph trace showed only a ripple of activity interspersed with ECG signals. At 14 min the myograph trace showed almost no activity, and breathing appeared to be entirely intercostal. At 20 min respiration was forced and gasping. The myograph record showed very little activity, but the phrenic volleys had increased markedly in frequency and amplitude. The arterial pressure was 130/105 mmHg, and the animal appeared cyanosed. As death approached at 37 min the phrenic volleys diminished until respiration became imperceptible. At this point the arterial pressure was 55/20 mmHg, and falling rapidly.

Effects on skeletal muscle

Neuromuscular blocking activity

The toxins of both species brought about complete blockade of the isolated phrenic nerve-diaphragm preparation of the rat at concentrations equal to or greater than 0·1 M.U./ml. There was an initial potentiation of the isometric twitch by 15–35%, coincident with an increase in base-line tension. This was followed after a latent period of 6–15 min by a decline in tension which followed an exponential time course. The time to half decay of tension was dose-dependent, and also varied between different animals. Preparations recovered very slowly from the paralysis, and even after washing for 180 min a second blockade occurred more rapidly than the first. It was expedient therefore to compare effects during the first blockade in paired hemi-diaphragms, since they blocked at closely similar rates.

It was found that the muscle failed to respond to direct stimulation at the same rate as the response to indirect stimulation declined. An increase in stimulating voltage partially restored muscle excitability, but it was not possible to break through the block completely. Curarization of the preparation with 2×10^{-6}M (+)-tubocurarine did not affect the rate of blockade of the directly stimulated muscle.

The initial twitch potentiation and increase in base-line tension were independent of the rate of stimulation of the muscles; however, the decline in twitch tension after *C. magus* toxin was approximately 8 times faster at stimulation rates of 1 Hz than it was at 0·1 Hz. This effect was also seen when *C. achatinus* toxin was used. Both the initial twitch potentiation and the rate of decline of twitch tension were sensitive to temperature change. At 25° C *C. achatinus* toxin did not potentiate the twitch, but after the usual latent period twitch tension fell off rapidly. Twitch potentiation and increase in base-line tension were maximal at 35° C, and twitch tension decayed at approximately half the rate seen at 25° C.

The ability of the diaphragm muscle to sustain a tetanus (100 Hz for 5 s) was not impaired by partial blockade with *C. achatinus* toxin. Low doses of *C. achatinus* toxin (0·05 M.U./ml), which caused little increase or decrease in twitch tension, slightly increased the twitch to tetanus ratio from 3·7 to 4·3. Post-tetanic potentiation was unchanged.

Effects of caffeine on the action of C. achatinus *toxin*

Caffeine (1 mM) increased the twitch tension of diaphragm muscle by 30–40%, but had little or no effect on base-line tension. At a concentration of 5 mM both

twitch and base-line tension were increased by caffeine. As may be seen in Fig. 3 caffeine (5 mM) added to the organ bath during muscle blockade by 0·1 M.U./ml *C. achatinus* toxin markedly increased base-line tension, but did not alter the rate of decay of twitch tension. The increase in base-line tension was more than additive. This was further demonstrated when 1 mM caffeine was added to the bath after 0·05 M.U./ml toxin. Neither toxin nor caffeine alone altered base-line tension, but this was considerably increased when they were present together.

Further, the caffeine-induced contracture of the toad sartorius muscle was increased by 0·1 M.U./ml *C. achatinus* toxin although the muscle twitch was not blocked by this toxin concentration. Toad muscle was also less sensitive than rat diaphragm to the blocking action of *C. magus* toxin.

Action of toxins on K^+-induced contracture

High K^+ solutions induce a transient contracture in the rat diaphragm muscle. High doses (greater than 0·15 M.U./ml) of either *C. magus* or *C. achatinus* reduced this contracture, while low doses enhanced it. The contracture induced by 92 mM K^+ was potentiated by either 1 mM caffeine or 0·05 M.U./ml *C. achatinus* toxin. After 60 min wash the toxin and caffeine were added together, resulting in an increase in base-line tension. The K^+ contracture was considerably potentiated by this treatment. After a further 60 min wash 0·1 M.U./ml caused little or no potentiation of the K^+ contracture. It was noted that treatment with *C. achatinus* toxin (0·05 M.U./ml) increased the depolarization of diaphragm muscle brought about by 46 mM K^+ solution. The control resting potential was $72{\cdot}6 \pm 0{\cdot}6$ mV (S.E. of 52 observations). K^+ (46 mM) reduced this potential by $29{\cdot}1 \pm 1{\cdot}0$ mV (S.E. of 21 observations). After washing 0·05 M.U./ml toxin was added; the resting potential was not altered by the toxin, but the subsequent addition of 46 mM K^+ reduced it by $33{\cdot}7 \pm 0{\cdot}7$ mV (S.E. of 21 observations). The difference between these two sets of data was significant ($P<0{\cdot}001$).

Effect of the toxins on muscle membrane potentials

The effect of *C. magus* toxin on the resting potential of rat diaphragm was determined by adding the toxin (0·2 M.U./ml) to the organ bath after obtaining a set of control readings. The depolarization due to the toxin was followed for periods of 45 minutes. The average control resting potential was $74{\cdot}4 \pm 0{\cdot}7$ mV (S.E. of 14 observations). This declined linearly to $51{\cdot}0 \pm 2{\cdot}3$ mV (S.E. of 8 obser-

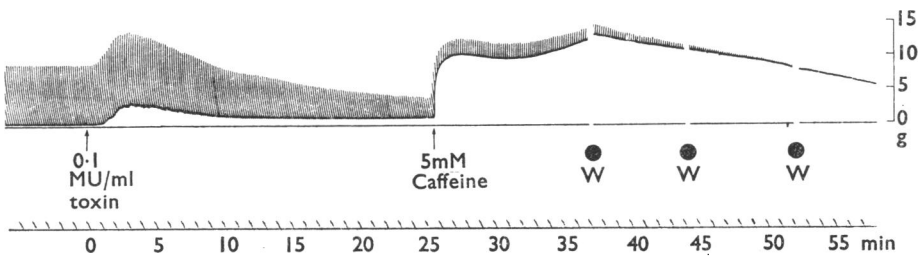

FIG. 3. The effect of *Conus achatinus* toxin on the directly stimulated diaphragm muscle of the rat. Addition of 0·1 M.U./ml toxin as shown caused an increase in base-line tension and twitch potentiation prior to blockade of twitch. Caffeine potentiated base-line contracture but did not affect blockade of twitch.

tions) at 45 minutes. The resting potential recovered slowly on washing the preparation. The effects of *C. achatinus* toxin were examined in more detail. Resting and action potentials were recorded, and the action potential was differentiated to obtain the maximum rate of rise (Jenerick, 1963). Table 1 shows the effects of 0·2 M.U./ml *C. achatinus* toxin on the rat diaphragm. Readings were taken between 9 and 40 min after addition of toxin to the solution perfusing the organ bath. Towards the end of the period of observation a number of the fibres penetrated would not fire in response to stimulation. Both resting and action potentials decreased, as did the maximum rate of rise of the spike. All parameters returned to the control level after 2–3 h washing.

The sartorius muscle of the toad was not significantly depolarized by 0·2 M.U./ml *C. magus* toxin.

Effects of ions and neurotoxins on depolarization of muscle membrane by C. achatinus *toxin*

The depolarization of rat diaphragm muscle by *C. achatinus* toxin resembles in many ways that reported for batrachotoxin (Albuquerque, Warnick & Sansone, 1971); the effects of low Na^+ solutions and tetrodotoxin confirmed this similarity. *C. achatinus* toxin at a dose level of 0·5 M.U./ml reduced the resting potential from 73·2±0·8 mV (12 fibres) to 41·3±1·1 mV (6 fibres) over a period of 25 min (Fig. 4a). It was necessary to wash the preparation for more than 100 min before there was a detectable recovery of the resting potential which had not entirely returned to the control level even after 3·5 h wash. The rate of recovery of the resting potential was unaffected by raising the level of Ca^{++} in the bathing solution to 15 mM, or by removal of the external K^+ or increasing the K^+ level to 18·4 mM (4 × normal). However, reduction of the external Na^+ level to 5 mM, with the addition of sucrose to maintain the osmolarity of the solution, caused a prompt repolarization of the muscle membrane (Fig. 4b). Similarly, the addition of tetrodotoxin (1 μg/ml) to the wash solution repolarized the membrane, as did saxitoxin (1 μg/ml). The time relationships of these effects are shown in Figures 4, a & b.

Effects on the nerve action potential

Both *C. magus* and *C. achatinus* toxin appeared to be entirely without effect on the desheathed toad sciatic nerve at doses up to 1 M.U./ml for 4 hours.

TABLE 1. *The effect of* Conus achatinus *toxin on membrane potentials of rat diaphragm*

	Control	Toxin (0·2 M.U./ml)	*P*
Action potential (mV)	95·1±2·3 (7)	77·5±5·8 (8)	0·02
Resting potential (mV)	71·4±1·0 (7)	62·3±2·9 (8)	0·02
Overshoot (mV)	23·7±2·1 (7)	15·3±3·2 (8)	0·05
Maximum rate of rise (V/s)	367 ±20 (7)	268 ±27 (8)	0·01

In this and subsequent tables, figures are shown ±S.E.M. The number of observations is shown in parentheses. *P* values refer to the significance of differences between control and treated values, using Student's *t* test. Readings in the presence of the toxin were taken between 9 and 40 min after addition of toxin to the bath.

Effects of the toxins on synaptic transmission

The finding that the toxins block the directly stimulated muscle and do not affect the nerve action potential suggests that they are myotoxins. The possibility exists, however, that they may also affect transmission across the neuromuscular junction. The chronically denervated rat diaphragm (Freeman & Turner, 1969b)

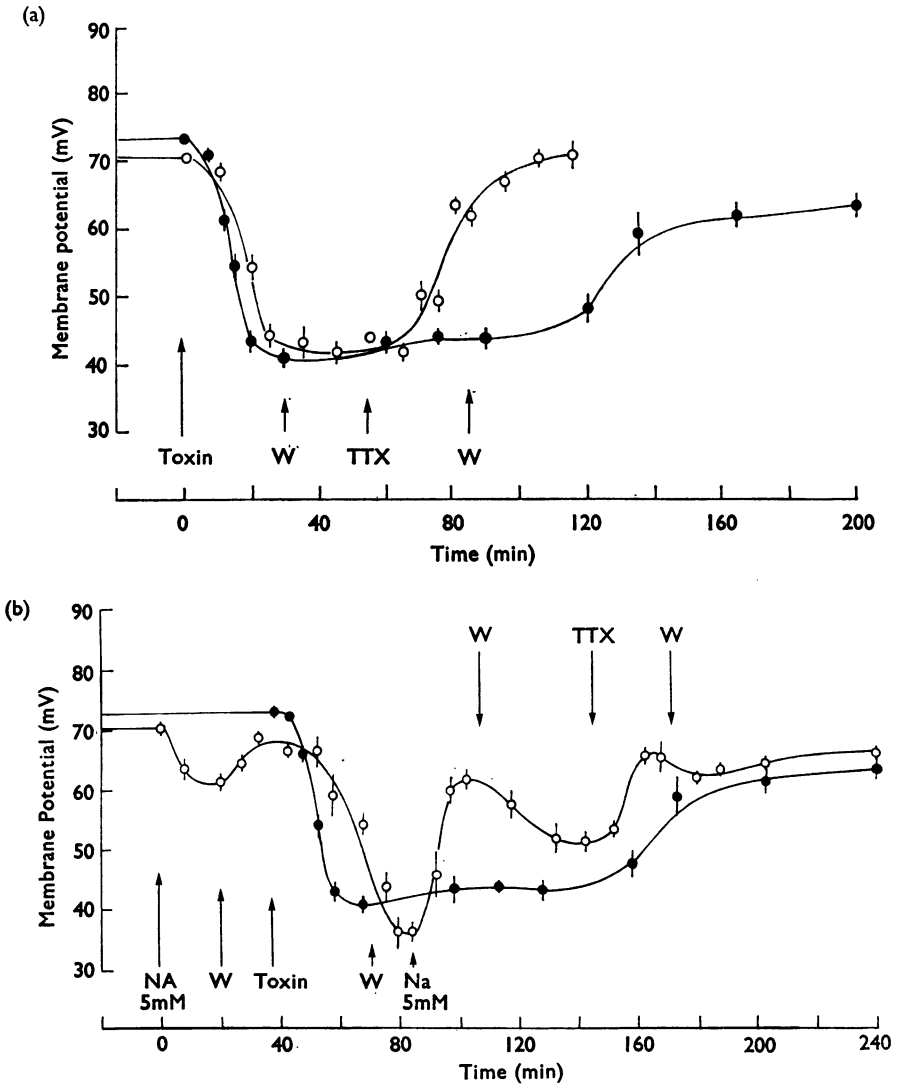

FIG. 4. (a), The effect of *Conus achatinus* toxin on the resting potential of rat diaphragm. Both diaphragms received the toxin (0·5 M.U./ml) at time zero; it was washed out at 30 minutes. The control preparation (filled circles) repolarized slowly over the next three hours. The treated preparation (open circles) received tetrodotoxin (1 μg/ml) at 55 min; this was washed out at 85 minutes. (b), The effect of *Conus achatinus* toxin and of low Na^+ concentration on the resting potential of the rat diaphragm. The control preparation (filled circles) was treated as in (a). The treated preparation (open circles) received 5 mM Na^+ solution at time zero, and depolarized slightly. The Na^+ level was restored to normal at 20 min, and both preparations received the toxin (0·5 M.U./ml) at 38 minutes. This was washed out at 70 min, and the treated preparation received 5 mM Na^+ solution at 85 minutes. A wash period at 105 min was followed by tetrodotoxin (1 μg/ml) between 145 and 168 minutes. Vertical bars represent ± S.E.M.

was used as a model of the postsynaptic receptor, and presynaptic function was determined from the estimate of miniature endplate potential frequency, and the quantal content, *m*, of the endplate potential (del Castillo & Katz, 1954).

Dose-response curves to acetylcholine were plotted for the chronically denervated rat diaphragm, and were repeated after the addition of *C. magus* toxin to the organ bath. Doses of 0·2 M.U./ml caused a slow reversible decline in tension in response to acetylcholine. On washing the preparation the response was potentiated for 60 min, suggesting that low doses of toxin might have potentiated the acetylcholine contraction. The depolarization of the preparation by acetylcholine was also reduced by *C. magus* toxin (0·5 M.U./ml). Acetylcholine (10^{-5}M) depolarized the denervated diaphragm by $16{\cdot}3 \pm 1{\cdot}3$ mV (15 observations), *C. magus* toxin (0·5 M.U./ml) depolarized the preparation by $8{\cdot}1 \pm 1{\cdot}1$ mV (15 observations). Acetylcholine added after the toxin brought about a further depolarization of $9{\cdot}7 \pm 2{\cdot}4$ mV (14 observations). After 35 min wash acetylcholine depolarized the membrane by $20{\cdot}7 \pm 1{\cdot}2$ mV (15 observations). After 60 min wash the acetylcholine depolarization returned to the control level of $16{\cdot}0 \pm 1{\cdot}7$ mV (14 observations).

Miniature endplate potentials (m.e.p.ps) were recorded from endplate regions of the rat diaphragm for periods of 100–400 seconds. In one experiment the control frequency was 1·9 s^{-1}; this was increased to 4·6 s^{-1} by 13·8 mM K^+, or to 2·7 s^{-1} by 4·5 mM Ca^{++}. At another junction the control rate was 2·2 s^{-1}. This fell to 1·6 s^{-1} during the first seven min after the addition of 0·2 M.U./ml *C. achatinus* toxin. Between 16 and 60 min after toxin addition the m.e.p.p. frequency was constant at 0·6 s^{-1}. At this time the K^+ level was raised to 13·8 mM; this increased the rate to 1·3 s^{-1}. Similarly, 4·5 mM Ca^{++} in the presence of the toxin increased the rate to 1·2 s^{-1}. At no time was the m.e.p.p. frequency increased by the toxin.

These findings suggest that the presynaptic release of acetylcholine is reduced by *C. achatinus* toxin. Determination of the quantal content of the endplate potential (e.p.p.) supported this suggestion.

Endplate regions of the rat diaphragm were located by repeated insertion of the microelectrode to find the site of largest miniature endplate potentials. The amplitude of the e.p.p. was then reduced by increasing the external Mg^{++} concentration to 12–13 mM and two series of e.p.ps obtained in response to approximately 200 nerve impulses were photographed; these were a control group, and a second group was obtained during a 25–30 min exposure to *C. achatinus* toxin.

The quantal content (*m*) of the e.p.p. was calculated from the equation

$$m = \log_e \frac{\text{number of nerve impulses}}{\text{number of failures of e.p.p. response}}$$

(del Castillo & Katz, 1954). The standard deviation of *m* was obtained from the expression $\frac{1-P}{NP}$ where P is the proportion of failures and N is the number of nerve impulses (Edwards & Ikeda, 1962). Statistical analysis of the results showed that there was no significant alteration in *m* ($P > 0{\cdot}25$) at the lower toxin level (0·1 M.U./ml) but there was a highly significant reduction ($P < 0{\cdot}001$) at the higher level (0·2 M.U./ml).

Effects on the rat ileum preparation

The acetylcholine dose-response curve of the rat ileum preparation was found to be entirely insensitive to *C. magus* toxin (0·2 M.U./ml) over a period of 45 minutes. The toxin at a level of 1 M.U./ml caused a small, transient increase in tension of the ileum and was either without effect on the response to acetylcholine, or in some experiments slightly potentiated it.

Effects of the toxins on cardiac muscle

Isolated, perfused guinea-pig heart

Isolated guinea-pig hearts were perfused with solutions containing the toxins at 0·2 M.U./ml or 0·5 M.U./ml. At both levels of the toxin there was an initial increase in coronary flow, which was maintained over the period of perfusion at the lower dose. At the higher dose the initial increase declined to below the control level after 5 minutes. Amplitude of contraction was reversibly increased over the period of toxin perfusion at both doses. Heart rate was not affected by the lower dose, but the high level caused a marked decrease in rate. Observation of the heart suggested that the bradycardia was the result of atrioventricular blockade. *C. achatinus* toxin (0·5 M.U./ml) caused similar changes in coronary flow, amplitude of contraction and rate.

Effects on atrial and nodal potentials of guinea-pig and rabbit

Since the toxins of both species of *Conus* reduced the rate of the isolated guinea-pig heart, it was decided to study the effect of *C. achatinus* toxin on the genesis and conduction of the action potential in the isolated atrium. The technique developed by Paes de Carvalho *et al.* (1959, 1969) was used to record potentials from sinoatrial and atrioventricular nodes of young rabbits. Atrial potentials were recorded from the isolated right atria of guinea-pigs; these preparations were electrically driven at 2 Hz. Maximum diastolic potentials, action potentials, and the rate of rise of the spike were recorded before and during perfusion of the organ bath with solutions of *C. achatinus* toxin.

Table 2 shows that potentials recorded from the guinea-pig atrium between 10 and 25 min after starting perfusion with *C. achatinus* toxin (0·5 M.U./ml) showed a significant decrease in spike height and maximum rate of rise of the spike. Neither the maximum diastolic potential nor the overshoot were significantly affected by the toxin over the period of observation.

TABLE 2. *Effect of* 0·5 *M.U./ml* Conus achatinus *toxin on guinea-pig atrium*

Treatment	Action potential (mV)	Max. diastolic potential (mV)	Overshoot (mV)	Max. rate of rise (*V*/s)
Control	89·3±1·7 (9)	68·7±1·6 (9)	20·7±1·3 (9)	173±8 (9)
0·5 M.U./ml toxin	81·6±2·5 (9)	62·7±2·4 (9)	19·6±1·0 (9)	117±5 (9)
P	0·03	0·06	0·5	<0·001

Data were obtained between 10 and 25 min after the addition of toxin to the perfusate. The atrium was stimulated at 2 Hz.

Tables 3 and 4 show the effects of the toxin on sinoatrial and atrioventricular potentials recorded from the rabbit atrium. It is notable that the lower toxin dose (0·3 M.U./ml) significantly increased spike height, maximum diastolic potential, and the rate of rise of the spike at the S-A node. At 0·8 M.U./ml the toxin did not affect the node; possibly an even higher dose might have depressed it.

Atrioventricular potentials were unaffected by 0·3 M.U./ml of toxin; 0·5 M.U./ml caused a just significant increase in the rate of rise of the spike.

C. achatinus toxin caused a dose-dependent decrease in the rate of the spontaneously beating rabbit atrium. At a dose of 0·8 M.U./ml this decrease was approximately 20%.

TABLE 3. *The effect of* Conus achatinus *toxin on rabbit sinoatrial potentials*

Treatment	Action potential (mV)	Max. diastolic potential (mV)	Overshoot (mV)	Max. rate of rise (*V*/s)
Control	63·0±3·6 (10)	48·8±1·9 (10)	14·2±2·9 (10)	13·5±1·1
0·3 M.U./ml toxin	78·3±3·4 (7)	57·7±2·2 (7)	19·3±3·0 (7)	17·3±1·1 (7)
P	0·01	0·01	0·25	0·04
Control	58·0±2·0 (12)	53·8±1·9 (12)	4·2±2·1 (12)	6·2±0·6 (12)
0·8 M.U./ml toxin	53·7±3·6 (7)	51·1±2·6 (7)	2·6±2·4 (7)	6·1±0·4 (7)
P	0·25	0·4	0·65	0·9

Data were obtained between 10 and 30 min after the addition of toxin to the perfusate. The preparation was allowed to beat spontaneously.

TABLE 4. *The effect of* Conus achatinus *toxin on rabbit atrioventricular potentials*

Treatment	Action potential (mV)	Max. diastolic potential (mV)	Overshoot (mV)	Max. rate of rise (*V*/s)
Control	65·6±2·5 (10)	55·2±1·4 (10)	10·4±2·3 (10)	8·0±0·3 (10)
0·3 M.U./ml toxin	61·4±2·9 (7)	57·1±3·8 (7)	4·3±1·4 (7)	7·4±0·6 (7)
P	0·3	0·6	0·07	0·3
Control	66·2±2·3 (12)	55·7±1·2 (12)	10·5±2·0 (12)	8·6±0·6 (12)
0·5 M.U./ml toxin	70·3±3·3 (8)	58·6±2·5 (8)	10·0±1·7 (8)	11·5±1·3 (8)
P	0·3	0·25	0·9	0·04

Data were obtained between 10 and 25 min after addition of toxin to the perfusate. The preparation was allowed to beat spontaneously.

Discussion

The toxins secreted by the venom ducts of *C. magus* and *C. achatinus* have similar pharmacological properties. They cause death in animals by producing a characteristic spastic paralysis. The animals die of respiratory failure; the heart does not fail until the animals become hypoxic. Nerve conduction appears to be unimpeded. The phrenic nerve of the rabbit remained capable of conduction until death, and the respiratory centre responded to hypoxia by increasing the amplitude and frequency of phrenic volleys. High levels of either toxin were also without effect on the toad sciatic nerve.

Striated skeletal muscle is more sensitive to the toxin than cardiac or smooth muscle. The rat diaphragm is more sensitive than the toad sartorius muscle. We confirmed the finding of Endean & Izatt (1965) that *C. magus* toxin causes a reversible increase in the base-line isometric tension of the rat diaphragm. There was an initial potentiation of the isometric twitch, followed by a frequency-dependent decline in twitch tension. Although a common factor may mediate the effects on base-line tension and the isometric twitch, there were differences in their response to various experimental procedures.

1. The contracture was independent of the frequency of stimulation of the preparation, but twitch tension declined more rapidly at high stimulation frequencies than at low rates.

2. The contracture was inhibited by low temperature, although blockade of the isometric twitch was more rapid at 25° C than at 35° C.

3. Caffeine greatly potentiated the contracture, but did not alter the rate of the blockade of the twitch.

4. Recovery from the contracture was more rapid on washing the preparation than was recovery of the twitch.

The K^+-induced contracture of diaphragm muscle was potentiated by low doses of toxin and/or caffeine. The potentiation of the K^+ contracture was associated with an increase in K^+ depolarization, although the low dose of toxin did not alter the resting potential *per se*.

The toxins of both Conidae depolarized the diaphragm muscle. Amplitude and maximum rate of rise of the action potential were both reduced. Spike propagation failed when the resting potential had fallen to about 60 mV.

The decreases in miniature endplate potential frequency and quantal content of the endplate potential after administration of 0·2 M.U./ml toxin suggest an impairment of transmitter release. In so far as the acetylcholine receptor of the denervated diaphragm may be considered an analogue of the postsynaptic receptor, it appears that low toxin levels (0·05 M.U./ml) may facilitate the acetylcholine-induced depolarization while higher, depolarizing doses (0·1 M.U./ml) may reduce it. Membrane depolarization may be the basis of twitch blockade, although the contracture induced by the toxin, and its potentiation by caffeine, suggests the involvement of the process of excitation-contraction coupling.

These actions on diaphragm muscle resemble those of batrachotoxin (Warnick, Albuquerque & Sansone, 1971 ; Albuquerque *et al.*, 1971). Both *Conus* toxin and batrachotoxin cause depolarization, contracture and a frequency dependent twitch blockade. In addition, the depolarization produced by batrachotoxin or *Conus* toxins is rapidly reversed by low Na^+ solutions and tetrodotoxin, suggesting a similarity in their action on the muscle membrane.

There are, however, several important differences. Whereas batrachotoxin causes an irreversible muscle blockade, *Conus* toxin has a slowly reversible effect. Further, we did not observe an initial increase in m.e.p.p. frequency prior to the decrease as was a feature of batrachotoxin action. Such an increase would be expected if the effects of *Conus* toxins at the neuromuscular junction were simply due to depolarization. Cardiac muscle is more sensitive to batrachotoxin than is skeletal muscle (Hogan & Albuquerque, 1971) whereas the converse is true for *Conus* toxin.

The difference in molecular weight of the two toxins (batrachotoxin 538, *C. achatinus* toxin approximately 10,000) need not indicate a difference in action, since we have no information about a possible prosthetic group on the *Conus* toxin molecule.

Many of the actions of *C. achatinus* toxin are consistent with an alteration of Ca^{++} binding in the muscle membrane. The stabilizing effects of Ca^{++} on biological membranes are well known, and specific interaction with a stabilizing site could lead to depolarization by Na^{+} entry. An increased lability of the muscle membrane was seen with low doses of toxin, which increased the depolarization by a given level of K^{+}. Interference with Ca^{++} binding by the sarcotubular system would also be consistent with the contracture-producing effects of *Conus* toxin. Further, the increase in isotonic contractility of the isolated guinea-pig heart could result from Ca^{++} release from a membrane binding site into the myoplasm. Paes de Carvalho *et al.* (1969) have suggested the importance of Ca^{++} in the genesis of pacemaker potentials. The potentiation of sinoatrial potentials by *Conus* toxin would therefore be consistent with an increase in Ca^{++} permeability. The specific antagonism of the depolarization of the muscle membrane by tetrodotoxin, saxitoxin and low Na^{+} solutions suggests that Ca^{++} movements may be secondary to an alteration in Na^{+} permeability. This would agree with the reasoning of Albuquerque *et al.* (1971) as to the role of batrachotoxin. However, it is known that tetrodotoxin can reduce Ca^{++} entry into squid axons (Baker, Hodgkin & Ridgeway, 1971), so that its antagonism of the depolarization by *Conus* toxin could involve a change in the binding of membrane Ca^{++}.

Consequently the tentative hypothesis is put forward that the primary action of *Conus achatinus* toxin is to bind to a Ca^{++} site on the muscle membrane, reducing Ca^{++} binding and making the membrane unstable. The action of tetrodotoxin and saxitoxin would therefore be that of stabilizing agents rather than of specific antagonists. Results of future experiments may or may not support this hypothesis; however, it is clear that *C. achatinus* toxin is an interesting addition to the range of compounds which act on excitable membranes.

REFERENCES

ALBUQUERQUE, E. X., WARNICK, J. E. & SANSONE, F. M. (1971). The pharmacology of batrachotoxin II. Effect on electrical properties of the mammalian nerve and skeletal muscle membranes. *J. Pharmacol. exp. Ther.*, **176**, 511–528.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.*, **218**, 709–755.

CLELAND, J. B. & SOUTHCOTT, R. V. (1965). *Injuries to man from marine invertebrates in the Australian region.* Commonwealth of Australia, Canberra, A.C.T.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.*, **124**, 560–573.

EDWARDS, C. & IKEDA, K. (1962). Effects of 2-PAM and succinylcholine on neuromuscular transmission in the frog. *J. Pharmac. exp. Ther.*, **138**, 322–327.

ENDEAN, R. & IZATT, J. (1965). Pharmacological study of the venom of the gastropod *Conus magus*. *Toxicon*, **3**, 81–93.

ENDEAN, R. & RUDKIN, C. (1963). Studies on the venoms of some Conidae. *Toxicon*, **1**, 49–64.

FREEMAN, S. E. (1968). Ionic influences on succinylcholine blockade of the mammalian neuromuscular junction. *Br. J. Pharmac.*, **32**, 546–566.

FREEMAN, S. E. & TURNER, R. J. (1969a). A pharmacological study of the toxin of a cnidarian *Chironex fleckeri* Southcott. *Br. J. Pharmac.*, **35**, 510–520.

FREEMAN, S. E. & TURNER, R. J. (1969b). Ionic interactions in the acetylcholine contraction of the denervated rat diaphragm. *Br. J. Pharmac.*, **36**, 510–522.

FREEMAN, S. E. & TURNER, R. J. (1970). Facilitatory drug action on the isolated phrenic nerve-diaphragm preparation of the rat. *J. Pharmac. exp. Ther.*, **174**, 550–559.

HALSTEAD, B. W. (1965). *Poisonous and venomous marine animals of the world. Vol.* 1. Washington D.C.: U.S. Govt. Printing Office.

HOGAN, P. M. & ALBUQUERQUE, E. X. (1971). The pharmacology of batrachotoxin III. Effect on the heart Purkinje fibres. *J. Pharmac. exp. Ther.*, **176**, 529–537.

JENERICK, H. (1963). Phase plane trajectories of the muscle spike potential. *Biophys. J.*, **3**, 363–378.

KOHN, A. J., SAUNDERS, P. R. & WIENER, S. (1960). Preliminary studies on the venom of the marine snail *Conus*. *Ann. N.Y. Acad. Sci.*, **90**, 706–725.

PAES DE CARVALHO, A., DE MELLO, W. C. & HOFFMAN, B. F. (1959). Electrophysiological evidence for specialized fiber types in rabbit atrium. *Amer. J. Physiol.*, **196**, 483–488.

PAES DE CARVALHO, A., HOFFMAN, B. F. & DE PAULA CARVALHO, M. (1969). Two components of the cardiac action potential. I. Voltage-time course and the effect of acetylcholine on atrial and nodal cells of the rabbit heart. *J. gen. Physiol.*, **54**, 607–635.

TURNER, R. J. & FREEMAN, S. E. (1969). Effects of *Chironex fleckeri* toxin on the isolated perfused guinea-pig heart. *Toxicon*, **7**, 277–286.

WARNICK, J. E., ALBUQUERQUE, E. X. & SANSONE, F. M. (1971). The pharmacology of batrachotoxin I. Effects on the contractile mechanism and on neuromuscular transmission of mammalian skeletal muscle. *J. Pharmacol. exp. Ther.*, **176**, 497–510.

WHYTE, J. M. & ENDEAN, R. (1962). Pharmacological investigation of the venoms of the marine snails, *Conus textile* and *Conus geographus*. *Toxicon*, **1**, 25–31.

(*Received April* 19, 1972)