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EFFECT OF PHOSPHOLIPASE A ON ACTIONS OF COBRA VENOM CARDIOTOXINS ON ERYTHROCYTES AND SKELETAL MUSCLE

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The actions of two phospholipase-free cardiotoxins from the venom of the cobra *Naja naja siamensis* were compared to phospholipase-contaminated cardiotoxins in terms of their ability to lyse human erythrocytes and to depolarize and contract skeletal muscle. The presence of 3–5% (w/w) phospholipase caused a 20–30-fold increase in the haemolytic activity of the two cardiotoxins, the pure cardiotoxins being virtually without haemolytic activity at 10^{-7}–10^{-6} M. Phospholipase contamination did not enhance the ability of the cardiotoxins to cause contracture of chick biventer cervicis muscles and it caused less than a 2-fold increase in the depolarizing activity of the cardiotoxins on cultured skeletal muscle. Phospholipase-free cardiotoxins were about 10–20-times more active on cultured skeletal muscle fibres than on erythrocytes. These results support the hypothesis that some cardiotoxins have more affinity for the membranes of excitable cells than for those of other cells such as erythrocytes.

Introduction

In addition to the postsynaptic neurotoxins that bind to nicotinic cholinoceptors of skeletal muscle, cobra venoms also contain another group of homologous polypeptides known as cardiotoxins or membrane-active toxins (for review, see Ref. 1). The mode of action of the cardiotoxins has not yet been established but they produce a range of effects, including haemolysis and depolarization of excitable cells [2–4]. It is often assumed that death following administration of a cardiotoxin is caused by ventricular fibrillation which results from depolarization of cardiac muscle cells by a direct action of the cardiotoxin reinforced by depolarization induced by release of K^+ from lysed erythrocytes. However, there are a number of reports that are not consistent with this view. For example, three cardiotoxins from the venom of the Cape cobra, *Naja nivea* had the same LD_{50} value as cardiotoxin $V^{11}2$ from *Naja mossambica mossambica* but only 1–2% of the haemolytic activity [5]. Similarly, lethal cardiotoxins with little haemolytic activity are found in venom of *Naja naja siamensis* [6].

A complicating factor in the determination of the mechanism of action of cardiotoxins is the possibility of contamination with phospholipases and subsequent enhancement of the biological action of the cardiotoxins. Traces of phospholipase A_2 can be removed from cardiotoxins by gel filtration in urea [7], hydrophobic chromatography [8] or immunoaffinity chromatography [9]. Previously, it has been shown that even 0.1% phospholipase A_2 greatly enhances the haemolytic activity of some cardiotoxins [10,11], but it is not known whether this synergistic action is important for the lethality of the cardiotoxins or their action on excitable cells. High concentrations of phospholipase A have been shown to accelerate the de-

polarizing effects of a cardiotoxin from the Taiwan cobra, *Naja naja atra* [12], although it was not demonstrated in this study that the cardiotoxin fraction itself was devoid of phospholipase activity.

In view of the proposal that the cardiotoxin group of polypeptides may contain lytic and non-lytic subgroups [6], we wanted to establish clearly the effect of phospholipase contamination on the action of cardiotoxins on erythrocytes and on excitable cells. Our results with cardiotoxins from *Naja naja siamensis* demonstrate that phospholipase A_2 greatly enhances the haemolytic potency of the cardiotoxins but does not markedly increase their ability to depolarize and produce contracture of skeletal muscle cells.

Methods

Isolation and purification of cardiotoxins

A complete description of the purification procedure has been published elsewhere [13]. *Naja naja siamensis* (Thailand cobra) venom, purchased from Sigma Chemical Co. (U.K.), was separated on Sephadex G-50 and the toxin fraction was subjected to ion exchange chromatography on SP Sephadex C-25. The cardiotoxin fractions were seriously contaminated with phospholipase activity in contrast to the neurotoxin fractions. This contamination was successfully removed by two cycles of either hydrophobic chromatography on phenyl-Sepharose CL-4B [8] or immunoaffinity chromatography [9].

Assay for phospholipase A_2 activity

The procedure was based on that reported by Zahler and co-workers [14]. Phosphatidylcholine (0.3 μmol) made up to a specific activity of about $1.8 \cdot 10^5$ dpm · μmol^{-1} with [*N-methyl*-^{14}C]phosphatidylcholine, was dried under reduced pressure. Enzyme containing solution was added in the presence of Tris-HCl (10 mM, pH 8) and $CaCl_2$ (4 mM). The mixture was incubated at 37°C for 60 min with continuous shaking. The reaction was stopped by the addition of EDTA (30 μl, 0.2 M). The lipids present in the mixture were then extracted as described by Renkonen et al. [15]. Aliquots of the separated phospholipids were chromatographed on silica gel plates (Kieselgel 60 G) with chloroform/methanol/water/acetic acid (14 : 6 : 1 : 0.5, v/v). After drying, the plates were stained with iodine vapour. Individual lipid spots were scraped directly into scintillation vials, and the vials were counted for ^{14}C content. The percentage of [^{14}C]phosphatidylcholine converted to [^{14}C]lysophosphatidylcholine was estimated and the phospholipase activity expressed as μmol phosphatidylcholine hydrolysed per min.

Haemolysis assay

Fresh heparinised human blood was washed three times with NaCl (130 mM), containing $CaCl_2$ (1 mM) and buffered with Tris-HCl (20 mM, pH 7.4). The washed cells were resuspended 1 : 500 (by volume) in buffered NaCl. Aliquots of the cell suspensions (12 ml) were incubated at 37°C with cardiotoxins (10^{-8}–10^{-4} M) for up to 90 min. Samples were removed after various incubation times, centrifuged for 30 s on a Beckmann microfuge and the absorption of the supernatant was monitored at 578 nm.

Chick biventer cervicis nerve-muscle preparation

Biventer cervicis nerve-muscle preparations were isolated from chicks aged 2–10 days and mounted in 10 ml organ baths with a resting tension of approx. 0.5 g in physiological salt solution (NaCl, 6.92; KCl, 0.35; $MgSO_4$, 0.29; $CaCl_2$, 0.28; KH_2PO_4, 0.16; $NaHCO_3$, 2.1; glucose, 2.0 g · l^{-1}). The solution was maintained at 32°C and aerated with 95% O_2 and 5% CO_2. The preparations were stimulated indirectly via the motor nerve in the tendon (0.1 Hz, 0.2 ms pulse width and voltage greater than that required for maximal contractions). Contractions and contractures were recorded isometrically on a Grass 79B Polygraph using Grass FT03 force displacement transducers. Preparations were allowed to stabilise for about 20 min before addition of cardiotoxin fractions. The actions of the cardiotoxins were quantified by measuring the delay, i.e. the time from injection to the first perceptible increase in resting tension; the time taken for the twitch response to be reduced to 50% of the control value; the time to peak contracture measured from the time of addition of the cardiotoxin; and the contracture height, expressed as a percentage of the height of the pre-toxin twitch responses (Fig. 1).

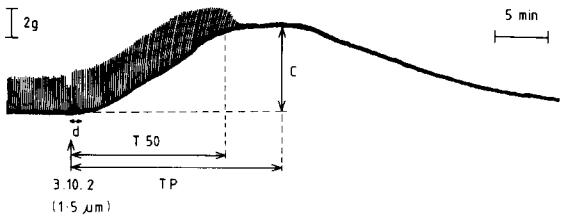

Fig. 1. Effect of cardiotoxin 3.10.2 on chick biventer cervicis muscle preparation. The muscle was stimulated indirectly through its motor nerve once every 10 s. Fraction 3.10.2 (1.5 μM) was added at the arrow. Delay (d), time to 50% block of twitches ($T50$), time to peak contracture (TP) and contracture height (C) were measured as indicated.

Skeletal muscle cultures

Suspensions of myoblasts were obtained from the leg muscles of 10–11-day chick embryos by exposure to 0.1% trypsin for 30 min at 37°C. Two ml of medium containing $5 \cdot 10^5$ cells $\cdot$ ml^{-1} were added to a 35 mm plastic Petri dish that had previously been coated with collagen. The growth medium was Eagle's minimum essential medium [16] containing 5% of a 50% (v/v) whole embryo and 15% horse serum. In order to prevent overgrowth of replicating fibroblast cells the medium was replaced on the second day of culture, after the beginning of myoblast fusion, by one containing 10 μM cytosine arabinoside, a DNA synthesis inhibitor. Thereafter, the medium was replaced on every third day with fresh medium. Cultures were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Myotube formation occurred at 2–3 days and cultures were 5–10 days old when used.

For experiments, cultures were mounted on an inverted phase-contrast microscope. Most experiments were performed at room temperature (20–22°C) but in some experiments the cultures were maintained at 34°C on a heated stage. Membrane potentials were recorded with an intracellular glass microelectrode filled with 3 M potassium chloride (electrode resistance 10–20 MΩ) and mounted obliquely on a Leitz micromanipulator. Potential differences between the penetrating microelectrode and a chlorided silver wire bath electrode were monitored via a d.c. preamplifier on an oscilloscope and on a pen recorder. All recordings were made in Eagle's minimum essential medium or an equivalent salt solution, which was maintained at pH 7.4 by bubbling with CO_2. Membrane potentials were sampled at random to estimate control levels, the bathing solution removed and replaced with one containing the required concentration of cardiotoxin. Membrane potentials were measured from as many cells as possible, with values obtained in each 2-min period being averaged.

Results

Toxin isolation and phospholipase activity

Following ion-exchange chromatography on SP Sephadex C-25, two major (3.10 and 3.12) and one minor (3.11) cardiotoxin fractions were isolated [13]. Fractions 3.10 and 3.12 were used in subsequent experiments. Both of these fractions had some phospholipase activity. This activity was not readily detectable by assays based on pH stat methods, but could be measured from the hydrolysis of ^{14}C-labelled phosphatidylcholine. Fractions 3.10 and 3.12 contained phospholipase activity equivalent to 0.9 ± 0.3 and 1.8 ± 0.3 μmol phosphatidylcholine hydrolysed per min per mg protein, respectively. This represents a contamination of the cardiotoxins with about 3–5% (w/w) phospholipase.

Following one or two cycles of hydrophobic chromatography, cardiotoxins 3.10.2 and 3.12.1 were isolated. Phospholipase contamination of these two fractions was $< 0.005\%$ (w/w).

From amino acid analysis, 3.10.2 was found to be identical to cardiotoxin F8 [17], CM6 [18], and CTXII [19], and 3.12.1 was identical to CM7 [18] and CTXIV [19] isolated previously from the same venom.

Haemolytic activity

The four fractions (3.10, 3.10.2, 3.12 and 3.12.1) were all haemolytic, although the two contaminated with phospholipase A were markedly more potent (Figs. 2 and 3). The influence of contaminating phospholipase is clearly illustrated by the EC_{50} values for haemolysis at 1 h, which shifts from $6 \cdot 10^{-7}$ M (for 3.10) to $3 \cdot 10^{-5}$ M (for 3.10.2) and from $2 \cdot 10^{-7}$ M (for 3.12) to $7 \cdot 10^{-6}$ M (for 3.12.1) (Fig. 3). The cardiotoxin 3.10.2 was virtually nonlytic at concentrations $\leqslant 10^{-5}$ M.

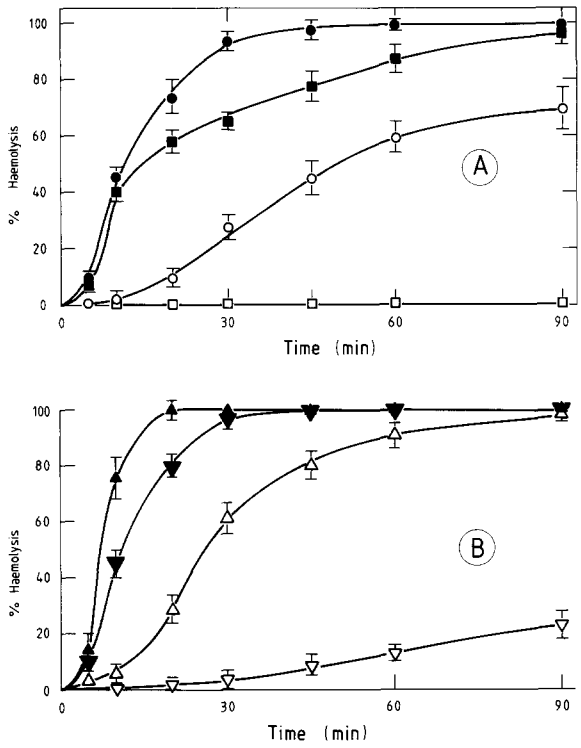

Fig. 2. Time response of human erythrocyte lysis in the presence of (A), 3.10: ●, 10^{-4} M; ○, 10^{-6} M; 3.10.2: ■, 10^{-4} M; □, 10^{-6} M; and (B), 3.12: ▲, 10^{-4} M; △, 10^{-6} M; 3.12.1: ▼, 10^{-4} M; ▽, 10^{-6} M. The medium consisted of NaCl (130 mM), $CaCl_2$ (1 mM) buffered to pH 7.4 by Tris-HCl (20 mM) and the packed cells were diluted 1:500 by volume. Each point represents the mean of three experiments. Standard errors are indicated by the vertical bars.

Effects on chick biventer cervicis preparations

The four fractions produced very similar effects on indirectly stimulated preparations. At concentrations of $1.5 \cdot 10^{-7}$ M and above, the cardiotoxins caused, after a slight delay, a contracture that was accompanied by inhibition of the responses to stimulation (Fig. 1). The contracture waned spontaneously but there was no recovery of twitch responses. With increases in concentration, the delay time to block twitches and the time taken to reach maximum contracture became less and the maximum size of the contracture increased (Fig. 4). The maximum effect was produced at 3–6 μM.

As judged by speed and size of contracture, there was no significant difference in the potency of the four fractions (Fig. 4). However, fractions

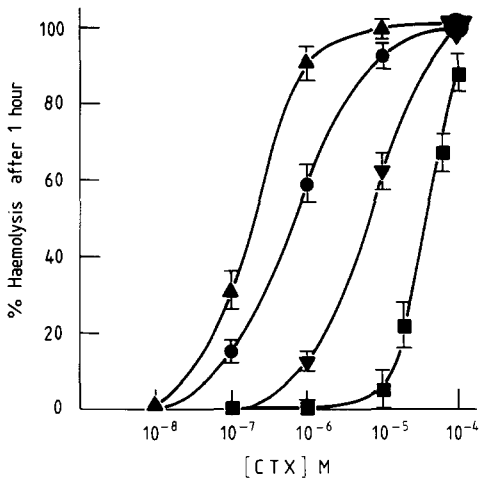

Fig. 3. Dose response curves for cardiotoxin-induced human erythrocyte lysis, (% haemolysis after 1 h). ●, 3.10; ■, 3.10.2; ▲, 3.12 and ▼, 3.12.1. Incubation conditions as for Fig. 2. Each point represents the mean of three experiments. Standard errors are indicated by the vertical bars, unless smaller than symbols. CTX, cardiotoxin.

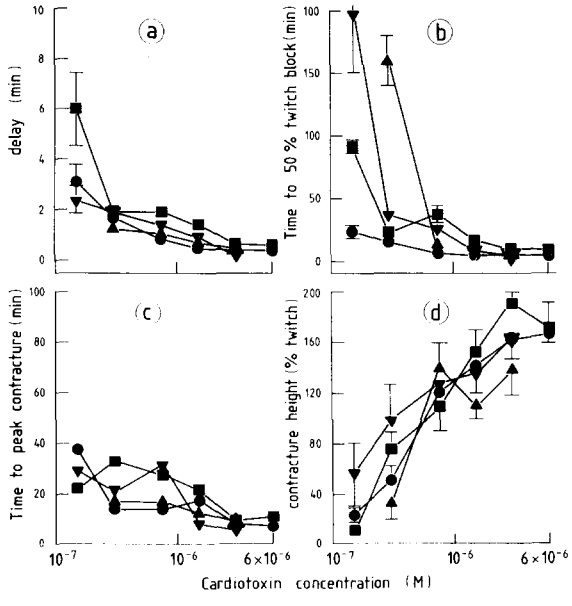

Fig. 4. Effects of the four cardiotoxin fractions on chick biventer cervicis muscle preparations. (a) Delay, (b) time to 50% twitch block, (c) time to peak contracture and (d) contracture height (as a percentage of the control twitch height). ●, 3.10; ■, 3.10.2; ▲, 3.12 and ▼, 3.12.1. Each point represents the mean of experiments on four to eight preparations. Standard errors are indicated by the vertical bars unless smaller than the symbols.

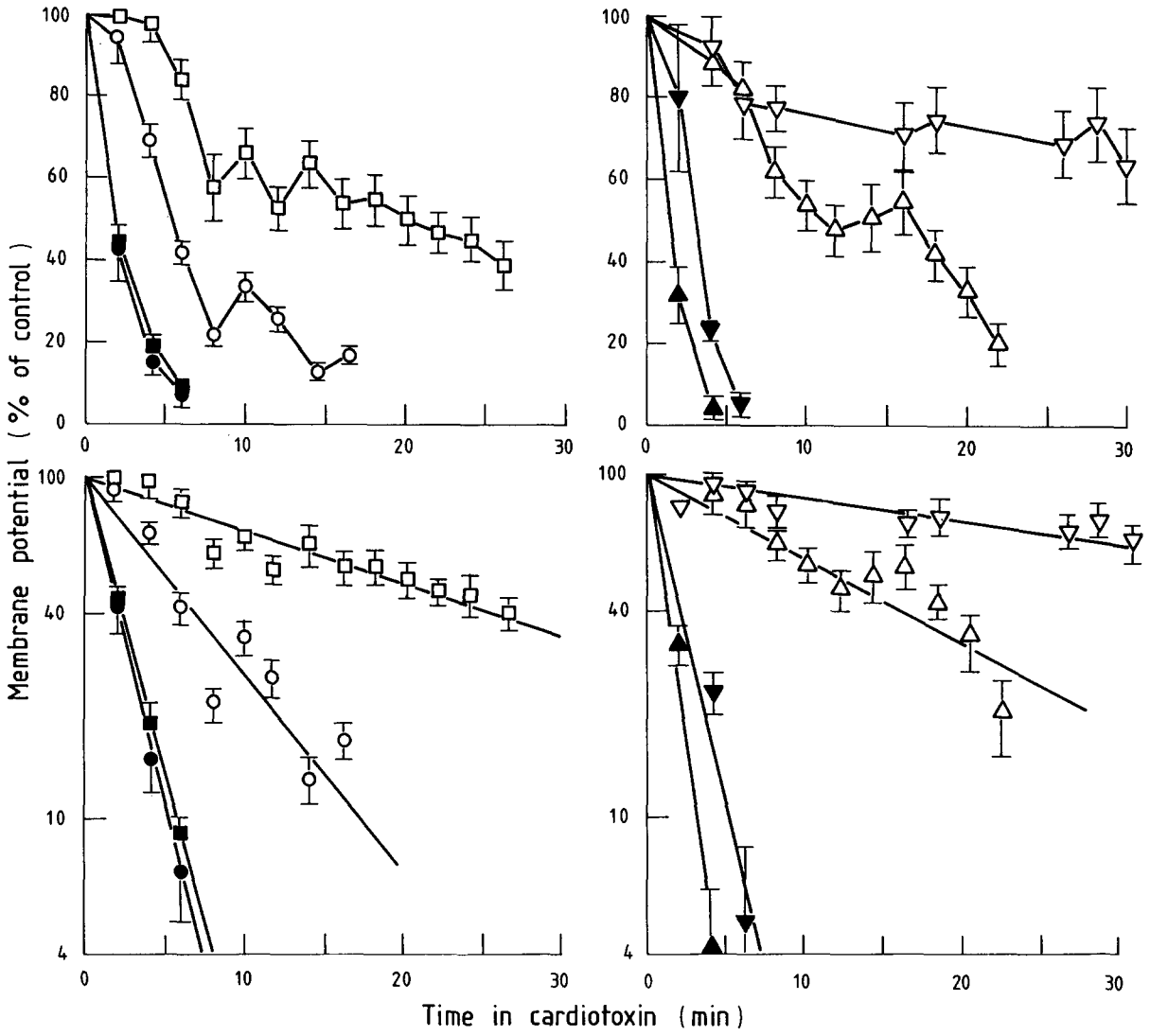

Fig. 5. Depolarization of cultured muscle fibres by the four cardiotoxin fractions. Upper graphs are plotted on an arithmetic scale, and the same results are plotted on a semilogarithmic scale on the lower graphs. 3.10: ●, $3 \cdot 10^{-6}$ M; ○, $0.75 \cdot 10^{-6}$ M; 3.10.2: ■, $3 \cdot 10^{-6}$ M; □, $0.75 \cdot 10^{-6}$ M. 3.12: ▲, $1.5 \cdot 10^{-6}$ M; △, 10^{-7} M. 3.12.1: ▼, $1.5 \cdot 10^{-6}$ M; ▽, 10^{-7} M. Each point represents the mean of measurement in at least six cells from two to three different cultures. The control membrane potentials have been averaged and taken as 100%; they ranged from −35 to −45 mV.

3.10 and 3.10.2 appeared to be slightly more effective at producing twitch block than fractions 3.12 and 3.12.1.

Effects on skeletal muscle cells in culture

All four fractions rapidly depolarized skeletal muscle fibres in culture (Fig. 5). The rate of depolarization increased with concentration of cardiotoxin, and the time course of depolarization was exponential (Fig. 5). The threshold for causing depolarization was 0.1 μM or lower for 3.12 and 3.12.1, but about 0.5 μM for 3.10 and 3.10.2. In order to compare the relative potency of the four fractions the rate at which they depolarized cultured myotubes was compared (Fig. 6). Maximal effectiveness for all four fractions was about 5 μM but fractions 3.12 and 3.12.1 were generally 2–3-times more potent than 3.10 and 3.10.2. The phos-

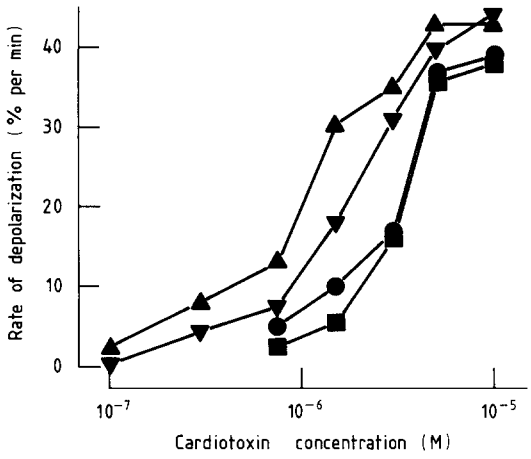

Fig. 6. Concentration dependence of the depolarizing activity of the four cardiotoxin fractions. At each concentration, the rate of depolarization was calculated by regression analysis from 100% to 10% of control membrane potential. ●, 3.10; ■, 3.10.2; ▲, 3.12 and ▼, 3.12.1

pholipase containing 3.12 was more active than the corresponding pure cardiotoxin, 3.12.1 but the difference was less than 2-fold. Fractions 3.10 and 3.10.2 at concentrations of 3 μM and above had indistinguishable potencies.

The cardiotoxin-induced depolarization was accompanied by visible cell damage. The first signs included loss of a clear cross-striated pattern within the cells and appearance of small bubbles of cytoplasm extruding from the cells (Fig. 7). Eventually the muscle fibres peeled off the surface of the petri dish or disintegrated. Damage induced by the cardiotoxins was more evident at high concentrations (1.5 μM and above) but even at these levels damage appeared to follow rather than precede depolarization. Although hard to quantify, fractions 3.12 and 3.12.1 were at least twice as effective as fractions 3.10 and 3.10.2 at damaging muscle fibres in culture.

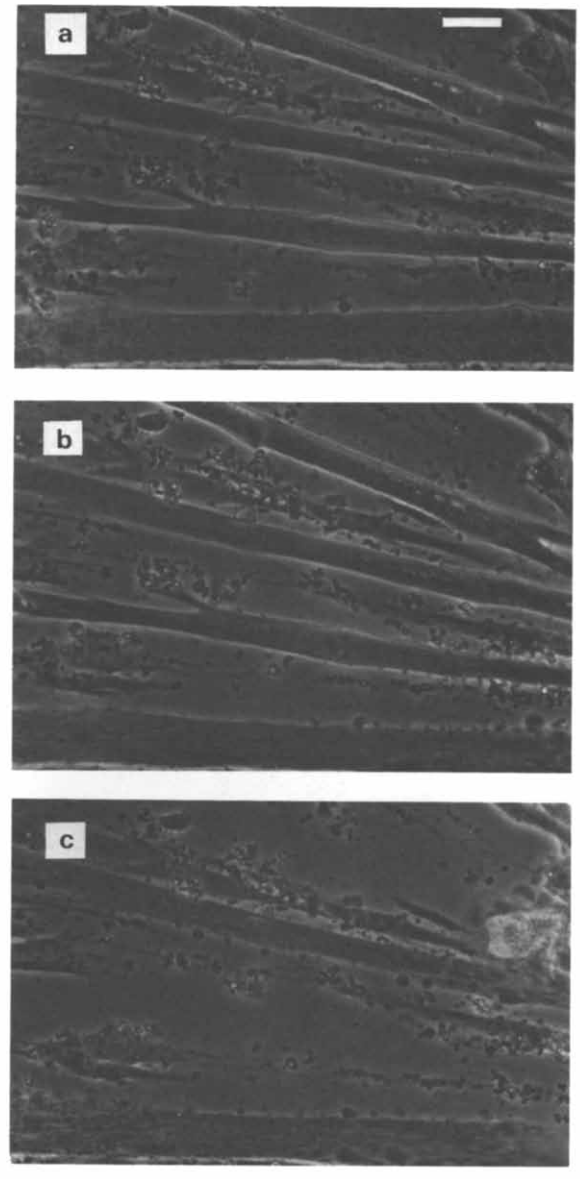

Fig. 7. Effect of cardiotoxin 3.12 on the structure of cultured muscle fibres. (a) control; (b) 80 s after addition of 1.5 μM 3.12; and (c) 2.5 min after addition of 3.12. Note the loss of cross striations in the bottom muscle fibres after 80 s. Scale marker = 50 μm.

Discussion

From our results, we can confirm that the presence of small amounts of phospholipase A can enhance the ability of cardiotoxins to lyse erythrocytes [10,11,20]. However, not all cardiotoxins have the same intrinsic haemolytic activity. In the present study, fraction 3.10.2 was almost completely devoid of haemolytic activity, and even the lytic 3.12.1 fraction is considerably weaker than some other cardiotoxins [13]. Taken with the earlier results of Botes and Viljoen [5], these findings form the basis for the proposal that cardiotoxins comprise two groups, one haemolytic and the other non-haemolytic [6].

Moreover, it is clear from the results of the

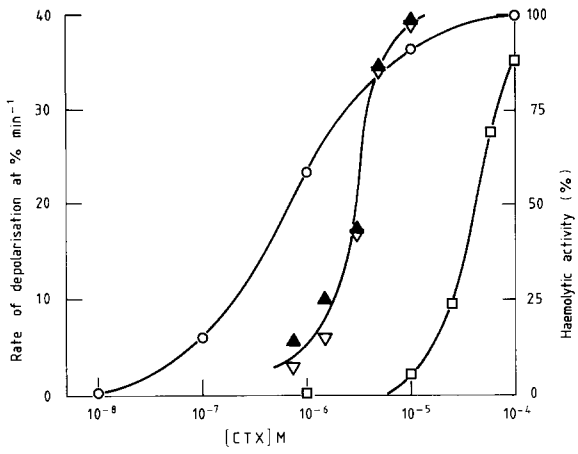

Fig. 8. Dose response curves of toxins 3.10 and 3.10.2 for haemolytic activity and ability to depolarize cultured skeletal muscle cells. Haemolytic activity (% haemolysis after 1 h): ○, 3.10; □, 3.10.2. Depolarizing activity: ▲, 3.10; ▽, 3.10.2.

present study that the presence of small amounts of phospholipase A activity does not markedly increase the ability of cardiotoxins to depolarise and cause contracture of skeletal muscle. Also, the haemolytic activity of the cardiotoxins used in this study does not correlate with their effects on muscle (Fig. 8). For example, the phospholipase-free fraction 3.10.2 had less than 5% of the haemolytic activity of the phospholipase-contaminated fraction 3.10 but it had virtually identical potency on skeletal muscle.

In addition to depolarizing cultured muscle fibres, the cardiotoxins also produced widespread muscle damage. The mechanism for this is unknown at present but it does not appear to be a nonspecific lytic action because fibroblast cells in the same cultures were not affected by concentrations of cardiotoxins that destroyed muscle fibres. Furthermore, the marked difference in sensitivity of human erythrocytes and cultured muscle cells towards cardiotoxins contrasts with the susceptibility of these two preparations towards the nonspecific lytic agent melittin. Melittin, the major honeybee toxin, lyses both cell populations with equal efficacy (Harvey, A.L., Kent, M. and Hider, R.C., unpublished data).

Muscle cell destruction by cardiotoxins is not a simple consequence of prolonged depolarization as depolarization of these cells by high concentrations of K^+ is readily reversible without causing cell damage [21]. Further studies on the mechanism of action of the cardiotoxins on excitable cell membranes are obviously necessary. It is likely that they have a specific binding site which directs them to excitable cells. After binding selectively to membranes of excitable cells, the cardiotoxins may modify the activity of endogenous ionic channels, or they may form ion-selective channels, as suggested by Harvey et al. [19] and demonstrated with δ-haemolysin on B cells from islets of Langerhans [22]. Experiments designed to distinguish between these two possibilities are in progress.

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