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Role of cardiotoxin and phospholipase A in the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra venom

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Summary

1. The effects of phospholipase A (PhA), cardiotoxin (CTX) and neurotoxin (cobrotoxin) isolated from Formosan cobra (*Naja naja atra*) venom on conduction of the rat phrenic nerve and membrane potential of the rat diaphragm were studied.

2. Phospholipase A, lysolecithin and cobrotoxin were without effect on the axonal conduction. Cardiotoxin was the only active agent in cobra venom, but it was less potent than the crude venom.

3. The blocking action of cardiotoxin was markedly accelerated by the simultaneous administration of phospholipase A. However, the minimum effective concentration of cardiotoxin (100 μg/ml), was not decreased by phospholipase A. Pretreatment of the nerve with phospholipase A, followed by washout, did not alter the activity of cardiotoxin.

4. Cardiotoxin (3 μg/ml) completely depolarized the membrane of superficial muscle fibres within 60 min, being 3 times more potent than the crude venom. Phospholipase A, on the other hand, needed a dose 30 times higher and a prolonged period of incubation to induce depolarization of similar extent. Cobrotoxin was without effect on membrane potentials.

5. $CaCl_2$ (10 mM) effectively antagonized the nerve blocking as well as the depolarizing effect of the crude venom, cardiotoxin or cardiotoxin plus phospholipase A. By contrast, the slow depolarizing effect of phospholipase A was enhanced by high concentrations of calcium.

6. Cardiotoxic fractions of Indian cobra venom affected both nerve conduction and diaphragm membrane potential in exactly the same way as cardiotoxin. Toxin A of the same venom was without effect.

7. It is concluded that the active agent in cobra venoms either on axonal conduction or on muscle membrane is cardiotoxin. The synergistic effect of phospholipase A on cardiotoxin appears to be due to acceleration rather than potentiation of its action. The mechanism of action of cardiotoxin and its synergism by phospholipase A are discussed.

Introduction

Cobra venom induces conduction blockade of amphibian nerves (see Meldrum, 1965a) and lobster and squid giant axons (Tobias, 1955, 1958, 1960; Narahashi & Tobias, 1964; Condrea, Rosenberg & Dettbarn, 1967; Condrea & Rosenberg, 1968).

Membrane properties such as resting potential, action potential and membrane resistance were changed. This blocking effect of cobra venom on nerve conduction was attributed to phospholipase A (PhA) in the venom, because both the enzyme activity and the blocking effect were heat stable in an acid medium (Tobias, 1955, 1958, 1960). Moreover, a phospholipase A-rich fraction, isolated from the ringhals venom by means of electrophoresis, blocked conduction of lobster axons (Condrea *et al.*, 1967) and squid giant axons containing adhering small nerve fibres (Condrea & Rosenberg, 1968), but other fractions were weaker in this respect. It is now known, however, that in addition to phospholipase A, both cardiotoxin (CTX) and cobra neurotoxin are also heat stable at 100° C in acid medium (see Lee, 1971).

Cobra venom also has a direct effect on skeletal muscles, producing fibrillation, contracture and depolarization of the membrane (see Lee, 1971). This effect, like the axonal blockade, was attributed to its phospholipase A content (Houssay, Negrete & Mazzocco, 1922 ; Houssay & Mazzocco, 1925 ; Tobias, 1955). On the other hand, cardiotoxic fractions isolated from cobra venoms cause depolarization and contracture of skeletal muscles (Meldrum, 1965b ; Chang & Lee, 1966 ; Lee, Chang, Chiu, Chiu, Tseng & Lee, 1968 ; Earl & Excell, 1971). Neurotoxin isolated from Formosan cobra venom had no such depolarizing effect (Chang & Lee, 1966) whereas that from Indian cobra venom still caused depolarization (Meldrum, 1965b). No purified phospholipase A from cobra venom has been so far investigated in this respect, although that from bee venom depolarized the rat diaphragm (Albuquerque & Thesleff, 1968).

Since phospholipase A has been used as an important tool for relating physiological functions of excitable membrane with its chemical structure, it is of prime importance to determine whether the observed effects of cobra venom on nervous tissue and skeletal muscle are due to phospholipase A, cardiotoxin, or to some other component. We therefore studied the effects of homogenous phospholipase A, cardiotoxin and neurotoxin (cobrotoxin) from Formosan cobra (*Naja naja atra*) venom (NNAV), and also cardiotoxic and neurotoxic (toxin A) fractions of Indian cobra (*Naja naja*) venom, on the conduction of the rat phrenic nerve and membrane potential of the diaphragm. The results indicate that these effects are mainly due to cardiotoxin. Phospholipase A was almost without effect when applied alone, but greatly potentiated or accelerated the effect of cardiotoxin. Neurotoxins, cobrotoxin and toxin A, were without effect in both respects.

Methods

Column chromatography

The Formosan cobra venom (NNAV) was first fractionated into twelve fractions (Fig. 1) on a column of CM-Sephadex (C-50) at 4° C with increasing gradients of ammonium acetate buffer (0·005–0·9 M) and of pH (5·0–7·0) as previously described (Lee *et al.*, 1968). Phospholipase A activity was found to be contained mainly in fractions, V, VI and VII. Fraction VIII was the most toxic and contained cobrotoxin. Both fractions XI and XII were cardiotoxic, but only the latter was used for further purification of cardiotoxin because it was more potent and more abundant.

Purification of cardiotoxin and cobrotoxin

Fractions VIII and XII were further purified by repeated rechromatography on a CM-cellulose column, to yield respectively cobrotoxin and cardiotoxin, using gradients of ammonium acetate buffer (0·15–0·9 M). After this procedure, each of them appeared as a single band on acrylamide gel electrophoresis, and traces of phospholipase A activity originally present in the CM-Sephadex fractions were removed.

Purification of phospholipase A

Fractions V, VI and VII were further purified for phospholipase A according to the method described by Braganca, Sambray & Ghadially (1969). Briefly the pooled fractions were treated with 4 volumes of 6% $HClO_4$, the precipitate redissolved in water, pH adjusted to 9·5, and chromatographed on CM-cellulose using ammonium acetate buffer. The major fraction was precipitated again with ammonium sulphate at 80% saturation, redissolved in water, heated at 100° C for 5 min at pH 5·0 and chromatographed on Sephadex G-50 column. On microzone electrophoresis it migrated to the anode at pH 7·4 in a single band. A homogeneous preparation of phospholipase A, isolated from the same cobra venom by means of repeated chromatography on CM-Sephadex, DEAE-cellulose and Sephadex G-50 columns, was kindly supplied by Dr. T. B. Lo, Department of Chemistry, National Taiwan University and compared with our phospholipase A preparation. The biological activities of both preparations of phospholipase A appeared to be exactly the same in every respect so far investigated.

*Indian cobra (*Naja naja*) neurotoxin and cardiotoxin*

Indian cobra neurotoxin (toxin A) (Nakai, Nakai, Sasaki, Kakiuchi & Hayashi, 1970) and two cardiotoxic fractions (CM-11 and CM-12) (Takeuchi, Sasaki & Hayashi, 1971), isolated by means of CM-cellulose column chromatography, were kindly supplied by Dr. K. Hayashi, Department of Biological Chemistry, Faculty of Pharmaceutical Science, Kyoto University.

Lysolecithin and mugilin-β

Lysolecithin prepared from egg lecithin was purchased from Sigma Chemical Co. Mugilin-β, a protamine preparation isolated from *Mugil japonicus*, was kindly supplied by Dr. R. Hirohata, Yamaguchi Medical School.

Measurement of phospholipase A activity

The method described by Yang, Huang & Tung (1954) was followed. After incubation of samples with 0·05% egg yolk in isotonic saline containing 10 mM $CaCl_2$ for 30 min at 38° C, 2% suspension of washed rabbit erythrocytes in normal saline was added and kept at 38° C for 60 minutes. Haemolysis was determined by the optical density at 578 nm of the supernatant. The activity of phospholipase A was calculated from the concentration (HD50) of test sample which would cause 50% haemolysis.

Nerve action potentials

The phrenic nerve-diaphragm preparation was isolated from Long Evans rats, mounted horizontally in an organ bath containing 20 ml Tyrode solution (mM: NaCl, 137; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1·1; NaH_2PO_4, 0·33; $NaHCO_3$, 11·9; Dextrose, 11·2) at 30–32° C and aerated with 95% O_2 + 5% CO_2. The phrenic nerve was lifted into a paraffin oil layer in a separate compartment for extracellular recording of compound action potentials elicited by a pair of stimulatng electrodes placed on the distal portion of the nerve in the main compartment.

Membrane potentials

Resting membrane potentials were measured by the conventional microelectrode recording method using the isolated rat diaphragm. The organ bath contained 20 ml Tyrode solution at 30–32° C and aerated with 95% O_2 + 5% CO_2. The microelectrodes filled with 3 M KCl had a resistance of 15–20 MΩ. Recordings were made only from the superficial muscle fibres at non-endplate zones.

Results

Phospholipase A activity

The relative phospholipase A activities of the crude Formosan cobra venom and its purified components as determined from the concentrations (HD50) which caused 50% haemolysis of rabbit erythrocytes after preincubation with egg yolk are shown

TABLE 1. *Phospholipase A activity of Formosan cobra venom and its components*

	Crude NNAV	PhA	CTX	Cobrotoxin
HD50, μg/ml	0·89	0·049	200	200
Relative potency	1·00	17·8	0·005	0·005

HD50 represents the concentration which caused 50% indirect haemolysis according to the procedure described in **Methods.**

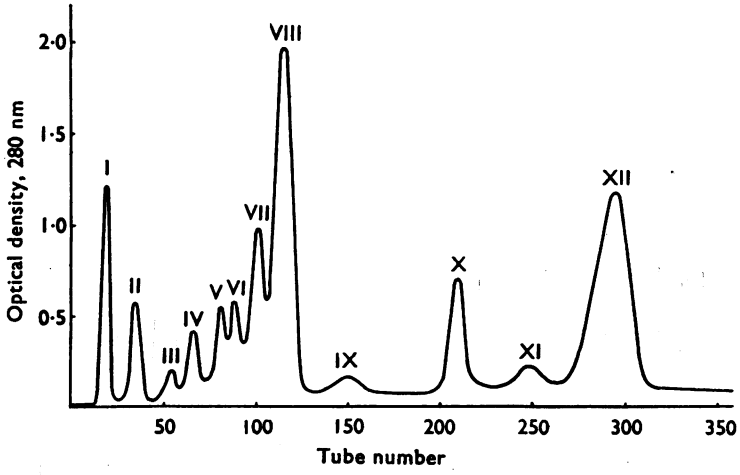

FIG. 1. Chromatography of Formosan cobra venom (NNAV, 500 mg) on CM-Sephadex (C-50) column (1·6×80 cm) by gradient elution with ammonium acetate buffer, from 0·005 M, pH 5·0 to 0·9 M, pH 7·0.

in Table 1. The activity of purified phospholipase A was increased to about 18 times that of the crude venom. By contrast, the enzyme activities of purified cardiotoxin and cobrotoxin were less than 1/200 of the crude venom.

Neuromuscular blocking action

Cobra neurotoxin (cobrotoxin) blocks neuromuscular transmission in a similar manner to tubocurarine (Chang & Lee, 1966; Su, Chang & Lee, 1967). Fractions V, VI and VII (*cf.* Fig. 1) obtained by CM-Sephadex chromatography also affected neuromuscular transmission by a curare-like action (Lee *et al.*, 1968). By contrast, phospholipase A purified from these fractions was found to be free from such activity. At a high dose of 100 μg/ml, phospholipase A caused a depression of muscle contraction after a 4 h period of incubation, and the contractor responses to both indirect and direct stimulation were equally affected (Fig. 2). A slight degree of contracture was also observed. This result indicates that the muscle itself, but none of the presynaptic or synaptic events of neuromuscular transmission was affected. Cardiotoxin acted on the preparations in a similar manner at a 10 times lower concentration (Fig. 2). Details of the effect of cardiotoxin on skeletal muscles have been described (Lee *et al.*, 1968).

Effects on nerve conduction

The blocking actions on axonal conduction by the crude Formosan cobra venom, cardiotoxin, phospholipase A, cobrotoxin and lysolecithin as measured from the reduction of the amplitudes of nerve action potentials are compared in Fig. 3. At 200 μg/ml, the crude venom abolished conduction in about 90 minutes. Cobrotoxin, phospholipase A and lysolecithin, a hydrolysis product of phospholipase A, were found to have no effect even at high concentrations ranging from 500 to 1,000 μg/ml. Toxin A, a neurotoxin from Indian cobra venom, was also without effect on nerve conduction. By contrast, rechromatographed cardiotoxin, the original two cardiotoxic fractions XI and XII obtained from Formosan cobra venom, as well as

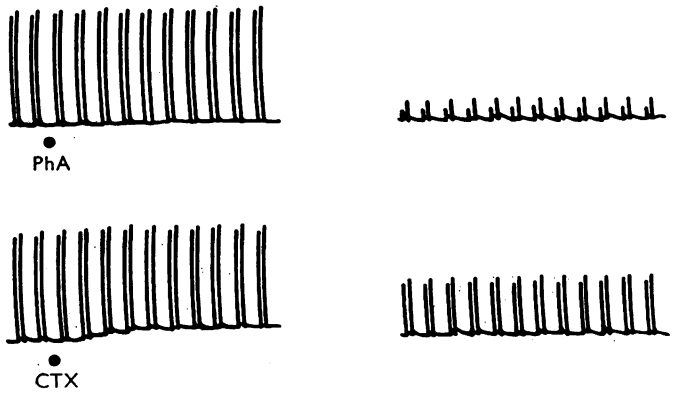

FIG. 2. Effects of PhA and CTX on contractor responses of rat phrenic-nerve diaphragm preparations. Contractions were elicited with a pair of pulses every 40 s, consisting of an indirect stimulus, followed 10 s later, by direct stimulation. At the dot, PhA (100 μg/ml) or CTX (10 μg/ml) was added. Right hand panels show the contractions 240 min after addition of each agent.

CM-11 and CM-12 from Indian cobra venom, were all able to abolish the nerve action potentials; the potencies, however, were less than that of the crude venom on a weight basis. In both cobra venoms, the more basic cardiotoxic components fraction XII and CM-12, were more potent than their less basic components, fraction XI and CM-11, respectively. None of the other fractions obtained from CM-Sephadex chromatography of Formosan cobra venom showed any appreciable effect on the nerve action potential at 200 μg/ml.

It is evident from these results that cardiotoxin is the only component which is by itself able to affect the nerve conduction, but it probably is not the only factor in the crude venom which leads to axon blockade.

Interaction between phospholipase A and cardiotoxin

Condrea, De Vries & Mager (1964) and Vogt, Patzer, Lege, Oldigs & Wille (1970) have shown that the haemolytic effect of phospholipase A could be potentiated by a direct lytic factor (DLF) of cobra venom, which has recently been proposed to be identical to, or at least a homologue of, cardiotoxin (Meldrum, 1965a; Slotta & Vick, 1969; Lee, Lin & Wei, 1970). On the conduction of the squid and lobster axons, however, no such synergism was observed (Condrea *et al.*, 1967, 1968). Since the axonal blocking effect of cardiotoxin on the phrenic nerve could not sufficiently account for the effect of the crude venom, the combined effect of phospholipase A and cardiotoxin was investigated in order to see whether the effect of cardiotoxin could be potentiated by phospholipase A. As illustrated in Fig. 4, a striking synergism between these two agents was observed. No preincubation was necessary for this synergism. The combined effect of phospholipase A (50 μg/ml) and cardiotoxin (100 μg/ml) far exceeded those obtained by phospholipase A (1,000 μg/ml) or cardiotoxin (500 μg/ml) alone. Preincubation of the nerve with phospholipase A (50 μg/ml) for 40–80 min followed by washout, however, did not enhance the blocking effect of cardiotoxin (Fig. 4).

When the concentration of cardiotoxin was fixed at 100 μg/ml and that of phospholipase A changed, the time needed for the axonal blockade was found to be

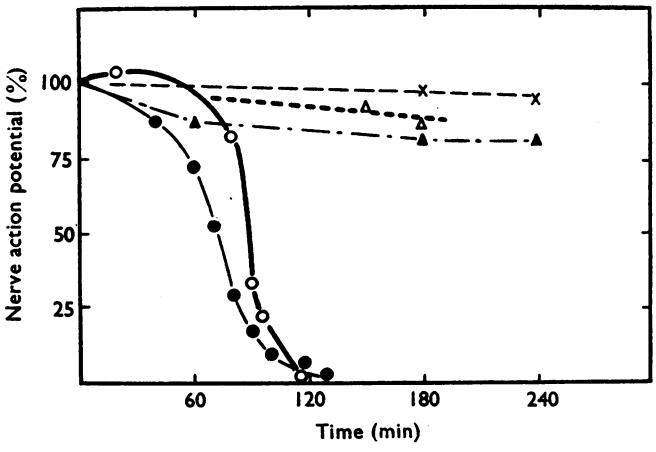

FIG. 3. Effects of NNAV, 0·2 mg/ml (●——●), CTX, 0·2 mg/ml (○——○), PhA, 1 mg/ml (▲– – –▲), cobrotoxin, 0·5 mg/ml (△– –△) and lysolecithin, 0·5 mg/ml (×– – –×) on axonal conduction. The compound action potentials of the rat phrenic nerve, expressed as percentage of controls, are plotted against the time (min) after application of the agents.

dependent on the concentration of phospholipase A (Fig. 5); the minimum effective concentration was 12·5 μg/ml. On the other hand, when phospholipase A was fixed at 50 μg/ml and the concentration of cardiotoxin varied, the blocking effect disappeared suddenly as it was reduced to 50 μg/ml (Fig. 5). The result indicates that the axonal blocking effect is critically dependent on the concentration of cardiotoxin. In fact, the minimum effective concentration of cardiotoxin for blockade was the same whether phospholipase A was present or not (see Fig. 5). It follows that the effect of phospholipase A is to accelerate rather than to potentiate the axonal blocking action of cardiotoxin. In other experiments, mugilin-β, a protamine pre-

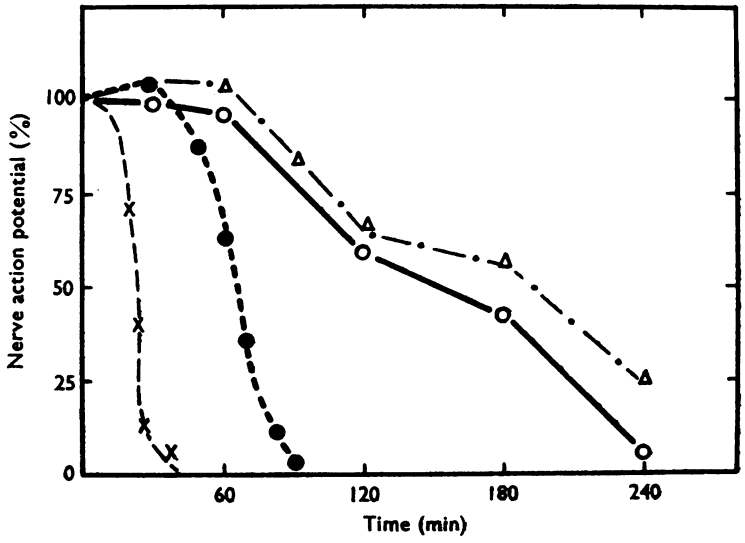

FIG. 4. Synergism between PhA and CTX on axonal conduction blockade. Experiments similar to those in Fig. 3. PhA, 0·05 mg/ml, added simultaneously with CTX, 0·1 mg/ml (×---×); PhA, 0·05 mg/ml, added 40–80 min and washed out 5 min before addition of CTX, 0·1 mg/ml (△-·-△); CTX, 0·5 mg/ml (●---●); CTX, 0·1 mg/ml (○——○).

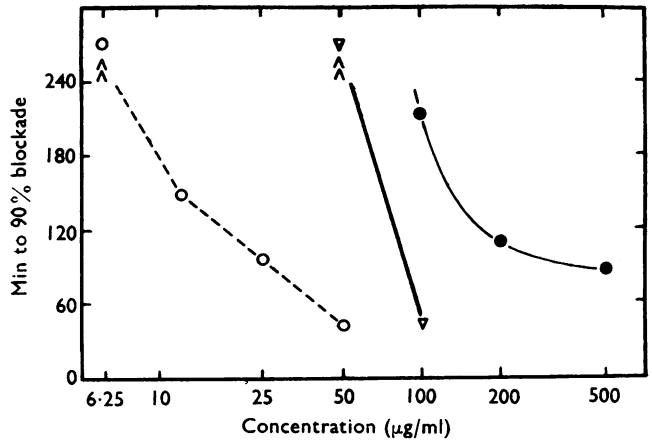

FIG. 5. Relation between doses of CTX and PhA and conduction-blocking activity. The times (min) needed for 90% depression of compound action potentials at various doses. Cardiotoxin alone (●——●); PhA, 0·05 mg/ml, plus various doses of CTX (▽——▽); CTX, 0·1 mg/ml, plus various doses of PhA (○---○).

paration isolated from *Mugil japonicus*, having no disulphide group nor surface activity (unpublished observation), did not appreciably affect the axonal conduction either added alone at a concentration of 500 μg/ml, or in combination with phospholipase A. This experiment suggests that high basicity alone is not the cause of cardiotoxin's effect.

Antagonism of cardiotoxin by calcium

When the concentration of $CaCl_2$ in the Tyrode solution was increased from 1·8 mM to 10 mM, the axon blocking effect of cardiotoxin either alone or in combination with phospholipase A was completely abolished (Fig. 6). The same antagonism was also observed for the crude cobra venom. Since the enzymatic action

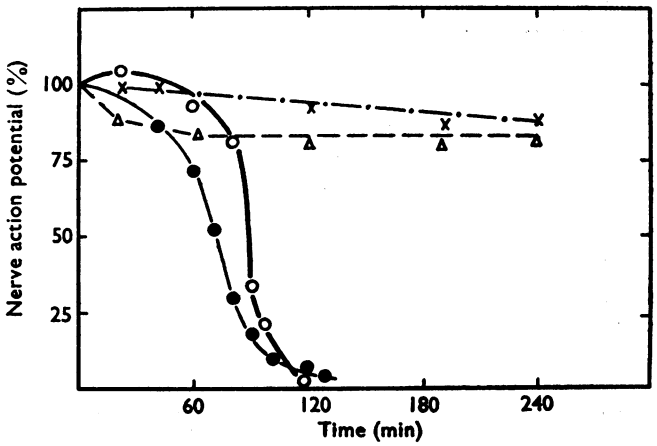

FIG. 6. Antagonism of axon blocking action of NNAV and CTX by Ca^{++}. Experiments similar to those in Fig. 3. NNAV, 0·2 mg/ml+Ca 10 mM (△——△); CTX, 0·2 mg/ml +Ca 10 mM (×-·-×); CTX, 0·2 mg/ml (○——○); NNAV, 0·2 mg/ml (●——●).

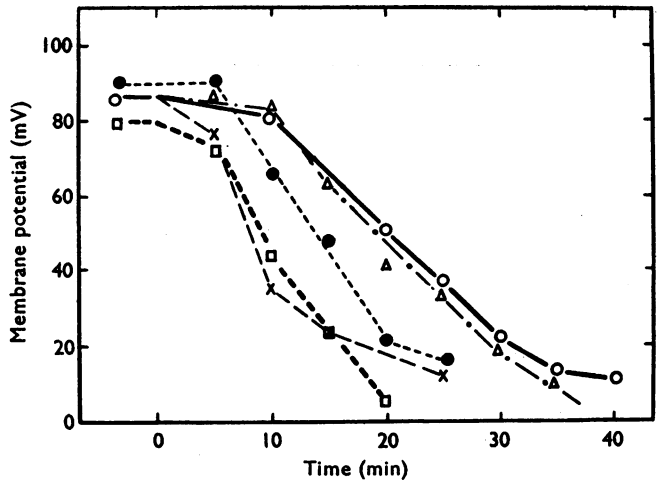

FIG. 7. Effects of NNAV and CTX, alone or in combination with PhA (25 μg/ml), on the membrane potential (mV) of the rat diaphragm. All agents were added at 0 minutes. NNAV, 30 μg/ml (●---●); NNAV, 10 μg/ml (△-·-△); CTX, 3 μg/ml (○——○); CTX, 10 μg/ml (□--□); PhA, 25 μg/ml+CTX, 3 μg/ml (×---×).

of phospholipase A is enhanced by increase of Ca^{++} concentration (Mohamed, Kamel & Ayobe, 1969; Condrea, Barzilay & Mager, 1970), it is obvious that cardiotoxin is the main target of Ca^{++} antagonism. This finding supports the view that cardiotoxin is the primary agent blocking nerve conduction.

Effects on muscle membrane potentials

At 3–10 μg/ml, cardiotoxin rapidly and irreversibly depolarized the membranes of superficial fibres of the rat diaphragm in 20–60 min (Fig. 7). The muscle fibres of deeper layers, however, were depolarized at a much slower rate. The crude *Naja naja atra* venom also affected the diaphragm in the same manner, but was 3 times less potent than cardiotoxin (Fig. 7). Since the cardiotoxin content in the crude venom is about 36% (Lee *et al.*, 1968), it may be reasonably inferred that the depolarizing effect of the crude venom is due mainly to cardiotoxin.

Cardiotoxic components of Indian cobra venom, CM-11 and CM-12, were also found to depolarize the diaphragm at the same concentrations as cardiotoxin, while purified cobra neurotoxins, cobrotoxin and toxin A, were without effect on the membrane potentials, although they are potent inhibitors of neuromuscular transmission (Chang & Lee, 1966; Su *et al.*, 1967). Phospholipase A (100 μg/ml), on the other hand, was found to slowly depolarize the muscle, taking 3 h for complete depolarization of the superficial fibres (Fig. 8).

As in the experiment on axonal conduction, the depolarizing effect of cardiotoxin, 3 μg/ml, was accelerated in the presence of phospholipase A, 25 μg/ml (Fig. 7). The synergism, however, was not as striking as that observed in the effect on nerve conduction.

Effect of high Ca^{++} concentration on membrane depolarization

In confirmation of Earl & Excell (1971), it was found that a high concentration of Ca^{++} (10 mM) effectively antagonized the depolarizing effect of 10 μg/ml cardiotoxin or 30 μg/ml *Naja naja atra* venom (Fig. 9), as it did the blocking effect on

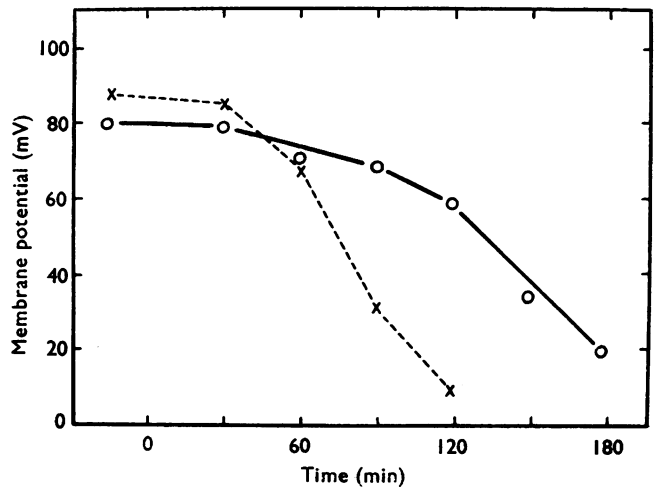

FIG. 8. Effect of PhA on the membrane potential (mV) and its enhancement by Ca^{++}. $CaCl_2$, 1·8 mM+PhA 100 μg/ml (O——O); $CaCl_2$, 10 mM+PhA 100 μg/ml (×– – –×).

axonal conduction. Contracture of the diaphragm induced by cardiotoxin was also prevented. When cardiotoxin was removed and the concentration of $CaCl_2$ restored to normal by washings with plain Tyrode solution after exposure of the diaphragm to cardiotoxin (10 μg/ml) for 150 min, no subsequent depolarization ensued in the next 30–60 min (Fig. 9). This experiment indicates that no cardiotoxin was bound to the membrane in the presence of high Ca^{++}. It may be inferred, therefore, that Ca^{++} interferes with the binding of cardiotoxin to the membrane. The depolarizing action of phospholipase A was, on the contrary, accelerated by the presence of high Ca^{++} (Fig. 8), as was its enzymatic hydrolysis of phospholipids (Mohamed *et al.*, 1969).

Discussion

The present study demonstrates that cardiotoxin is the only active component of cobra venom, when applied alone, that blocks axonal conduction in the rat phrenic nerve. Phospholipase A and neurotoxins were not effective by themselves. Cardiotoxin is not as potent as the crude venom, however. It is likely that there may be a potentiator of cardiotoxin in the crude venom. The high degree of synergism between cardiotoxin and phospholipase A indicates that, in the crude venom, these two components may act synergistically in blocking nerve conduction.

Our results are at diametrical variance with those obtained by Condrea *et al.* (1967, 1968) who found that phospholipase A was the active conduction blocking agent on squid and lobster axons, while direct lytic factor was much less effective in this respect and did not potentiate the effect of phospholipase A at all. The failure of direct lytic factor to block axonal conduction and to act synergistically with phospholipase A in their experiments, however, can be easily explained on the basis of their experimental conditions. We have found that the effect of cardiotoxin on nerve conduction is nullified in the presence of 10 mM $CaCl_2$. The artificial sea water used by them to test the effect of direct lytic factor contained 9·27 mM $CaCl_2$ and 48·4 mM $MgCl_2$. It is also possible that this high calcium enhanced the

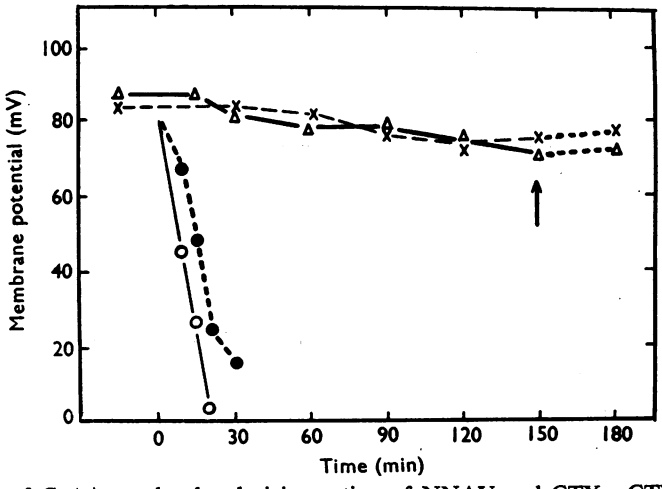

FIG. 9. Effect of Ca^{++} on the depolarizing action of NNAV and CTX. CTX, 10 μg/ml+$CaCl_2$ 10 mM (×---×; NNAV, 30 μg/ml+$CaCl_2$ 10 mM (△——△). At arrow, CTX or NNAV was removed and $CaCl_2$ reduced to 1·8 mM simultaneously. NNAV, 30 μg/ml+$CaCl_2$ 1·8 mM (●--●); CTX, 10 μg/ml+$CaCl_2$ 1·8 mM (○——○).

activity of phospholipase A in their experiments. The phenomenon of synergism between phospholipase A and cardiotoxin on the blocking effect on axonal conduction is rather analogous to that observed in the direct haemolysis with these agents as previously reported by Condrea *et al.* (1964) and Vogt *et al.* (1970). In view of the potentiation by direct lytic factor of phospholipid hydrolytic activity of phospholipase A (Condrea *et al.*, 1970 ; Klibansky, London, Frenkel & De Vries, 1968) the question arises as to whether it is cardiotoxin or phospholipase A which is the primary agent involved in the axonal conduction blockade. Phospholipase A has been regarded as the primary agent for the direct haemolysis in their experiment, since the haemolysis paralleled the hydrolysis of phospholipids (Condrea *et al.*, 1964, 1970) and it was potentiated by Ca^{++}, a phospholipase A activator (Vogt *et al.*, 1970). By contrast, the following evidence of the present experiments indicates that cardiotoxin is the primary agent for blockade of axonal conduction: (1) it is the only agent active by itself, (2) high Ca^{++}antagonizes the blocking effect of cardiotoxin, alone or in combination with PhA, whereas it enhances the enzymatic effect of phospholipase A, and (3) the minimum effective concentration of cardiotoxin is the same either in the presence or absence of phospholipase A. In view of the latter finding, it is possible that the synergistic effect of phospholipase A is only to accelerate the action of cardiotoxin rather than to augment it. The marked depolarizing effect of cardiotoxin on muscle fibres also indicates the potential of cardiotoxin as a poison of excitable membranes.

The mechanism of synergism by phospholipase A is not clearly understood. Since no preincubation with the enzyme was needed before addition of cardiotoxin and the synergism was lost when phospholipase A was washed out, even after a prolonged period of preincubation, it is obvious that in order to show a synergism the two agents must be present together at the same time. It may be assumed that the phospholipid of axonal membrane is not accessible to phospholipase A because of the outermost layer of protein of the membrane. The highly basic cardiotoxin (Narita & Lee, 1970), having surface activity (unpublished observation), may bind with this protein layer resulting in alteration of the membrane structure as well as its physiological functions. Consequently phospholipids in the membrane may be exposed and made accessible to phospholipase A. Since a nerve trunk is composed of many layers of phospholipid-protein membranes surrounding individual axons, phospholipase A might not be able to hydrolyze its substrate without the aid of cardiotoxin. Hydrolysis of phospholipids by phospholipase A, in turn, might remove the lipid barrier and accelerate the penetration of cardiotoxin. It remains to be determined which one acts primarily in the case of direct haemolysis caused by a combination of direct lytic factor and phospholipase A. The recent observation made by Condrea *et al.* (1970) that direct lytic factor enhanced both the haemolytic and phospholipid-hydrolytic activities of phospholipase A on washed red cells, whereas calcium ion enhanced only the hydrolytic but not the haemolytic activity, indicates that hydrolysis of phospholipids may not be a primary factor for the haemolysis.

The hypothesis that the disulphide bonds of direct lytic factor or other basic polypeptides may be involved in its pharmacological effect by interacting with the membrane sulphydryl groups (Vogt *et al.*, 1970), was not put to the test in the present experiments. It appears unlikely, however, since melittin and other synthetic basic compounds, having no disulphide in their molecule, still acted synergistically with

phospholipase A (Klibansky *et al.*, 1968 ; Vogt *et al.*, 1970). Besides, no evidence of such an interaction has been observed. Basic polypeptides having lipophilic groups are generally active in potentiating hydrolysis of erythrocyte-ghost phospholipids by phospholipase A (Klibansky *et al.*, 1968). It might be the ionic character, together with the specific conformation with regard to their hydrophobic group, that leads these molecules to be surface active and enables them to bind with the membrane protein.

There seems to be no doubt about the primary effect of cardiotoxin in the crude venom on membrane potentials of skeletal muscle, since it is so potent and can account quantitatively for the effect of the crude venom. The different time-courses and potencies between the effect of cardiotoxin on the nerve trunk and on the superficial muscle fibre may be due to the fact that the latter is exposed while the nerve axons are concealed under phospholipids. On the other hand, the effect of phospholipase A on this exposed muscle fibre was still very slow, suggesting that even the phospholipids in the superficial membrane, being covered with protein, are still not easily accessible to phospholipase A. That the depolarization by phospholipase A of diaphragms is due to its enzymatic action is supported by the finding that the enzyme activator, Ca^{++}, also potentiated the depolarization. The interference by 10 mM $CaCl_2$ of the effect of cardiotoxin on skeletal muscle is striking. It appears to interfere with the binding of cardiotoxin to the membrane. It is interesting to note that the interaction between cardiotoxin and acetylcholinesterase in solution is likewise prevented by high Ca^{++} (Lee, Liao & Lin, unpublished observation). The depolarizing action of neurotoxic fraction isolated by Meldrum (1965b) from Indian cobra venom was evidently due to contamination of cardiotoxic components as judged from the present study with the more purified neurotoxin, toxin A.

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