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The mode of inotropic action of ciguatoxin on guinea-pig cardiac muscle

*†Asami Seino, *Masaki Kobayashi, †Kazutaka Momose, ‡Takeshi Yasumoto & *[1]Yasushi Ohizumi

*Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan, †School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan and ‡Faculty of Agriculture, Tohoku University, Sendai 980, Japan

1 Ciguatoxin (CTX) caused a dose-dependent increase in the contractile force of the guinea-pig isolated left atria at concentrations ranging from 0.1 to 10 ng ml^{-1} with the ED_{50} value of 0.5 ng ml^{-1}.

2 In the atria, tetrodotoxin (5×10^{-7} M) inhibited markedly the inotropic action of CTX. The inotropic effect of CTX at low concentrations was abolished by practolol (10^{-5} M) and reserpine (2 mg kg^{-1} daily, for 3 days), whereas that of CTX at high concentrations was partially inhibited by both drugs.

3 In single atrial cells, CTX (3 ng ml^{-1}) produced a marked increase in the amplitude of longitudinal contractions.

4 CTX (3 ng ml^{-1}) caused marked prolongation in the falling phase of action potentials of atrial strips without affecting the maximum rate of rise of action potentials and membrane resting potentials. The effect of CTX on action potentials was abolished by tetrodotoxin (10^{-6} M).

5 The whole-cell patch-clamp experiments on myocytes revealed that CTX (20 ng ml^{-1}) shifted the current-voltage curve of Na inward currents by 40 mV in the negative direction. CTX caused a small sustained Na inward current even at resting membrane potentials.

6 These results suggest that the inotropic action of lower concentrations of CTX is primarily due to an indirect action via noradrenaline release, whereas that of higher concentrations is caused not only by an indirect action but also by a direct action on voltage-dependent Na channels of cardiac muscle. It is also suggested that CTX activates cardiac muscle Na channels by modifying the voltage-dependence of channel activation to increase Na inward currents, thus producing cardiotonic actions.

Introduction

Ciguatoxin (CTX) is found in a variety of fish associated with tropical reefs and causes a type of food poisoning known as ciguatera (Gillespie *et al.*, 1986; Anderson & Lobel, 1987). CTX is one of the most potent marine toxins known. Although the chemical structure of CTX is still unknown, the toxin has been revealed to be an oxygenated polyether compound with a molecular weight of 1112 (Nukina *et al.*, 1984). It has been reported that CTX is produced by the benthic dinoflagellate *Gambierdiscus toxicus* and transmitted to a variety of reef fish through the food chain (Yasumoto *et al.*, 1977). Since CTX was isolated from the moray eel (Scheuer *et al.*, 1967), the pharmacological actions of CTX have been extensively studied by numerous investigators using various cells and organs. CTX increases the permeability of the frog skin membranes to Na^+ (Setliff *et al.*, 1971) and depolarizes the cell membrane of frog skeletal muscle (Rayner & Kosaki, 1970; Rayner, 1972), cardiac muscle (Lewis & Endean, 1986) and neuroblastoma N1E115 (Bidard *et al.*, 1984). CTX induces contraction of the guinea-pig vas deferens (Ohizumi *et al.*, 1981) and ileum (Lewis & Endean, 1984) mediated through transmitter release from autonomic nerve endings and causes tetrodotoxin (TTX)-sensitive supersensitivity in the contractile response of the vas deferens to various agonists (Ohizumi *et al.*, 1982). Recently, it has been revealed that CTX acts in synergy with other neurotoxins specific for the Na channels to increase Na^+

[1] Author for correspondence.

influx into neuroblastoma cells and rat muscle cells (Bidard *et al.*, 1984). CTX has been shown to cause positive chronotropic and inotropic actions in the isolated atria of rats or rabbits (Ohshika, 1971) and guinea-pigs (Miyahara *et al.*, 1979). Furthermore, it has been reported that in guinea-pig atria CTX at high concentrations causes a biphasic positive inotropic effect consisting not only of a neurogenic component but also of a myogenic component (Lewis & Endean, 1986). In spite of the availability of pharmacological information concerning the positive inotropic action of CTX, there still remains a lack of understanding of the underlying mechanisms involved. In this paper the detailed mechanism of the inotropic action of CTX has been investigated by electrophysiological and pharmacological techniques.

Methods

Measurement of mechanical response

Male guinea-pigs (250–350 g) were used. The method of preparing the tissues and the technique for measurement of contractions were as described in the preceding paper (Ohizumi *et al.*, 1988). CTX dissolved in 50% dimethylsulphoxide was added cumulatively. Tetrodotoxin (TTX) and practolol were applied to the bath solution 15 min before the application of CTX. Reserpine (2 mg kg^{-1}, i.p., daily, for 3 days) was administered to guinea-pigs before the experiment, which was performed 24 h after the last administration.

Mechanical response of cardiac cells

Cardiac myocytes were isolated from atrial muscle of guinea-pigs (250–300 g) by digestion with collagenase (Cooper Biochemical) as described by Kobayashi *et al.* (1987). The enzyme solution was washed out with a high-K^+, low-Cl^- solution containing (mM): K glutamate 70, KCl 25, KH_2PO_4 10, taurine 10, oxalic acid 10, EGTA 0.5, glucose 11 and HEPES 5 (pH 7.4). The left atrium was dissected, chopped up and stored at 4°C in the same solution. The beating activity of isolated atrial cells was measured as described in the previous paper (Ohizumi *et al.*, 1988) under a phase contrast microscope equipped with thermostated stage (Kobayashi *et al.*, 1986).

Action potential experiments

The action potential (AP) experiments on the guinea-pig left atrial strip were carried out as described in the preceding paper (Ohizumi *et al.*, 1988). The muscle was stimulated electrically at a frequency of 2 Hz with rectangular pulses.

Voltage-clamp experiments

Voltage-clamp experiments were performed in guinea pig atrial cells by a patch-clamp method in the whole-cell recording configuration as described previously (Ohizumi *et al.*, 1988). Giga-ohm seals were obtained with patch-clamp pipettes filled with a solution containing (mM): Cs aspartate 115, NaCl 15, EGTA 5, ATP-Tris 5 and HEPES-Tris 5 (pH 7.1). After the membrane patch was disrupted by suction, the perfusion medium was then changed to a solution in which K^+ was replaced by equimolar Cs^+.

Statistical analysis of the data

The data are expressed as the mean ± s.e.mean. Statistical analysis was performed by Student's *t* test.

Extraction and purification of ciguatoxin

CTX was extracted and purified as described previously (Yasumoto, 1980). Muscle and liver of moray eel (1000 kg) were extracted with acetone. The acetone extract was distributed between hexane and aqueous methanol. The aqueous methanol extract was further distributed between ether and water. The ether extract residue containing the toxin was chromatographed over silicic acid and LiChroprep RP-8 column to yield CTX (1.1 mg) having an LD_{50} of 2 $\mu g\,kg^{-1}$ (i.p.) in mice. In the present experiments, the concentration of CTX is expressed on a nanogram per milliliter basis because its molecular weight has not yet been determined.

Drugs

The following drugs were used: TTX (Sankyo Company, Ltd., Tokyo, Japan), practolol (Imperial Chemical Industries, Ltd., Macclesfield, UK) and reserpine (apoplon; Daiichi-Seiyaku Company, Ltd., Tokyo, Japan).

Results

Mechanical responses

CTX caused a positive inotropic effect on the guinea-pig isolated left atria without affecting the resting tension. As shown in Figure 1, the contractile force in the atria increased with increasing CTX concentrations in the range of 0.1 to 10 ng ml^{-1} in a dose-dependent manner. The 50% effective dose (ED_{50}) of CTX was 0.5 ng ml^{-1} and the maximal response was obtained with 10 ng ml^{-1}. Figure 1 and Table 1 also shows the effect of treatment of the atria with various blocking agents on the positive inotropic

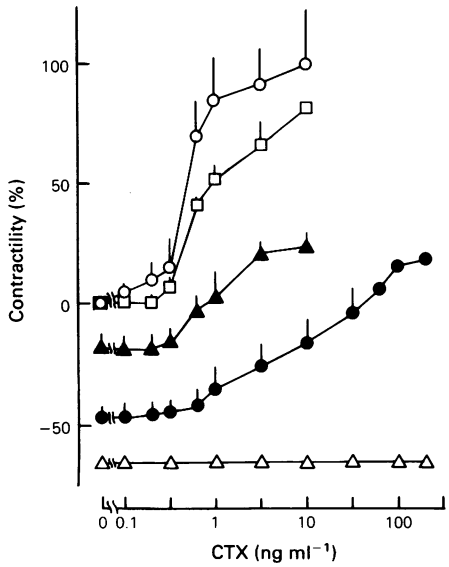

Figure 1 Inhibitory effects of tetrodotoxin (TTX), practolol and reserpine on the log dose-response curves for ciguatoxin (CTX) in the guinea-pig left atria: (○) control; (●) TTX (5×10^{-7} M); (△) TTX (3×10^{-5} M); (▲) practolol (10^{-5} M); (□) reserpine pretreatment. The maximum response to CTX in control was expressed as 100%. Each point and vertical bar represent the mean ± s.e.mean ($n = 4$–8).

action of CTX. In the presence of TTX (5×10^{-7} M), the inotropic effect of CTX at concentrations used was markedly inhibited and the maximum response to CTX was obtained with the concentration of 200 ng ml^{-1}. But, increasing the concentration of TTX to 3×10^{-5} M abolished the positive inotropic action of CTX (Figure 1). The inotropic action of CTX at lower concentrations (0.1–0.3 ng ml^{-1}) was completely inhibited by practolol (10^{-5} M) or reserpine (2 mg kg^{-1} daily, for 3 days), whereas that of higher concentrations (0.6–10 ng ml^{-1}) was partially inhibited by both the drugs (Figure 1 and Table 1).

Contractile response for a single atrial cell

The time course change in the movement of the isolated atrial cells was observed by video recording. Almost all the cells beat regularly in response to electrical stimulation. CTX (3 ng ml^{-1} or more) induced a time-dependent increase in the degree and rate of longitudinal contractions of the electrically stimulated myocytes. Figure 2 shows the effect of CTX (3 ng ml^{-1}) on the time course of contraction-relaxation cycles in single beating myocytes. At 5 min after the application of CTX, the degree of contractions was increased by 110%. After 8 min the cellular motion was changed into irregular beating and the myocytes then gradually shortened. These transformations induced by 3 ng ml^{-1} of CTX were observed reproducibly in 5 different atrial cells.

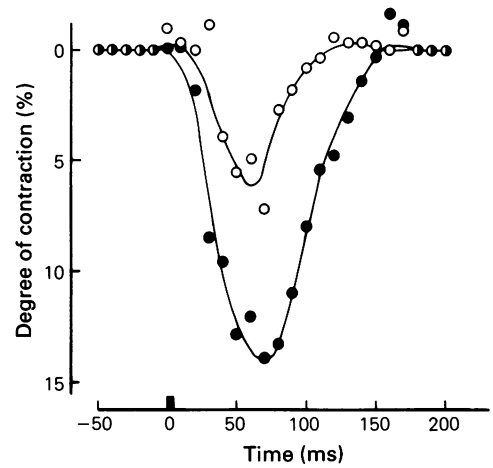

Figure 2 Effects of ciguatoxin (CTX, 3 ng ml^{-1}) on the time course of contraction-relaxation cycles in a single beating action of an atrial cell. The degree of longitudinal contraction was expressed as a ratio to the cell length in the resting state at time zero: (○) control; (●) 5 min after the addition of CTX. The application of the stimulation pulse is indicated as a solid bar on the abscissa scale. Similar results were obtained reproducibly from at least 5 different cells.

Table 1 Effects of tetrodotoxin (TTX), practolol and reserpine on the positive inotropic effect of ciguatoxin (CTX) in the guinea-pig left atria

Drug	*Dose*	*Before CTX* (%)	*After CTX* (%)	*Increase in tension by CTX* (%)	ED_{50} (ng ml^{-1})
None (8)		0	100.0 ± 22.4	100.0 ± 22.4	0.5 ± 0.2
TTX (4)	5×10^{-7} M	−47.1 ± 5.5	21.5 ± 0.1*	68.6 ± 5.5*	8.9 ± 3.8*
Practolol (4)	10^{-5} M	−18.4 ± 5.9	22.4 ± 5.7*	40.8 ± 5.0*	0.6 ± 0.1
Reserpine (4)	2 mg kg^{-1} day^{-1}	0	80.1 ± 1.5*	80.1 ± 1.5*	0.6 ± 0.1

The maximum response to CTX in control is expressed as 100%. Each value indicates the mean ± s.e.mean and numbers in parentheses are number of experiments.

* Significantly different from the maximum response in the absence of CTX at $P < 0.05$.

Action potential experiments

CTX at concentrations above 1 ng ml^{-1} caused an increase in the duration of the AP of guinea-pig left atrial strips, which was accompanied by a positive inotropic effect. Table 2 summarizes the effect of CTX at 3 ng ml^{-1} on the AP. A prolongation of the AP duration and an increase in contractile force were evoked within 2 min after the addition of CTX and reached a maximal level after 4 to 5 min. The resting membrane potential, the maximum rate of depolarization and overshoot remained unchanged. Addition of TTX (10^{-6} M) inhibited the effect of CTX on the AP and the contractility. TTX (10^{-6} M) slightly decreased the maximum rate of rise of AP, but did not modify the AP duration and the resting membrane potential in control muscle (data not shown). In addition, when the concentration of CTX was elevated to 20 ng ml^{-1}, the resting membrane potential was slightly depolarized by 5.0 ± 0.3 mV.

Voltage clamp experiments

The whole-cell patch clamp technique was applied to isolated guinea-pig atrial cells. As shown in Figure 3a, in the control experiment Na inward currents were evoked in response to the step changes from the holding potential (−90 mV) to −50 mV or more positive potentials. Exposure to CTX for 5 min shifted the voltage-dependence of Na channel activation. Twenty-five and 45 min after application of CTX (20 ng ml^{-1}), the Na currents were observed at −60 mV and −85 mV, respectively (Figure 3a). The time course of inactivation and the peak current amplitude of Na currents were almost unaffected by treatment with CTX. CTX (6 to 20 ng ml^{-1}) shifted the current-voltage curve by 10 to 40 mV in the negative direction (Figure 3b), indicating a shift of the voltage-dependence of channel activation toward more hyperpolarized potentials. The maximum response was obtained with concentrations of CTX around 20 ng ml^{-1}. The maximum amplitude of peak current was slightly reduced by CTX at 6 and 20 ng ml^{-1} from 4.7 ± 0.3 nA (control) to 4.5 ± 0.4 nA and to 3.9 ± 0.4 nA, respectively. As shown in Figure 4a, in the control recording Na inward currents were evoked by 20 ms test depolarizing pulses from −90 mV to −30 mV and then were completely inactivated within 20 ms. In the presence of CTX (20 ng ml^{-1}) the Na currents were inactivated after the depolarizing pulse and then a small sustained inward current was evoked (Figure 4b). In addition, after treatment with CTX the peak amplitude of the inward currents elicited by depolarizing pulses was reduced by shifting the voltage-dependence of Na channel activation to more negative potentials. As shown in Figure 5a, when the 20 ms hyperpolarizing pulse was applied from a holding potential (−90 mV) to −130 mV, no current was observed. But, upon returning to −90 mV, CTX (20 ng ml^{-1}) caused a large transient inward current followed by a small sustained inward current (Figure 5b). The time course of activation and inactivation of the CTX-induced transient currents was similar to that of the normal Na inward currents. The sustained inward currents induced by CTX reduced during the hyperpolarizing pulse.

Discussion

In the guinea-pig isolated left atria, CTX induced an increase in the contractile force in a dose-dependent manner. The positive inotropic action of CTX was markedly inhibited by low concentrations of TTX, an agent known to be highly selective in blocking the Na channel (Narahashi, 1972; Baer *et al.*, 1976) and was completely inhibited by high concentrations of TTX. Practolol, a β-adrenoceptor blocking agent and reserpine, a catecholamine depleting agent completely blocked the cardiotonic effect of CTX at lower concentrations (0.1–0.3 ng ml^{-1}), while that of

Table 2 The effect of ciguatoxin (CTX) on the action potential of the guinea-pig atria in the presence or absence of tetrodotoxin (TTX)

Drug	*MRP* (mV)	*MRD* (V s^{-1})	*APD_{25}* (ms)	*APD_{50}* (ms)	*APD_{75}* (ms)
None (control)	−71.7 ± 1.4 (5)	70.2 ± 11.8 (5)	27.6 ± 1.6 (6)	46.3 ± 2.4 (6)	67.5 ± 2.4 (6)
CTX (3 ng ml^{-1})	−70.8 ± 1.2 (5)	77.6 ± 11.9 (5)	40.0 ± 2.6 (6)*	62.6 ± 3.7 (6)*	79.9 ± 4.0 (6)**
CTX (3 ng ml^{-1}) + TTX (10^{-6} M)	−69.9 ± 3.3 (5)	60.2 ± 13.5 (5)	28.6 ± 1.5 (6)†	44.7 ± 2.1 (6)†	58.5 ± 3.5 (6)†

Each value indicates the mean ± s.e.mean and numbers in parentheses are number of experiments.
MRP, membrane resting potential; MRD, maximum rate of depolarization; APD_{25}, 25% repolarization time; APD_{50}, 50% repolarization time; APD_{75}, 75% repolarization time.
Significantly different from the untreated control value at *$P < 0.01$ and **$P < 0.05$.
Significantly different from the value in the group exposed to CTX alone at †$P < 0.01$.

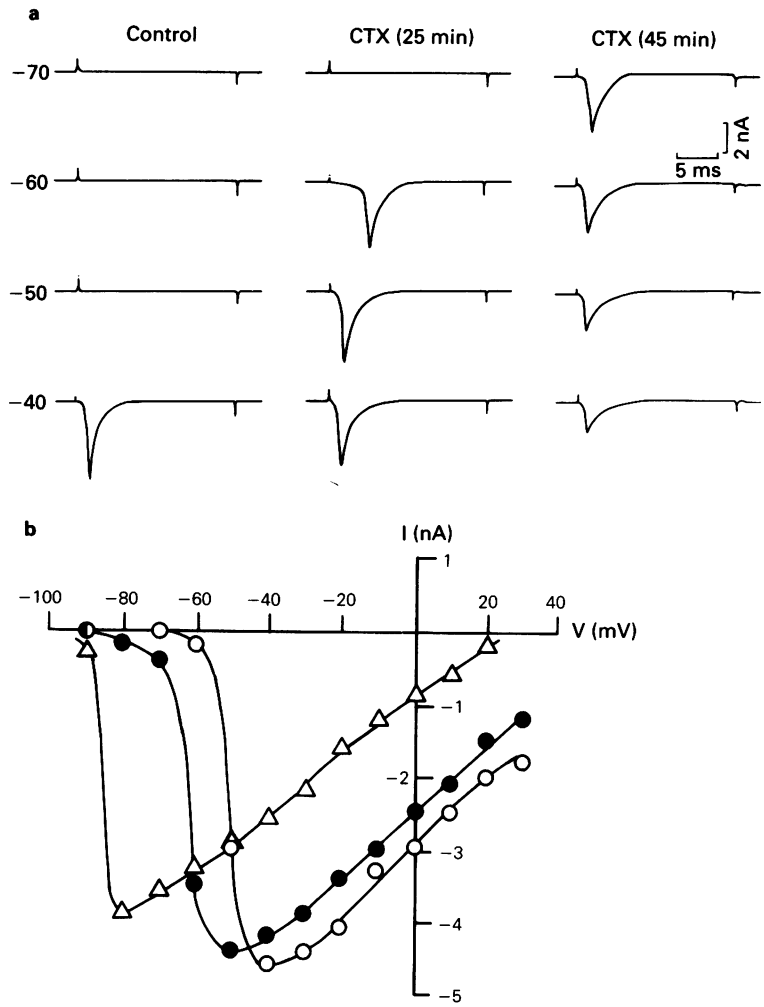

Figure 3 Effects of ciguatoxin (CTX) on Na inward currents in isolated atrial cells. The inward currents were evoked by 20 ms test depolarizing pulses from the holding potential (−90 mV) to various potentials at a frequency of 0.33 Hz. (a) Representative examples of patch-clamp recordings of Na currents. The numbers on the left of each trace indicate the membrane potentials (mV). (b) The current-voltage curve before (○) and after exposure to CTX at 6 (●) and 20 (△) ng ml^{-1}. Ordinate scale, peak current amplitude; abscissa scale, membrane potential during the pulse. Each point represents the mean of values from three different cells.

higher concentrations (0.6–10 ng ml^{-1}) was partially inhibited by both the drugs. In addition, CTX (3 ng ml^{-1}) was able to produce an increase in the degree and rate of longitudinal contraction of stimulated single atrial cells free from neural influence, suggesting that CTX acts directly on the cardiac cell membrane. These results suggest that an increase in contractility of the atria induced by lower concentrations of CTX is primarily the result of an indirect action mediated through the noradrenaline release from adrenergic nerve endings, whereas that induced by higher concentrations occurs mainly through a direct action on Na channels in cardiac muscle membrane.

In the current clamp conditions, CTX produced marked prolongation of the AP duration without affecting the resting membrane potential and the maximum rate of rise in AP. This effect was accompanied by an increase in contractile force, and was reversed by the addition of TTX. Therefore, it is suggested that the positive inotropic action of CTX in the atria is associated with prolongation of the AP

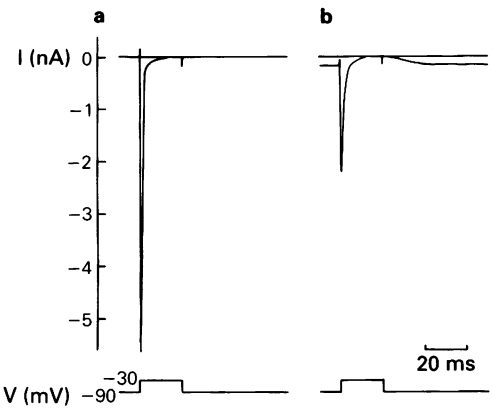

Figure 4 Na inward currents evoked by 20 ms test depolarizing pulses before (a) and 45 min after (b) treatment of the isolated atrial cells with ciguatoxin (CTX, 20 ng ml^{-1}). Twenty ms depolarization pulses from a holding potential (−90 mV) to −30 mV were applied to the cells at a frequency of 0.33 Hz. In the presence of CTX, the peak amplitude of Na currents was reduced by shifting the voltage-dependence of Na channel activation to more negative potential.

duration. Our electrophysiological data obtained from whole-cell clamp experiments showed that CTX shifts the voltage-dependence of Na channel activation to more negative membrane potentials without affecting the inactivation kinetics of the current, suggesting that CTX modifies the activation process of fast Na currents. These results suggest that CTX-induced prolongation of the AP duration is attributed to an increase in the Na current through the Na channels. This may result in an increase in Ca^{2+} availability in the cardiac muscle cell, which is thought to be mediated through the Na–Ca exchange mechanism (Mullins, 1979; Langer,

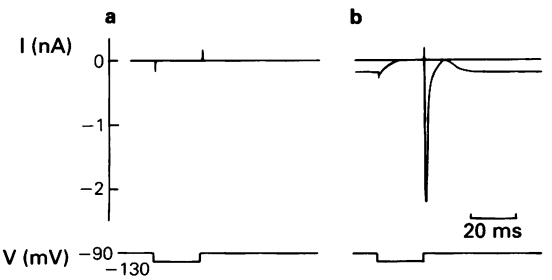

Figure 5 Na inward currents evoked by 20 ms test hyperpolarizing pulses before (a) and 45 min after (b) treatment of the isolated atrial cells with ciguatoxin (20 ng ml^{-1}). Twenty ms hyperpolarization pulses from a holding potential (−90 mV) to −130 mV were applied to the cells at a frequency of 0.33 Hz. The currents were recorded at slow sweep speed.

1982), thus inducing positive inotropic effects. Furthermore, the Na current at −40 mV in the presence of CTX was less than that in control (Figure 3a), suggesting that the voltage-dependence of Na channel inactivation is also shifted by CTX.

It is an interesting observation that CTX induced a sustained inward current both before and after the depolarizing pulse (Figure 4). Furthermore, on returning to the resting potential following hyperpolarizing pulses, CTX caused a large transient Na inward current probably due to depolarization from −130 to −90 mV, and this was accompanied by a sustained inward current (Figure 5). It is possible that the sustained inward current induced by CTX at high concentrations contributes to small depolarization and the prolongation of the AP duration in the cardiac cell membrane. Also an interesting observation is that the Na current decayed to zero level and then recovered to the resting current value (Figures 4 and 5). This cannot be accounted for by a simple shift in the activation curve of Na currents. One possible explanation for this phenomenon is that CTX is being displaced when the channels enter the open state and then is rebinding in the inactivated state.

There are a large number of toxins specifically affecting Na channels. These toxins have been divided into 4 classes according to their binding sites (Catterall, 1986). (i) The Na channel blockers, TTX and saxitoxin; (ii) the lipid-soluble toxins (veratridine, batrachotoxin, aconitine and grayanotoxin) that cause persistent channel activation and alter the kinetics of channel inactivation; (iii) the polypeptide toxins including sea anemone toxins and α-scorpion toxins that slow channel inactivation; (iv) β-scorpion toxins that primarily produce channel activation, but do not bring about a persistent membrane depolarization. It has been shown that CTX cannot associate with the respective receptors of the above 4 toxin classes (Bidard *et al.*, 1984). Recently it has been revealed that brevetoxin, isolated from the dinoflagellate binds to the Na channels at the same site as CTX shares (Lombet *et al.*, 1987), suggesting that both toxins belong to a new class of toxins. The electrophysiological effects of CTX observed in the present study are closely similar to those obtained from squid and crayfish axons exposed to brevetoxin (Huang *et al.*, 1984; Atchison *et al.*, 1986). Therefore, it is possible that in the atria, CTX and brevetoxin bind to the channel site which affects the activation gating of the entire channel function. Toxins affecting Na channels such as aconitine or veratridine (Honerjäger & Reiter, 1975; Honerjäger & Meissner, 1983) and sea anemone toxins or α-scorpion toxins (Shibata *et al.*, 1976; 1978; Ravens, 1976), like CTX, produce a positive inotropic action. On the basis of these observations described above, it is suggested

that the mechanism of action of CTX is different from that of these cardiotonic toxins.

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