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# Crotoxin potentiates L-type calcium currents and modulates the action potential of neonatal rat cardiomyocytes

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ABSTRACT

Crotoxin (CTX), the major component of the venom from the South American rattlesnake (*Crotalus durissus terrificus*) has diverse toxic effects, including pre-synaptic neurotoxicity. Among these, the effect of CTX in the heart is the least understood. In this study, we explored the effect(s) of CTX on the electrophysiological activity of neonatal rat cardiomyocytes (NRCM). By using patch, voltage-, and current-clamping techniques, we found that, in NRCM, CTX strongly potentiates L-type $Ca^{2+}$ currents, an important contributor to the cardiac action potential (AP) and excitation-contraction (EC) coupling. External addition of CTX produced a rapid increase in L-type $Ca^{2+}$ currents. Addition of verapamil (10 μM) an L-type $Ca^{2+}$ channel blocker, completely blocked the CTX-induced increase in the currents. A detailed kinetic analysis of AP in NRCM revealed that CTX caused both an elongation of AP duration (APD) and an increase of its amplitude, while a decrease of firing frequency. In addition, increasing concentrations of CTX completely blocked the AP as well as the beating of NRCM. The data indicate that CTX is a potent modulator of L-type $Ca^{2+}$ currents, which provides a possible mechanistic correlation for the cardiotoxicity of the toxin, and may offer potential clinical implications in the control of cardiac function.

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## 1. Introduction

Nature has been a source of medicinal products for thousands of years, among which snake venoms form a rich source of bioactive molecules, such as peptides, proteins and enzymes with important pharmacological activities (Marcinkiewicz, 2005; Koh et al., 2006). Snake envenomation also present serious health problems, since there are about 2.5 million incidents of snakebite worldwide each year, resulting in over 100,000 deaths (Koh et al., 2006). Snake venom is a natural biological resource that contains several components of potential therapeutic value, and has been used in the treatment of a variety of pathophysiological conditions (Patlak, 2003; Koh et al., 2006).

Among the best-studied snake venoms are α-neurotoxins that bind to nicotinic acetylcholine receptors (nAChRs). These toxins reversibly block nerve transmission by competitive binding to the nAChR located at the post-synaptic membrane of skeletal muscles and neurons, preventing neuromuscular transmission and thereby leading to death by asphyxiation (Tsetlin and Hucho, 2004). Neurotoxins that recognize the muscarinic acetylcholine receptors (mAChRs) have also been isolated from green mamba venom (Harvey et al., 2002). These toxins have been useful pharmacological tools for investigating the physiological role of muscarinic receptor subtypes (Bradley, 2000; Potter, 2001; Jerusalinsky et al., 2000) as well as the

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treatment of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Mulugeta et al., 2003). CTX is the major toxic component of the venom from the South American rattlesnake (*Crotalus durissus terrificus*) (Hendon and Fraenkel-Conrat, 1971). CTX is a pre-synaptic neurotoxin (β-neurotoxin), with similar function to other neurotoxins of the same group, including β-bungarotoxin from *Bungarus multicinctus*, notexin from *Notechis scutatus*, and taipoxin from the Australian taipan (*Oxyuranus scutellatus*) (Rossetto et al., 2004).

CTX (~24.0 kDa) is comprised of two non-identical subunits (B and A) that are joined by non-covalent interactions. Subunit B of CTX is a peptide of approximately 14.5 kDa molecular weight and phospholipase $A_2$ ($PLA_2$) activity, which is essential for its toxicity. Subunit A (~9.5 kDa) is a nontoxic protein, which contains no $PLA_2$ activity, despite the fact that it exhibits sequence homology to subunit B of CTX and other $PLA_2$. Interestingly, CTX displays no toxic effect in its heterodimer form, while dissociation is important for its toxicity. The dissociation seems to be tissue-specific, where the subunit A may play an important role in the recognition that triggers the dissociation. CTX effects include neurotoxicity, myotoxicity and cytotoxicity. CTX exerts its neurotoxic effect by blocking the neuromuscular transmission from the pre-synaptic membrane (Brazil and Excell, 1971; Chang and Lee, 1977). As a result, the toxic manifestations of CTX include flaccid paralysis and respiratory failure. The myotoxicity of CTX can cause local muscle damage (Gopalakrishnakone et al., 1984; Mebs and Ownby, 1990; Salvini et al., 2001) as well as a more generalized cytotoxic effect toward a variety of human and mouse cell lines (Corin et al., 1993; Newman et al., 1993; Yan et al., 2006, 2007). Due to its toxic effect toward tumor cells, CTX has been used in phase I clinical trials on advanced cancer patients, where it is believed to act through a novel mechanism of action (Cura et al., 2002).

The toxicity of CTX in the cardiac system is little understood and remains controversial. Current data suggest that CTX can interfere with cardiac function. For example, myocardial damage was observed in a human case after fatal *C.d. terrificus* envenomation (de Siqueira et al., 1990). Patients suffering from *C.d. terrificus* envenomation show changes in electrocardiogram recordings (Cupo et al., 2003), and changes in the level of serum enzymes that are markers of muscle damage, resembling a pattern found in acute myocardial infarction (Cupo et al., 1990). Moreover, in mice, CTX induces ultrastructural changes of cardiac autonomic neurons (Hernandez et al., 2007), while in the guinea pig heart, CTX significantly reduces the contractile force (Santos et al., 1990). These observations would suggest a potent effect of CTX in cardiac function. However, the cardiotoxicity and the mechanisms underlying these effects of CTX remain largely unknown.

In the present study, we investigated the effect(s) of CTX on the electrical activity of NRCM, particularly the L-type $Ca^{2+}$ channels. L-type $Ca^{2+}$ currents are a major $Ca^{2+}$ entry pathway that triggers the release of $Ca^{2+}$ from the sarcoplasmic reticulum and subsequently cardiac contraction (Bers, 2002). L-type $Ca^{2+}$ currents play an important role in maintaining the plateau phase of AP and modulate the refractory period between AP (Bers, 2002; Boyett and Jewell, 1980). An increase in L-type $Ca^{2+}$ currents elongates the AP duration (APD). In rat, an increase in APD results in the decrease of contraction (Babuty et al., 1998; Schouten, 1986; Schouten and ter Keurs, 1991). Using various electrophysiological techniques, herein we found that CTX significantly increased L-type $Ca^{2+}$ currents of NRCM, which profoundly affected the kinetics of cardiac AP. Increasing concentrations of CTX completely blocked NRCM beating. The encompassing data suggest that CTX affects NRCM electrical activity and thus provide both potential uses in cardiac pharmacology and a possible molecular mechanism for its cardiotoxicity.

## 2. Materials & methods

### 2.1. Purification of CTX

CTX (NSC-624244) was obtained from Ventech Research (Cambridge, MA), which was purified with a method previously described (Newman et al., 1993). Briefly, dried venom of *C.d. terrificus* was obtained from the Butantan Institute (Sao Paulo, Brazil), which contained approximately 50% CTX, 20% crotamin, 3–5% convulsin and a few other components. CTX was purified by gel filtration (Sephacryl S-200 and Sephadex G-75) and anion exchange chromatography (DEAE Sephadex A-50). The purified CTX contained both subunit A and subunit B and was stored at 4 °C.

### 2.2. Isolation and culture of NRCM

Primary cultures of NRCM were obtained with procedural modifications to a commercial isolation kit (Worthington Biochemicals, Freehold, NJ) as previously described (Lader et al., 1999). Briefly, beating hearts were harvested from less than 24-h-old neonatal rats and immediately placed in a $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS, Worthington). The hearts were minced and subjected to trypsin digestion (100 μg/mL in HBSS) for 16–18 h at 4 °C. Trypsin digestion was stopped by addition of trypsin inhibitor (Worthington). Further collagenase digestion (type II collagenase, 150 U/ml; Worthington) was conducted at 37 °C on a shaking bath for 45 min. Cell clumps were filtered through a 70-μm nylon filter, centrifuged, and washed with fresh Leibovitz L-15 medium. Cell pellets were resuspended in Ham's F-10 medium with L-glutamine (BioWhittaker, Walkersville, MD), also containing 5% bovine serum and 10% horse serum (BioWhittaker). Cells were seeded onto glass coverslips and allowed to grow at 37 °C in an incubator gassed with 5% $CO_2$. Cells that attached to the glass and spread after one day in culture were usually used for the patch-clamping experiments. Cells maintained in culture within one week after harvest had no electrical differences.

### 2.3. Electrophysiology

Electrophysiological data were acquired with an Axon Patch 200B amplifier, low-pass filtered at 10 kHz, and digitized with 1400A Digidata (Axon Instruments, Union City, CA). The pCLAMP 10.0 software (Axon Instruments) was used to acquire and to analyze the data. Usually traces

were further filtered with a low-pass 8-pole Bessel filter at 100–300 Hz for display purpose only. Patch pipettes were pulled from borosilicate glass (Garner Glass Co, Claremont, CA) with a two-stage Narishige PB-7 vertical puller and then fire-polished to a resistance between 6 and 12 MΩ with a Narishige MF-9 microforge (Narishige International USA, East Meadow, NY). The whole-cell patch clamping of L-type $Ca^{2+}$ currents was performed as previously described (Lader et al., 1998, 1999). Briefly, the pipettes were filled with an intracellular solution containing (in mM): 125 CsCl, 20 Tetraethylamonium-Cl (TEA-Cl), 10 Hepes (free acid), 5 MgATP and 5 EGTA, pH was adjusted to 7.3 with CsOH. The NRCM were placed in a chamber containing the following extracellular solution (in mM): 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (free acid), pH was adjusted to 7.4 with NaOH. TEA and the substitution of $K^+$ with $Cs^+$ inhibited endogenous $K^+$ channel activity. NRCM were viewed using the 40× DIC objective of an Olympus IX71 microscope (Olympus Inc., Tokyo, Japan). After a cell-attached gigaseal was established, a gentle suction was applied to break in, to attain the whole-cell configuration. Immediately upon breaking in, the cell was voltage clamped at −50 mV to inactivate both voltage-activated $Na^+$ and T-type $Ca^{2+}$ currents. L-type $Ca^{2+}$ currents were recorded every 30 s, using a protocol that stepped the voltage from −50 mV to +70 mV in 10 mV intervals (200 ms/step). L-type $Ca^{2+}$ currents were determined at different holding potentials, by subtracting the peak inward currents from the plateau currents measured at 190 ms. Cell-attached recordings and current-clamp recordings of AP were conducted with an intracellular (pipette) solution containing (in mM): 115 K-Aspartate, 15 NaCl, 0.8 $MgSO_4$, 1.0 ATP, 10 Hepes (free acid), and pH 7.4 with KOH. The bath solution contained (in mM): 135 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.8 $MgCl_2$, 10 Hepes (free acid), and pH 7.4 with NaOH. The cell-attached patch clamping was performed in voltage-clamp mode ($V_h = 0$ mV) immediately after a gigaseal was established. Spontaneous spikes in current were recorded, which were correlated to the spontaneous AP of the cells. For current-clamp experiments, the whole-cell configuration was first obtained as described above. AP was recorded with or without the injection of current. No differences were found between spontaneous AP and current-induced AP.

### 2.4. NRCM beating assay

Cultured NRCM were maintained in Ham's F-10 medium and viewed with 40× optics of an Olympus IX71 microscope. Most isolated NRCM beat spontaneously. NRCM beatings were recorded with an ORCA C4742-80-12AG camera (Hamamatsu, Hamamatsu City, Japan) and processed with IPLab 4.0 software (BD Bioscience, Rockville, MD). The instantaneous beating frequency was calculated during a window of five seconds, by dividing the number of beatings over time.

### 2.5. Chemicals and data analysis

All chemicals and drugs were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Verapamil (10 μM) was prepared in a ×10 stock saline solution, and added to that bathing solution immediately prior to addition of the toxin. Data were expressed as mean ± standard error (SEM). Paired or unpaired Student's $t$ tests were performed with Origin 7.5 (Northampton, MA) to assess statistical difference between experimental groups. Statistical significance was established at $p < 0.05$.

## 3. Results

### 3.1. Effect of CTX on whole-cell L-type $Ca^{2+}$ currents of NRCM

L-type $Ca^{2+}$ currents play an important role in cardiac AP and EC coupling. We first investigated the effect of CTX on the L-type $Ca^{2+}$ currents of cultured NRCM (Fig. 1). Using the whole-cell voltage-clamping technique, cells were held at −50 mV to inactivate T-type $Ca^{2+}$ currents and voltage-gated $Na^+$ currents, and L-type $Ca^{2+}$ currents were followed by changes in membrane potential from −50 mV to +70 mV. L-type $Ca^{2+}$ currents were recorded every 30 s and a slight rundown was observed with time, which was consistent with our previous findings (Lader et al., 1999). No $Na^+$ current contamination was observed by this method, as the addition of TTX (10 μM) had no effect on the elicited inward current (data not shown). By contrast, verapamil (10 μM), a specific L-type $Ca^{2+}$ channel blocker abolished these currents, suggesting that only L-type $Ca^{2+}$ currents were elicited under the experimental condition (see below). The addition of CTX (2 μM) to the bath significantly increased the magnitude of the NRCM L-type $Ca^{2+}$ currents (Fig. 1a–d, $n = 6$). L-type $Ca^{2+}$ currents showed peak current between −10 and −20 mV (Fig. 1c). CTX always increased L-type $Ca^{2+}$ currents doubling the original value in average (Fig. 1c, $n = 7$). This increase reached statistical difference ($p < 0.05$) by paired Student's $t$ test. In the presence of verapamil (10 μM), which completely blocked the basal L-type $Ca^{2+}$ currents, addition of CTX (6 μM) in the bathing solution, was without any effect (Fig. 1d), confirming the specificity of the drug. The I–V relationship showed no apparent change of other parameters, including voltage of the peak currents and reversal potentials (Fig. 1b). The increase in L-type $Ca^{2+}$ currents was usually quite rapid after addition of CTX, with latency of less than 1 min, and was rapidly reversible upon washout of the toxin (Fig. 1e).

### 3.2. Effect of CTX on the AP of NRCM

To determine the effect of CTX on the AP of NRCM, we performed both voltage clamping in the cell-attached configuration (Fig. 2) and current clamping in the whole-cell configuration (Fig. 3). Under cell-attached voltage clamping conditions, the spontaneous AP of cells resulted in transient current spikes. In a representative cell (Fig. 2a), AP fired spontaneously with an instantaneous frequency of 1.20 ± 0.06 Hz (mean ± SEM, $n = 104$ spikes) under control conditions. The addition of increasing concentrations of CTX (1–6 μM) modified the duration, amplitude and frequency of the current spikes (Fig. 2b). Exposure of cells to CTX changed the spike width ($APD_{50}$, the duration at 50% peak amplitude), which increased by nearly 75% (6 μM CTX,

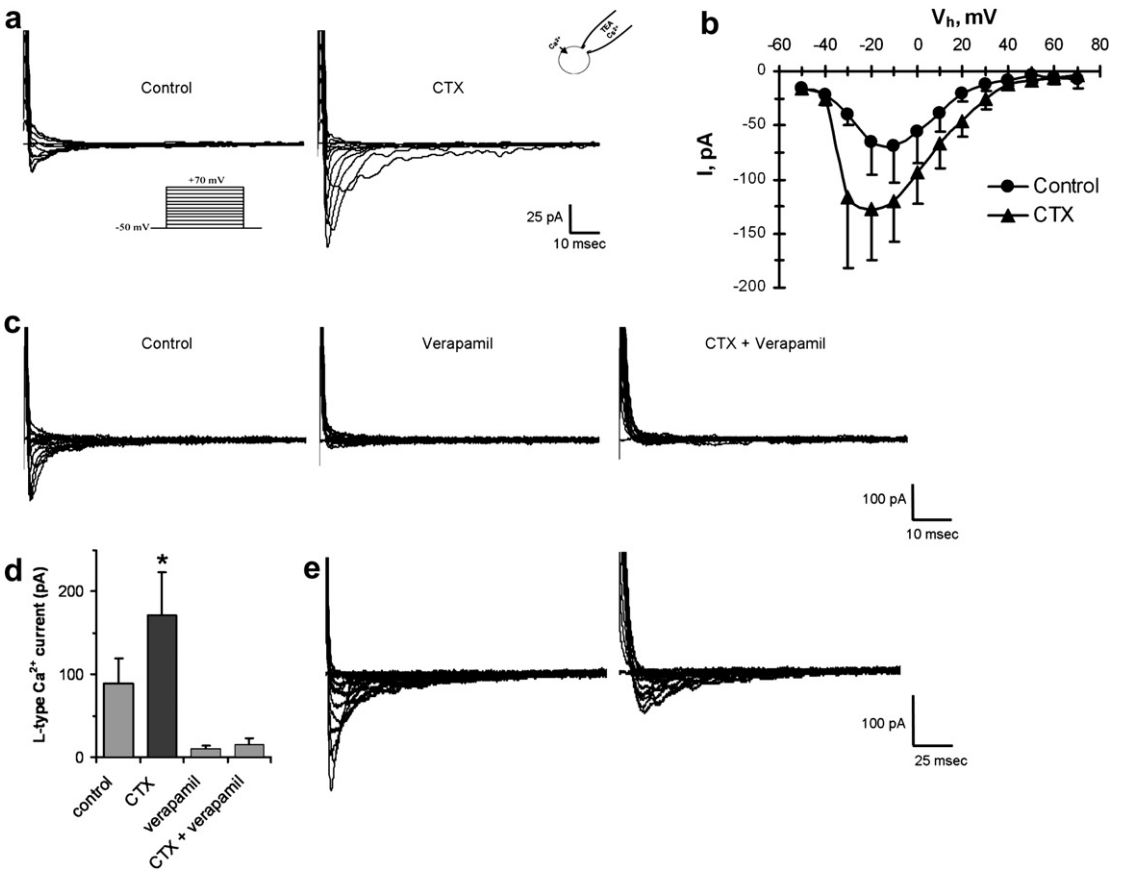


**Fig. 1.** Effect of CTX the whole-cell L-type $Ca^{2+}$ currents in NRCM. (a) Representative whole-cell L-type $Ca^{2+}$ currents in NRCM. L-type $Ca^{2+}$ currents were obtained with a voltage protocol that steps membrane potentials from −50 mV to +70 mV in 10 mV intervals (200 ms/step), as shown on the left. L-type $Ca^{2+}$ currents significantly increased within 30 s after addition of CTX (2 μM). (b) Current-voltage relationships for L-type $Ca^{2+}$ currents in the absence (filled circles) and presence (filled triangles) of CTX. Data are expressed as the mean ± SEM, $n = 6$. (c) Addition of verapamil (10 μM) completely blocked the stimulatory effect of CTX (4 μM). Data representative of $n = 4$. (d) CTX (2 μM) increased the L-type $Ca^{2+}$ currents ($n = 7$, $^*p < 0.05$, paired Student's $t$ test). Verapamil (10 μM) blocked both, the basal and CTX-stimulated L-type $Ca^{2+}$ currents. (e) The CTX-stimulated L-type $Ca^{2+}$ currents reversed to control values upon washout. Data representative of $n = 3$.

Fig. 2b–c). The elongation of spikes was especially significant around their bases, suggesting that the toxin elongates the plateau of AP. The amplitude of spikes was slightly increased by CTX (1–6 μM), probably because the increased L-type $Ca^{2+}$ currents contributed to the cell depolarization. However, in cells exposed to increasing concentrations of CTX, the spike amplitude declined and eventually disappeared. The concentration needed to block the beating of NRCM was slightly different among cells, suggesting different sensitivity to the toxin. The inhibition of spikes by CTX is in agreement with the NRCM beating experiments (Fig. 4). In addition, CTX significantly decreased the frequency of AP (Fig. 2c). This is consistent with the increase in L-type $Ca^{2+}$ currents, which elongated the plateau of AP and the refractory period between AP.

To directly assess the effect of CTX on the AP of NRCM, current clamping was also performed in the whole-cell configuration (Fig. 3). A lower concentration of CTX (2 μM) elongated the duration and slightly increased the amplitude of AP. Increasing the concentration of CTX further elongated the AP but decreased their amplitude. Finally, the AP was blocked by higher concentration of CTX (Fig. 3). These data are in agreement with the cell-attached patch-clamping data, and together show the dramatic effect of CTX on the electrophysiological properties of cardiomyocytes.

### 3.3. Effect of CTX on the beating rate of cultured NRCM

To further assess the effect of CTX on the electrical activity of NRCM, we explored the spontaneous beating of individual cells before and after addition of increasing concentrations of CTX to the bathing medium. A series of 70-s image recordings were obtained for each cell, at 5.7 frames/s (Fig. 4). Lower concentrations of CTX (1–2 μM) had no apparent effect on the beating rate of NRCM. Interestingly, addition of 2 μM CTX-induced a small and transient increase in the beating rate of some cells. However, a higher concentration of CTX (3–4 μM), quickly and dramatically inhibited the beating rate of most NRCM (Fig. 4), consistent with cell-attached recordings (Fig. 2) and current-clamping data (Fig. 3).

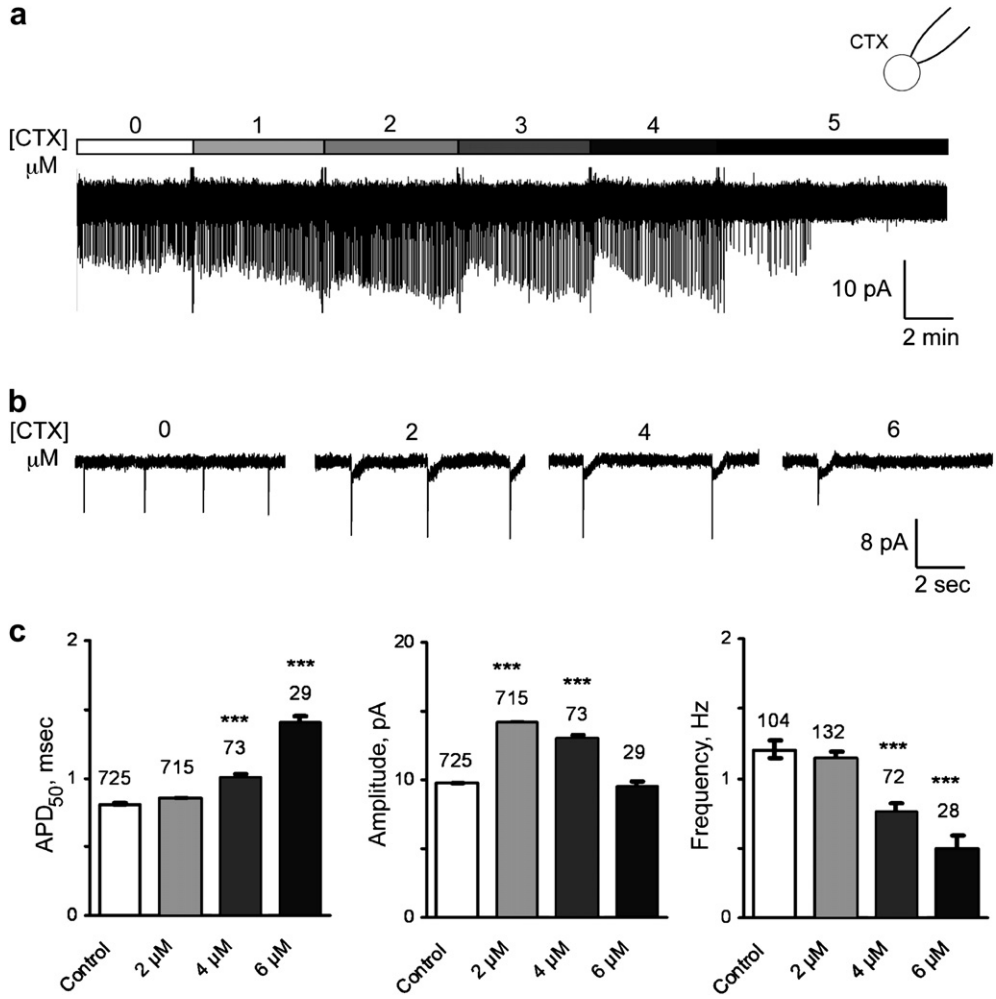


**Fig. 2.** Effect of CTX on AP shape and beating frequency of NRCM. (a) Representative experiment in which AP-evoked current transients were recorded in the cell-attached configuration. The NRCM spontaneously fired AP under control conditions. Concentrations of CTX between 1 and 4 μM slightly increased the AP spikes while they decreased the firing frequency. Increasing the CTX concentration (6 μM) blocked the AP ($n = 6$). (b) Expanded tracings at different concentrations of CTX. The changes in amplitude, frequency, and duration of the AP spikes can be observed. (c) Bar graphs of the effect of CTX on NRCM mean $APD_{50}$ (AP duration at 50% amplitude, Left), amplitude (Middle), and frequency of spikes (Right) at different concentrations of CTX. Each column represents the numbers of spikes averaged. Data are expressed as mean ± SEM of a number of spikes as indicated on top of the columns. *** indicates statistical significance at $p < 0.001$ with unpaired Student's $t$ test.

## 4. Discussion

The present studies strongly support a potent modulatory role of CTX on cardiac function, suggesting that the cardiotoxic effect of CTX may be mechanistically associated with the regulation of L-type $Ca^{2+}$ currents and thus cardiac function. We found that CTX potentiated the L-type $Ca^{2+}$ currents in NRCM. This may, in turn, play an important role in cardiac function. The CTX-induced increase in L-type $Ca^{2+}$ currents increased NRCM depolarization and thus the amplitude of the AP. CTX elongated the AP plateau and increased the width of AP. Moreover, the refractory period of AP was also increased by CTX, thus decreasing the frequency of AP firing. At higher concentrations, CTX inhibited the AP and the beating of NRCM, which could be due to its inhibitory effect on $K^+$ channels (Rowan and Harvey, 1988). This sequence of functional events provides a mechanistic explanation for the effect of CTX on cardiac function, as well as its cardiotoxicity. The fact that CTX affects both L-type $Ca^{2+}$ currents and the electrical activity of cardiomyocytes makes this toxin a potentially useful natural product that can be used, or from which other compounds may be developed, for the treatment of cardiac disease.

Snake venom forms a rich source of bioactive molecules, such as peptides, proteins and enzymes with important pharmacological activities (Koh et al., 2006). This has helped to the understanding of specific interactions leading to the development of effective drugs targeted to particular diseases. A number of drugs have been derived from snake venom proteins and have progressed to clinical uses (reviewed in Koh et al., 2006). Captopril, the first oral angiotensin converting enzyme (ACE) inhibitor was originally derived from observations on the toxic effect of

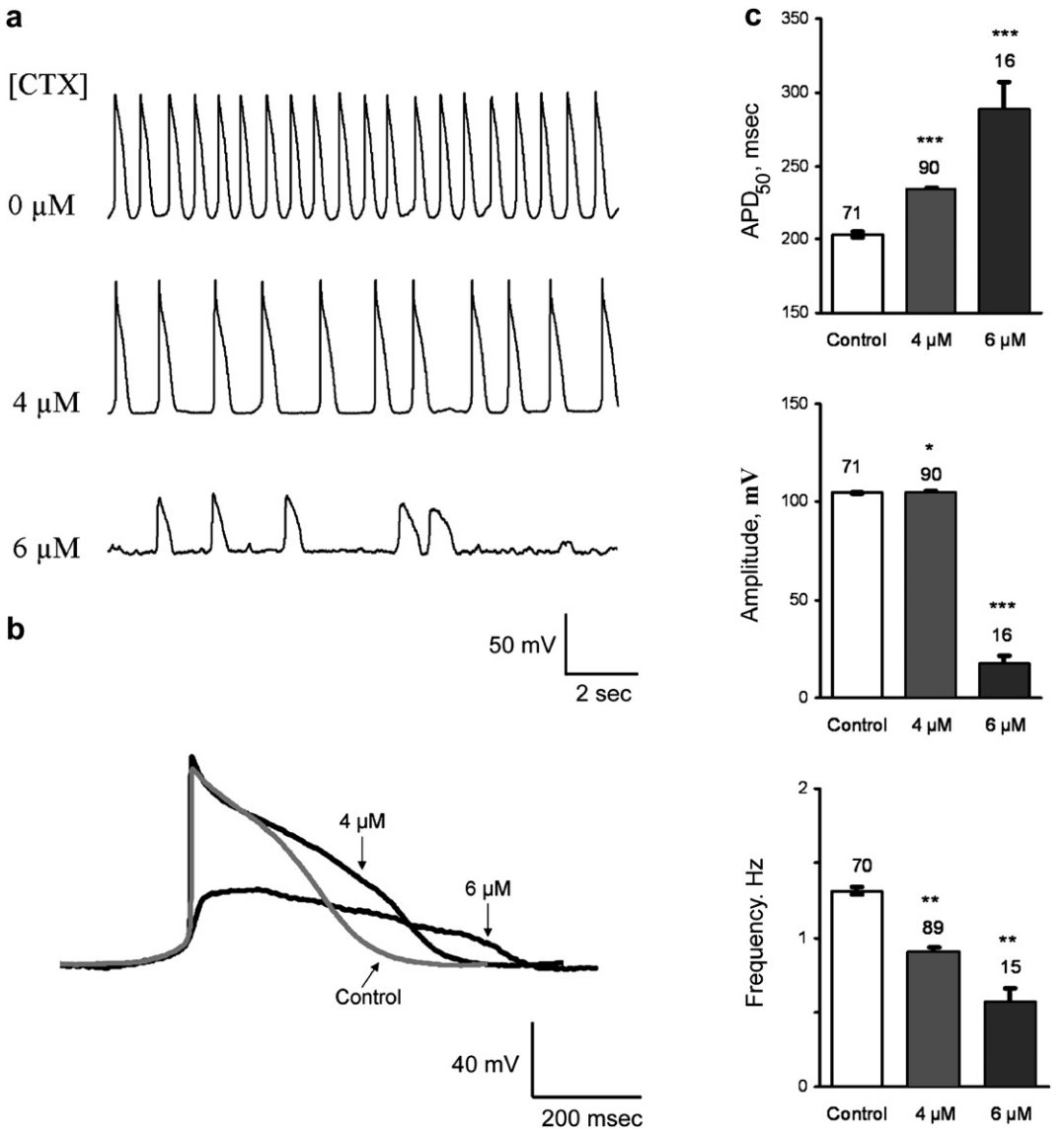


**Fig. 3.** The effect of CTX on current-clamp recordings of AP from NRCM. (a) Representative tracings of a cell spontaneously fired AP without current injection. Addition of CTX affected the width, amplitude and frequency of AP in a dose-dependent manner. (b) Expanded representative AP at different concentrations of CTX clearly shows the changes in AP shape. (c) Bar graphs of mean $APD_{50}$ (AP duration at 50% amplitude, Top), amplitude (Middle) and frequency of AP (Bottom) at different concentrations of CTX. Data are expressed as mean ± SEM. The number of spikes averaged is indicated on top of each column. * Indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ with unpaired Student's $t$ test.

venom from the Brazilian viper (*Bothrops jararaca*), whose bite causes a sudden, massive drop in blood pressure (Patlak, 2003). More recently, the clinical application of disintegrins for the potential treatment of human diseases such as cancer further suggests that snake venom components could have important clinical implications (Marcinkiewicz, 2005). Snake venom also has been used in the laboratory for the routine assay of coagulation factors and as reagents to study both coagulopathy and hemostasis (White, 2005). While the original native snake venom compounds may be usually unsuitable as therapeutic agents, derivatives and related compounds may be of relevant use in the diagnostic and treatment of human disease. The possible pharmacological use of CTX in the treatment of cancer is under investigation because of its high cytotoxic activity toward cancer cells (Cura et al., 2002). Phase I clinical trial of CTX with patients with advanced cancer has revealed no serious side effects when administrated at proper dose (Cura et al., 2002). Thus purified CTX or product thereof could prove promising as therapeutic agents in medicine. Specific mechanistic aspects of CTX function are thus required to further explore its specific function(s).

L-type $Ca^{2+}$ channels play an important role in cardiac function. Changes in cardiac L-type $Ca^{2+}$ currents are implicated in a number of pathological conditions,

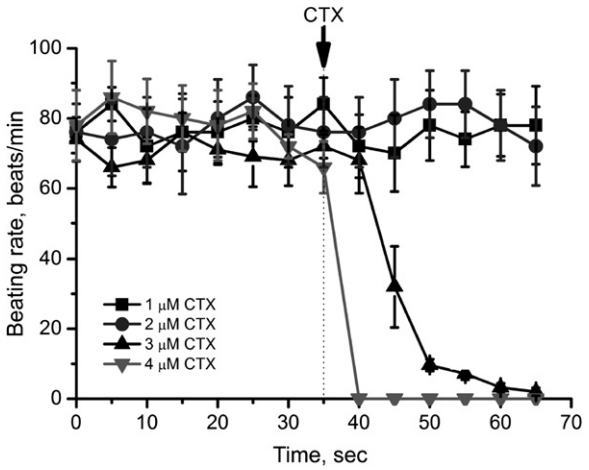


**Fig. 4.** Effect of CTX on the spontaneous beating rate of NRCM. NRCM spontaneously beat in culture. The beating rate was measured in a 5-s window. Each data point indicates the average ± SEM of six cells. The addition of 1 μM CTX had no apparent effect on the beating rate of NRCM. Addition of 2 μM CTX transiently increased the beating in most cells, while addition of 3–4 μM CTX completely inhibited the beating rate of NRCM.

including severe cardiac hypertrophy and congestive heart failure (Beuckelmann et al., 1992; Mukherjee and Spinale, 1998). Our data indicate that CTX was effective in the activation of the L-type $Ca^{2+}$ channels from the extracellular aspect of the cell membrane. CTX is a protein, and thus may not easily cross the plasmalemma. From the external side of the membrane, CTX likely affects cardiac function either by direct binding to the channel complex, or instead may exert its effect through the $PLA_2$-associated reaction product(s). CTX catalyzes the hydrolysis of membrane lipids to a variety of products (for example arachidonic acid), which can travel through the membrane easily and thus can work from either side of the plasma membrane. It seems plausible, therefore, that the downstream product(s) of the CTX's PLA2 enzymatic activity may also play a role in the modulation of L-type $Ca^{2+}$ channels. Further experimentation is warranted to explore the nature of the molecular interaction(s) between CTX and the L-type $Ca^{2+}$ channels, as well as the mechanistic pathways linking CTX function to cardiac function. CTX may be potentially used in the control of cardiac function, and/or relief the symptoms of cardiac disease. Together, the encompassed data revealed mechanistic steps underlying the cardiac effect(s) of CTX, and further study of its pharmacological properties, which may cast light to drug discovery.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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