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# Suppression of snake-venom cardiotoxin-induced cardiomyocyte degeneration by blockage of $Ca^{2+}$ influx or inhibition of non-lysosomal proteinases

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The incubation of $10^5$ single neonatal rat cardiomyocytes with 1 $\mu$M-cardiotoxin in a bath medium, Tyrode solution in the presence of 1 mM-$Ca^{2+}$, at 37 °C evoked the following chain of events. Firstly, there appeared a latent period of about 10 min during which the cells behaved normally. Neither lactate dehydrogenase nor ATP leaked from the cells. Cytosolic free $Ca^{2+}$ increased considerably, as measured by the fluorescence intensity of fura-2–$Ca^{2+}$ complex. At the same time a large portion of endogenous ATP was depleted. Secondly, after the latent period, the cell beating became irregular and eventually stopped. Thirdly, blebs appeared on the cell surface, leading to cell degeneration. If, before the appearance of blebs, the cells were washed with the bath medium exhaustively or incubated in the presence of the toxin antibody, cytosolic free $Ca^{2+}$ and endogenous ATP returned to normal levels and cells resumed regular beating. Preincubation of the cells with 3.75 $\mu$M-flunarizine or 3.75 $\mu$M-diltiazem (both are $Ca^{2+}$ antagonists), or 1.5 $\mu$M-fura-2 acetoxymethyl ester (a chelate for $Ca^{2+}$), or 200 $\mu$M-leupeptin or 50 $\mu$M-antipain (both are proteinase inhibitors) considerably suppressed the toxin's ability to degenerate the cells. On the other hand, lysosomal proteinase inhibitor, autophage inhibitor, serine proteinase inhibitor, phospholipase inhibitor and calmodulin antagonist did not inhibit the toxin's activity. The results suggest that the toxin may act on the extracellular surface of intact cardiomyocytes to increase cytosolic free $Ca^{2+}$. The subsequent cell degeneration may result from the activation of a $Ca^{2+}$-dependent non-lysosomal proteolytic system.

## INTRODUCTION

Snake venom-cardiotoxin (CTX), a basic polypeptide of $M_r$ 7000, acts on cell membranes of different origins and causes cardiac arrest, muscle contracture, membrane depolarization and cytolysis [1,2]. Thus, CTX has been described as a cytotoxin, cytolysin, direct lytic factor, membrane-active polypeptide and a membrane-disruptive polypeptide [3–6]. With regard to its toxic effect on cardiomyocytes, the electrophysiological study by Arm & McPheeters [7] showed that the toxin can irreversibly depolarize cultured cardiomyocytes of chicken embryos. However, the primary cause of these events has remained obscure. This work presents the CTX-induced degeneration of neonatal rat cardiomyocytes. We found that incubation of cells with CTX caused an increase in $[Ca^{2+}]_i$ and depletion of endogenous ATP in a period during which the membrane structure remained intact and the spontaneous activity of the cells was maintained. On further incubation the cells stopped contracting, changed morphology and eventually degenerated. Chelation of intracellular $Ca^{2+}$ with fura-2, or blockage of $Ca^{2+}$-influx with diltiazem or flunarizine, or inhibition of proteinases with antipain or leupeptin, could inhibit the CTX-induced cell degeneration partially, though not completely.

## MATERIALS AND METHODS

### Materials

Crude venom of the Taiwan cobra (*Naja naja atra*) was supplied by Chen Hsin Tong Chemical Co., Taiwan. CM-Sephadex C-25 and Protein A–Sepharose 4B were both acquired from Pharmacia. Antipain, leupeptin, methylamine, methyladenine, indomethacin, diltiazem, trifluoperazine and flunarizine were from Sigma. F10, horse serum and foetal bovine serum were obtained from Gibco. Fura-2 and its acetoxymethyl ester were acquired from Molecular Probes Inc, Oregon, U.S.A. All other chemicals were of reagent grade.

### Preparation of CTX and its antibody

CTX was purified from crude snake venom on a CM-Sephadex C-25 column [8]. IgG was isolated from the CTX antiserum [3] by a protein A-affinity column according to the manufacturer's recommendation.

### Culture of rat cardiomyocytes

Cardiomyocytes of neonatal Long Evans rats (0–3 days) were prepared by a modified method of Lau *et al.* [9]. Cells were collected and washed twice with a medium composed of 80% (v/v) F10/10% (v/v) horse serum/10% (v/v) foetal bovine serum/14 mM-$NaHCO_3$/

Abbreviations used: CTX, cardiotoxin; $[Ca^{2+}]_i$, concentration of intracellular free $Ca^{2+}$; fura-2 AM, fura-2 acetoxymethyl ester; LDH, lactate dehydrogenase.

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20 mM-Hepes, pH 7.4. To enrich cardiomyocytes in the cell preparation, the differential replating procedure of Blondel *et al.* [10] was followed. The cells were plated in a 100 mm Nauce dish and incubated in a 5% $CO_2$ incubator at 37 °C for 3 h. For single cell cultures, the unattached cells were replated at a density of $10^5$ cells/Petri dish (35 mm × 10 mm) or coverslip (18 mm × 18 mm). When the cells were cultured on a 96-well microplate, $10^4$ cells/well were replated. After a 2-day culture, 90% of the cells were cardiomyocytes. Cells were used within 2–3 days of preparation. The cell morphology was examined under a phase-contrast microscope. Cells beat rhythmically and independently with 6–140 beats/min at 37 °C. Most cells stopped beating at 21 °C. Throughout the study, Tyrode solution containing 1 mM-$CaCl_2$ was used as bath medium according to Piper *et al.* [11]. Cells in this medium maintained their spontaneous contractions. The incubation of the cells with CTX took place at 37 °C unless stated otherwise. Cell degeneration was indicated by the distortion of cell morphology and was confirmed also by Trypan Blue-exclusion test [12].

### Measurement of lactate dehydrogenase and ATP

Lactate dehydrogenase (LDH) activity was measured according to the method of Lejohn *et al.* [13]. Cellular ATP was determined after the cells were disrupted with nucleotide releasing reagent (Lumac company) for somatic cells. ATP was quantified on the basis of luminescence arising from the transformation of luciferin to decarboxyluciferin by luciferase. The luminescence was counted in a Lumac biocounter M2010.

### Measurement of $[Ca^{2+}]_i$

Cardiomyocytes on a coverslip were incubated with 1.5 μM-fura-2 acetoxymethyl ester (AM) at 37 °C for 1.5 h. Fura-2 AM is able to penetrate the cells [14]. The coverslip was rinsed three times with the bath medium to remove free fura-2 AM before the cells were incubated with CTX. The fluorescence of each cell was measured with a Leitz MPV 3 microscope spectrofluorometer using excitation filter E2, band-pass emission filter LP515 and a measuring diaphragm of 0.123 mm diameter. The emission intensities were counted 64 times at 1.8 s intervals and averaged.

### Scanning electron microscopy

Following the method of Kimes & Brandt [15], cardiomyocytes on a coverslip were fixed with glutaraldehyde and osmium tetraoxide. After the cells were dehydrated with ethanol, they were dried in a critical-point dryer (Hitachi HCP-2, Japan), sputter-coated with gold in an ion coater (model IB-2; Eiko Engineering, Japan) and observed under a Hitachi S-520 scanning electron microscope at 20 kV.

### Binding assay

Cardiomyocytes on a microplate well were incubated at 37 °C in the presence of 50 μM-antipain or 200 μM-leupeptin for 30 min, or 3.75 μM-diltiazem or 3.75 μM-flunarizine for 60 min, or 1.5 μM-fura-2 AM for 90 min. The solutions were decanted. Treated cells, except for those treated with fura-2 AM, were incubated with 1–20 ng of CTX in 0.1 ml of the same incubation solution at 21 °C for 10 min. In the case of cells treated with fura-2 AM, the cells were washed to remove free fura-2 AM before they were incubated with CTX. The amount of free CTX in the solutions was determined by conventional enzyme immunoassay [16] using horseradish peroxidase-conjugated goat anti-rabbit IgG as the second antibody. The amount of CTX bound to intact cells was determined by subtraction of the free CTX from the total CTX used for the assay.

### Statistical analysis

Data was analysed according to the Student's *t* test. Results were considered to differ significantly when a *P* value was $< 0.05$.

## RESULTS

### Morphological changes in cardiomyocytes during the CTX action

Figs. 1 and 2 display, under phase-contrast and scanning-electron microscopy respectively, the morphology of cells being incubated with 1 μM-CTX. A latent period was observed, which extended for at least 10 min for all cells, during which cell morphology was not distorted and cells beat normally (cf. Figs. 1*a* and 1*b*, Figs. 2*a* and 2*b*); the cells did not degenerate. Fura-2 in the bath medium was unable to enter the cells as examined under a fluorescence microscope. Neither LDH nor ATP was found in the bath medium. Apparently, the cells remained intact; note that LDH should not leak from and fura-2 should not penetrate an intact cell [12,17]. Further incubation of the CTX beyond the latent period evoked a series of changes. Despite individual variations in the timing of the changes, the changes observed in each cell seemed to possess similar features. Initially, the beating rate of cells accelerated temporarily and then became slow and irregular. The cells fibrillated quickly and stopped beating before blebs appeared on several regions of the cell borders (Figs. 1*c* and 1*d*). The blebs often enlarged gradually, and shrank abruptly as the cells degenerated. Increasing the CTX dosage shortened the latent period and accelerated cell degeneration (Fig. 3). In contrast, scanning-electron microscopy did not reveal any blebs. Instead, small holes appeared on the cell surfaces and hole size enlarged with increasing exposure to CTX (Figs. 2*c* and 2*d*). Hole formation might either be induced directly by CTX or formed indirectly during fixation for microscopy. Regardless, the blebs might have been so labile that they were easily broken or detached from the cell surface during the fixation. Occasionally, some cells beat irregularly even after bleb formation. If the CTX-treated cells, before the occurrence of fibrillatory motion, were either washed exhaustively with the bath medium or incubated with 125 μg of CTX/ml of antibody at 37 °C for 20 min, the cells could resume normal beating. However, once bleb formation appeared, the same treatments had little effect as the cells were permanently inhibited from resuming the normal contractions. It was interesting to note that both the originally quiescent cells at 37 °C and the cells at 21 °C could resume regular beating if they were incubated with CTX. However, the restoration was temporary. Further incubation with CTX resulted in cell degeneration following the aforementioned morphological changes.

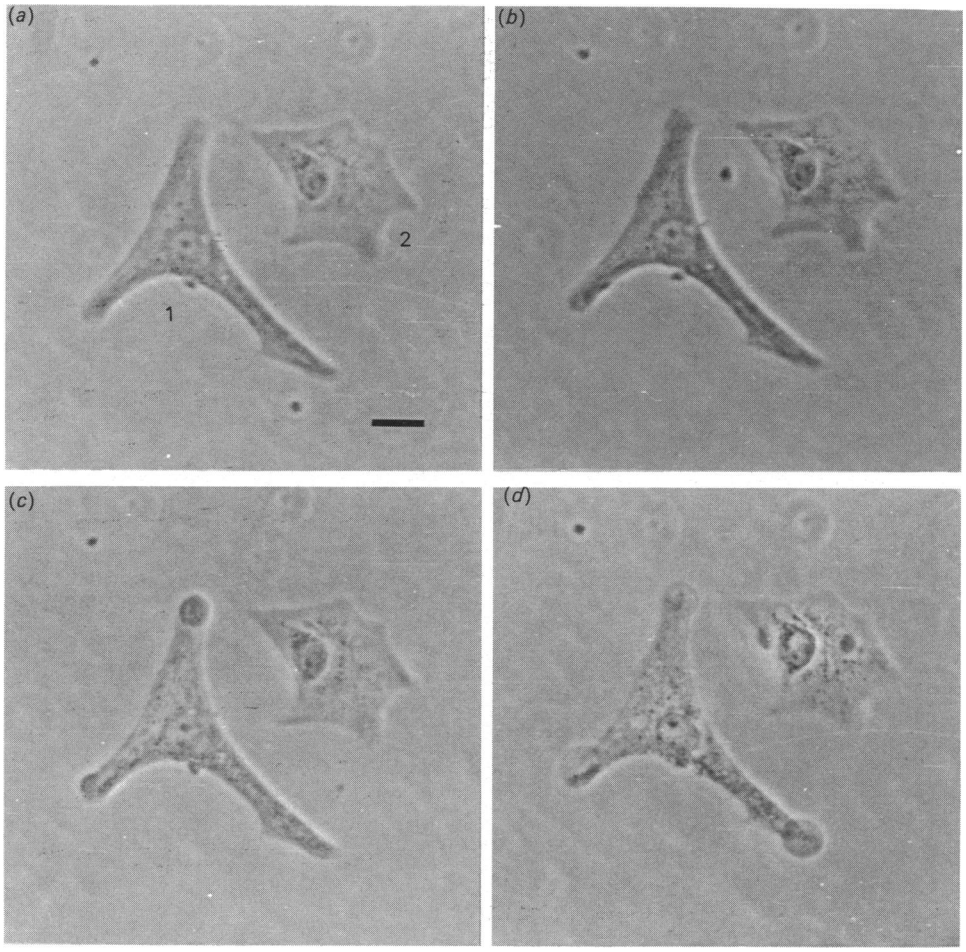


**Fig. 1. Cell morphology under phase-contrast microscope**

Cardiomyocytes on a Petri dish were incubated with 1 $\mu$M-CTX at 37 °C for 0 min (*a*), 10 min (*b*), 20 min (*c*) and 30 min (*d*). Number of beats/min was counted for cells 1 and 2 as denoted on the micrograph at the four incubation times. Cell 1: (*a*), 48; (*b*), 46; (*c*), no beats and bleb formation; (*d*), no beats and cell degeneration. Cell 2: (*a*), 36; (*b*), 37; (*c*), 36; (*d*), no beats and bleb formation. Bar represents 10 $\mu$m.

### Increase of $[Ca^{2+}]_i$ and depletion of cellular ATP in the latent period

We found that cardiomyocytes preloaded with fura-2 AM maintained their cellular ATP at a normal level at 37 °C for more than 2 h. The cell morphology was not distorted on incubation with 1 $\mu$M-CTX at 21 °C in the latent period.

Chelation of intracellular $Ca^{2+}$ of cardiomyocytes with fura-2 produces a characteristic fluorescence showing emission maximum at 510 nm with an excitation wavelength at 340 nm [18]. This optical property was exploited to measure the value of $[Ca^{2+}]_i$ in cardiomyocytes. We measured the fluorescence intensity of each cell and normalized the data obtained from at least 400 cells. As shown in Figs. 4(*a*) and 4(*b*), the CTX action in the latent period tended to shift the cells to a population having higher fluorescence intensities. The longer the CTX treatment, the higher the intensity. The mean values of fluorescence intensities were: control cells, $30.30 \pm 10.57$ (Fig. 4*a*); cells treated with CTX for 3 min, $40.01 \pm 13.31$; cells treated with CTX for 5 min, $44.60 \pm 14.42$; cells treated with CTX for 10 min, $48.93 \pm 17.98$ (Fig. 4*b*). The large S.D. might be due to the inevitable heterogeneity of $[Ca^{2+}]_i$ in a population of isolated cardiomyocytes. This was indicated in a study by Wier *et al.* [18]. According to their measurement, the values of $[Ca^{2+}]_i$ (mean $\pm$ S.D.) are $134 \pm 43$ nM for quiescent cells, $270 \pm 91$ nM for spontaneously contracting cells, and $955 \pm 800$ nM for hypercontracted cells. Regardless, the values of each kind of CTX-treated cells differed significantly from the value of the controlled cells ($P < 0.01$ by Student's $t$ test). Apparently, within 5 min, CTX caused elevation of $[Ca^{2+}]_i$ levels.

Meanwhile, a large portion of endogenous ATP was depleted. As shown in Fig. 5(*a*), the longer the CTX treatment or the larger the CTX dosage used, the more energy (ATP) was consumed. The endogenous ATP could be lowered to 50% of the original value without affecting membrane integrity. Deluca *et al.* [19] demonstrated also that cardiomyocytes remained viable even if their endogenous ATP dropped to 25% of the original value.

Both $[Ca^{2+}]_i$ and the endogenous ATP could be returned to normal levels if the CTX-treated cells were washed exhaustively with the bath medium or incubated

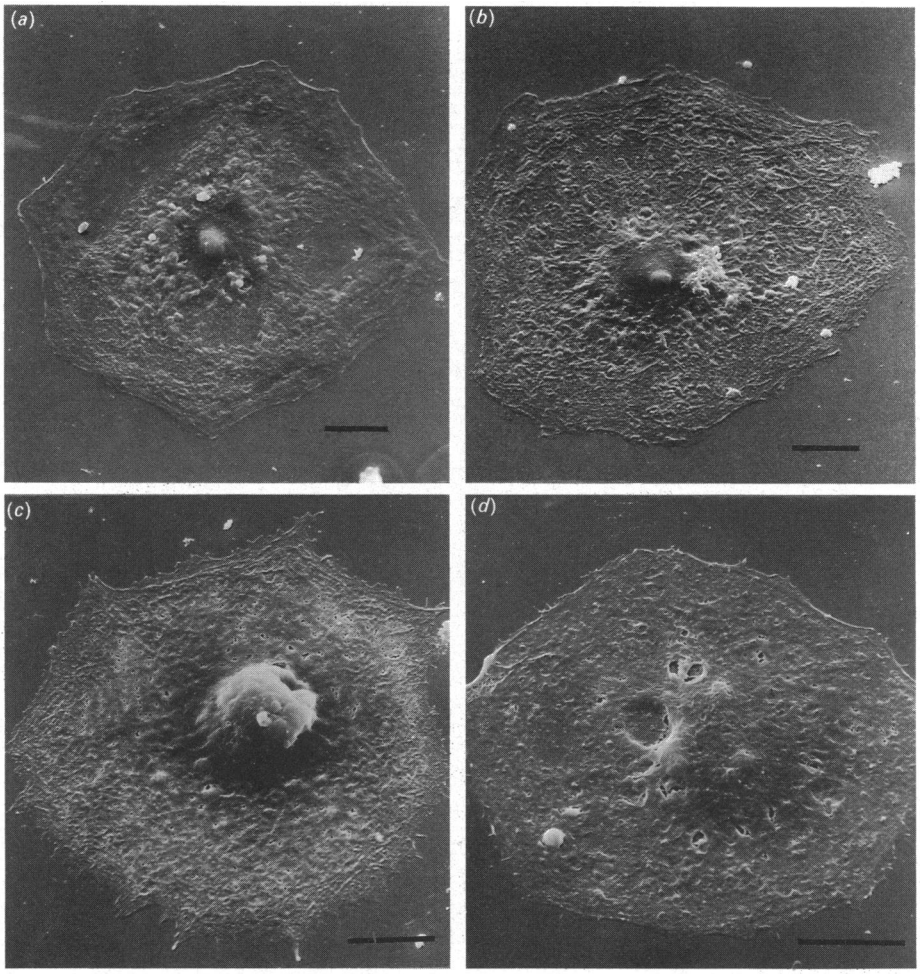


**Fig. 2. Cell morphology under scanning-electron microscope**

Cardiomyocytes on a coverslip were treated with cardiotoxin under the same conditions as in Fig. 1 (*a–d*). The cells were fixed according to conventional procedure (see text for details). Bar represents 10 $\mu$m.

with 125 $\mu$g of CTX/ml of antibody. In the latter situation, $[Ca^{2+}]_i$ decreased gradually and was restored to a normal level after a 20 min incubation (Figs. 4*c* and 4*d*). The mean values of fluorescence intensities of cells were: $38.06 \pm 14.26$ for a 10-min incubation period; $32.08 \pm 9.07$ for a 20-min incubation period. Meanwhile, ATP was regenerated, but a longer incubation was required to achieve a normal level (Fig. 5*b*).

Having acknowledged the ability of CTX to enhance $[Ca^{2+}]_i$, we decided to test whether diminution of $[Ca^{2+}]_i$ would affect the CTX degeneration of cardiomyocytes.

### Suppression of the CTX-induced cardiomyocyte degeneration by $Ca^{2+}$ antagonists and $Ca^{2+}$ chelation

Flunarizine and diltiazem are $Ca^{2+}$ antagonists and can block $Ca^{2+}$ influx [20]. We observed that cardiomyocytes did not degenerate when they were incubated in 3.75 $\mu$M-flunarizine or 3.75 $\mu$M-diltiazem or 1.5 $\mu$M-fura-2 AM at 37 °C for 2 h. Degeneration induced by 4 $\mu$M-CTX of these pretreated cells and a control sample was compared. The time course for the cell degeneration is shown in Fig. 6. After 30 min incubation, the extent of cell degeneration was 63 % for the control cells, 49 % for the flunarizine-pretreated cells, 50 % for the diltiazem-pretreated cells and 47 % for the fura-2 AM-pretreated cells. Apparently, diminution of intracellular $Ca^{2+}$ via blockage of $Ca^{2+}$-influx or chelation of intracellular $Ca^{2+}$ inhibited the CTX-induced degeneration of cells, although only partially.

### Effects of inhibitors of proteinase or phospholipase on the CTX-induced cardiomyocyte degeneration

All experiments were performed under conditions where the enzymic inhibitors used alone caused minimal degeneration of cardiomyocytes. Degeneration of cells in 4 $\mu$M-CTX in the presence or in the abscence of the inhibitors was compared.

Indomethacin is believed to inhibit platelet and leucocyte phospholipase $A_2$ [21]. Trifluoperazine, a calmodulin antagonist, may also inhibit endogenous phospholipase [22]. The CTX-induced cell degeneration was not influenced by the presence of 12.5 $\mu$M-trifluoperazine or 100 $\mu$M-indomethacin.

Leupeptin and antipain modify the active cysteine to inhibit lysosomal and non-lysosomal proteinases [23]. Both agents greatly suppressed the CTX-induced cell degeneration (Fig. 6). About 37–39 % of cells degenerated on treatment with CTX at 37 °C for 30 min in the presence of 200 $\mu$M-leupeptin or 50 $\mu$M-antipain.

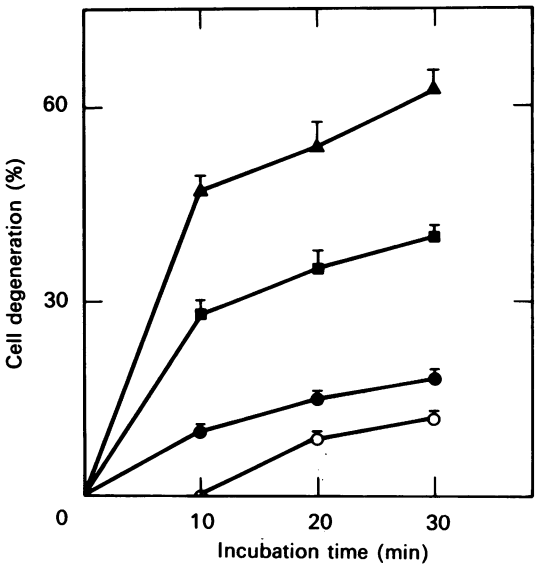


**Fig. 3. Dependence of cardiomyocyte degeneration on CTX dosage**

Cells on Petri dishes were treated with CTX at 37 °C. CTX concentrations: (○), 1 μM; (●), 2 μM; (■), 3 μM; (▲), 4 μM. Each point represents the mean ± S.D. of 15 determinations.

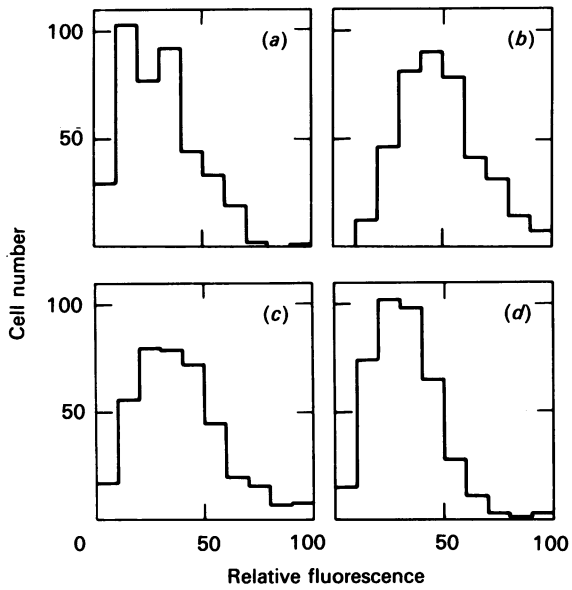


**Fig. 4. Relative concentration of $[Ca^{2+}]_i$ among cardiomyocytes**

The relative $[Ca^{2+}]_i$ in each cell is reflected by the fluorescence intensity shown on the abscissa. Cells on a coverslip were preloaded with 1.5 μM-fura-2 AM at 37 °C for 90 min. The cells were then treated with 1 μM-CTX at 21 °C for 0 min (*a*) and 10 min (*b*). Cells which had been treated with CTX at 21 °C for 10 min were incubated with CTX antibody (125 μg/ml) for 10 min (*c*) and 20 min (*d*). For each experiment 400 cells were selected at random and the fluorescence intensity of each cell, which was measured with a microscope fluorometer (see text for details), was represented by a value relative to 100, the highest intensity.

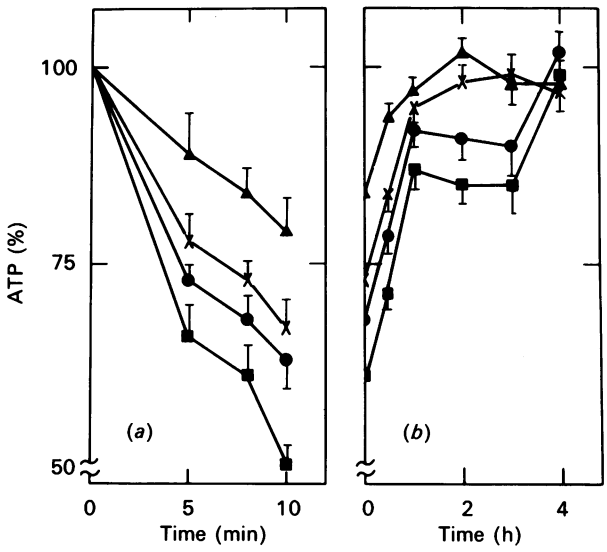


**Fig. 5. (*a*) ATP content of myocardiocytes during CTX treatment and (*b*) restoration of ATP in the CTX-treated cardiomyocytes with CTX antibody**

(*a*) Cells on a microplate were treated with CTX in the bath medium at 21 °C. Cardiotoxin concentrations: (▲), 0.14 μM; (×), 0.28 μM; (●), 0.42 μM; (■), 0.56 μM. (*b*) Cardiomyocytes were treated with CTX at several dosages as in (*a*) for 8 min. The CTX-treated cells were incubated with 125 μg of CTX antibody/ml at 21 °C for varying times. Percentage of ATP was estimated using the level of ATP in the control cells as 100. Each point represents the mean ± S.D. of 20 determinations. Each point differs significantly from the corresponding control samples at zero time.

Suppression of the cell degeneration was not enhanced by increasing concentrations of leupeptin to 400 μM or antipain to 100 μM.

Methylamine is an inhibitor of lysosomal proteinases and 3-methyladenine is an inhibitor of autophagy [23]. The CTX-induced cell degeneration was not affected by addition of 10 mM-methylamine or 5 mM-3-methyladenine (Fig. 6 for the effect of methylamine).

Trypsin inhibitor (20 μM), a serine proteinase inhibitor, did not affect the CTX-induced cell degeneration when added to the bath medium.

Since the results of binding assays indicated that leupeptin, antipain, fura-2 AM, flunarizine and diltiazem all had a very slight effect on the binding of CTX to the cells (results not shown), the inhibitory effect of these agents on the CTX-induced cell degeneration could not be due to decreased binding of CTX to the cells.

## DISCUSSION

That CTX bound to cardiomyocytes can be washed off and cardiomyocytes treated with CTX antibody to reduce the effectiveness of CTX suggests that the CTX-binding sites are on the outer cell surface. Several of our findings indicate that the immediate effect of CTX in the latent period, during which the membrane structure remains intact, can increase $[Ca^{2+}]_i$ levels and deplete endogenous ATP. These two events can be reversed (Figs. 4 and 5). If CTX indeed has a dual action on

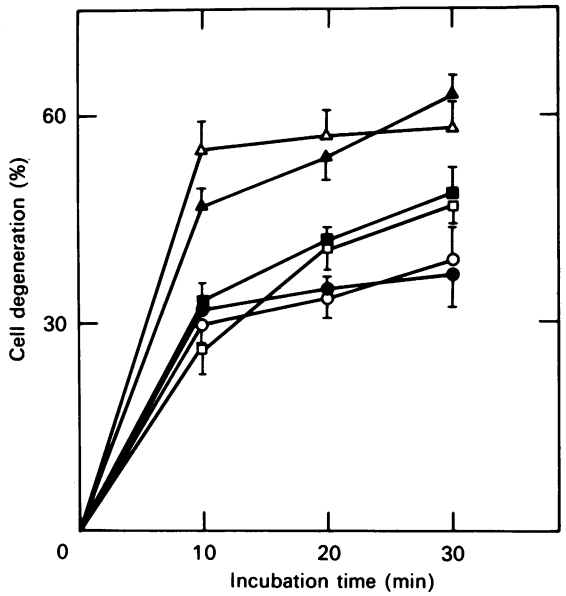


**Fig. 6. Inhibitory effect of several agents on cardiomyocyte degeneration caused by CTX treatment**

Cells on Petri dishes were treated with 4 $\mu$M-CTX at 37 °C. Pretreatment with the agents was at 37 °C. (▲), Control cells; (△), cells preloaded with 10 mM-methylamine; (●), cells preloaded with 50 $\mu$M-antipain for 30 min; (○), cells preloaded with 200 $\mu$M-leupeptin for 30 min; (■), cells preloaded with 3.75 $\mu$M-flunarizine for 60 min; (□), cells preloaded with 1.5 $\mu$M-fura-2 AM for 90 min. Each point is the mean ± S.D. of 15 determinations. Except for the methylamine-treated cells, each point differs significantly from the corresponding one in the control sample.

muscle suggested in previous studies [24], the CTX-induced increase of $[Ca^{2+}]_i$ in cardiomyocytes shown here may result from two mechanisms. Firstly, depolarization owing to increasing $Na^+$ influx leads to release of $Ca^{2+}$ from the sarcoplasmic reticulum. Secondly, increase of $Ca^{2+}$ influx may be through membrane pores or other physiological transport systems. Both quin-2 AM and fura-2 AM have been used as intracellular $Ca^{2+}$ indicators. Fura-2 may have the same ability as quin-2, which can inhibit $Ca^{2+}$ inflow via intracellular-$Na^+$/extracellular-$Ca^{2+}$ exchange [25]. Because attenuation of $Ca^{2+}$ influx by blockade of either the slow channel for $Ca^{2+}$ influx with flunarizine or diltiazem [20], or the intracellular-$Na^+$/extracellular-$Ca^{2+}$ exchange mode with $Ca^{2+}$ chelates [25], can suppress the ability of CTX to degenerate cardiomyocytes, the enhancement of intracellular $Ca^{2+}$ through the two physiological transport systems should not be ruled out. Our results suggest that the CTX-induced formation of membrane pores, as proposed by Harvey *et al.* [24], may appear after the latent period following CTX treatment (Figs. 2*c* and 2*d*). Depletion of ATP in the latent period suggests that endogenous ATP is consumed in pumping out the excess $Ca^{2+}$ resulting from the enhanced $Ca^{2+}$ influx. The ATP depletion may arise also from the inhibition of ATP regeneration. When endogenous ATP decreases to a certain level, the pumping-out process for $Ca^{2+}$ is insufficient to compensate for the increase in $[Ca^{2+}]_i$. Cells are therefore overloaded with $Ca^{2+}$ and some of the $Ca^{2+}$-dependent enzymes may be activated causing damage to the membrane structure. Indeed, we found that if mitochondrial calcium uptake was blocked with Ruthenium Red [26], thereby increasing $[Ca^{2+}]_i$ and maintaining mitochondrial ATP generation CTX-induced cardiomyocyte degeneration was enhanced (W.-F. Tzeng & Y.-H. Chen, unpublished observations). That leupeptin and antipain suppress the ability of CTX to degenerate cardiomyocytes suggests that activation of $Ca^{2+}$-dependent proteinases in lysosomes or/and in non-lysosomal fractions is relevant to the CTX-induced cell degeneration. Activation of lysosomal proteinases to impair the cells can be ruled out since lysosomotropic agents such as methylamine and autophage inhibitors such as methyladenine cannot suppress the CTX action. The results of Nicotera *et al.* [23] revealed that the formation of plasma-membrane blebs on hepatocytes by agents that increase $[Ca^{2+}]_i$ is mediated by the activation of a nonlysosomal proteolytic system. Recent reports indicate that $Ca^{2+}$-dependent proteinases are involved in $Ca^{2+}$-activated proteolysis in various pathological conditions in muscle [23,27–9]. In agreement with our results, the CTX-induced formation of blebs on cardiomyocytes may arise from the proteolysis attributed to the activation of $Ca^{2+}$-dependent non-lysosomal proteinases, which can be inhibited by antipain and leupeptin but cannot be inhibited by trypsin inhibitors.

Activation of $Ca^{2+}$-dependent phospholipase to attack membrane phospholipid [30] does not seem to be crucial for the CTX-induced cell degeneration, since neither indomethacin nor trifluoperazine suppress CTX action.

In conclusion, the action of CTX on the extracellular surface of intact cardiomyocytes increases $[Ca^{2+}]_i$ and cells become overloaded with $Ca^{2+}$. Thus, $Ca^{2+}$-dependent non-lysosomal proteinase(s) may be activated leading to damage of the membrane structure.

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