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Dual effects of extracellular Ca^{2+} on cardiotoxin-induced cytotoxicity and cytosolic Ca^{2+} changes in cultured single cells of rabbit aortic endothelium

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Abstract

The effects of extracellular Ca^{2+} on cytotoxicity induced by cardiotoxin (CTX), isolated from Chinese cobra venom, were investigated in cultured rabbit aortic endothelial cells (RAECs). In Hank's buffered saline solution (HBSS) containing 1.2 mM Ca^{2+}, CTX (1–30 μM) caused cell necrosis and cell death in a concentration-dependent manner, as determined by trypan blue exclusion test performed after a 20-min CTX treatment. The concentration of CTX that caused 50% cell death was about 6.5 μM. CTX (10 μM)-induced RAEC damage was also evident but less prominent in Ca^{2+}-free medium and almost completely prevented in medium containing 7–10 mM Ca^{2+}. Therefore, Ca^{2+} appears to provoke CTX-induced injury at physiological concentrations, but protects against it at high concentrations. The protection of RAECs from CTX-induced injury could also be achieved by high concentrations of Ni^{2+} and Mg^{2+}. Using the fura-2 fluorescence technique to measure the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) of single RAEC, it was shown that in 1.2 mM Ca^{2+}-containing HBSS, treatment of RAECs with 10 μM CTX for 7–35 min resulted in a tremendous and irreversible $[Ca^{2+}]_i$ elevation, suggestive of cell membrane damage and extracellular Ca^{2+} entry. Ni^{2+} could also enter the cytosol of these damaged RAECs. However, there was no $[Ca^{2+}]_i$ elevation or Ni^{2+} entry in RAECs that were preincubated in HBSS containing 7 mM Ca^{2+} or Ni^{2+} before CTX exposure. In RAECs protected with 7 mM Ca^{2+}, the intracellular Ca^{2+} signals triggered by 100 μM extracellular ATP or 10 μM bradykinin in CTX-treated groups were similar to those in the untreated control groups. Taken together, the results indicate that high extracellular Ca^{2+} concentrations protected RAECs from CTX-induced injury, and preserved the ability of CTX-treated RAECs to generate Ca^{2+} signals in response to physiological stimuli. © 1997 Elsevier Science B.V.

Keywords: Calcium; Cardiotoxin; Cytotoxicity; Endothelium; Direct lytic factor; Aorta

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1. Introduction

Cardiotoxin (CTX) is a membrane-active polypeptide that has been purified from cobra snake venom [1]. It exerts many effects, including membrane depolarization, contraction of cardiac and skeletal muscles, and modulation of a number of enzymes, such as phospholipase C and the plasmalemmal Ca^{2+} pump [2,3]. CTX has also been named cytotoxin or direct lytic factor because continuous exposure to this toxin leads to hemolysis and cytolysis [4–7]. The manner by which CTX interacts with the plasma membrane is not fully understood, but there is evidence that suggests that the positively charged amino acid residues of CTX interact with some anionic domains of the plasmalemmal lipid molecules, thus leading to disorganization of the plasma membrane [8–11]. In concordance with this notion, it has been shown that high concentrations of extracellular Ca^{2+} can prevent the action of CTX, presumably by competing with CTX for the same anionic sites. For instance, Chen et al. [12] and Jiang et al. [6] reported that 10 mM Ca^{2+} inhibited CTX-induced hemolysis. Leung et al. [4] also demonstrated that CTX-induced cytolysis of Ehrlich ascites tumor cells was attenuated by high Ca^{2+} concentrations. In addition, high Ca^{2+} concentrations also inhibited the contracture of skeletal and cardiac muscles that was triggered by CTX [13–15]. High concentrations of Ca^{2+} could also block the binding of radiolabeled CTX to membranes of axons [8], skeletal muscles [13] and erythrocytes [16].

Vascular endothelial cells respond to many vasoactive substances by releasing a number of factors, such as the endothelium-derived relaxation factor (now known as nitric oxide) and endothelin, which can modulate the tone of the underlying vascular smooth muscle [17]. The endothelium is in immediate contact with CTX, once this toxin invades the circulatory system. We previously showed that, in endothelium-intact rat aortic rings, CTX-induced contraction was preceded by a small and transient relaxation and that CTX could also transiently suppress phenylephrine-induced contraction in an endothelium-dependent manner [18]. These functional studies suggest that CTX damaged the endothelial plasmalemma, thus permitting Ca^{2+} influx and subsequent nitric oxide release. However, despite the well-recognized hemodynamic effects of CTX, direct toxic effects of CTX on isolated endothelial cells have not been demonstrated hitherto. Whether or not high levels of Ca^{2+} can also protect endothelial cells from CTX-induced injury is therefore unknown. Furthermore, previous studies on CTX effects used either cell membranes or a group (population) of cells, and there has not been any work reporting changes in single cells after CTX treatment. In this study, using the trypan blue exclusion test and the fura-2 microfluorimetric technique to measure $[Ca^{2+}]_i$ in single cells, it was shown that in 1.2 mM Ca^{2+}-containing HBSS, treatment of rabbit aortic endothelial cells (RAECs) with CTX led to a substantial $[Ca^{2+}]_i$ elevation prior to plasma membrane damage and eventual cell lysis. However, RAECs preincubated in medium containing high Ca^{2+} levels (7–10 mM) were protected from CTX-induced injury. Although there have been many reports showing that high Ca^{2+} (or divalent cation) concentrations could protect cells from CTX-induced structural damage, whether functional integrity is preserved or not in these protected cells has not been demonstrated hitherto. Here, we show that CTX-treated RAECs that were protected by high levels of Ca^{2+} could generate Ca^{2+} signals (in response to agonists), similar to those of untreated cells, suggesting that high levels of Ca^{2+} could maintain the functional integrity of CTX-challenged endothelium.

2. Materials and methods

2.1. Materials

Medium 199, penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Fura-2 AM, trypan blue, mepacrine, neomycin and ATP were purchased from Sigma (St. Louis, MO, USA). Bradykinin was purchased from Peninsula Laboratories, (Belmont, CA, USA). CTX was purified from the venom of *Naja naja atra* by a three-step column chromatography method, as previously described [15]. Phospholipase A_2 (PLA_2) activity was found to be very low (0.05–0.15%) in the purified CTX using a standard pH–stat assay [15]. Mepacrine, an inhibitor of PLA_2, did not prevent the cytotoxic action of CTX (see Section 3), suggesting that the CTX-induced cytotoxicity was not due to the trace amount of contaminating PLA_2.

Neurotoxin was also purified from the same snake venom by carboxymethyl (CM) Sephadex G-25 column chromatography [15]. All other chemicals were of reagent grade.

2.2. Culture of endothelial cells

Endothelial cells were isolated from the thoracic aorta of New Zealand white rabbits (weighing 1.5 to 2.5 kg) in the following manner. Under sterile conditions, the thoracic aorta was removed and placed in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco). After careful removal of the surrounding fat and connective tissue, the luminal surface was incubated in HBSS containing 0.25% trypsin and 1 mM EDTA (Gibco) for 5 min at 37°C. Thereafter, the luminal surface was gently flushed with Medium 199 supplemented with 20% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) (enriched Medium 199). The effluent was collected and centrifuged (200 *g*) for 10 min. The cell pellet was resuspended in enriched Medium 199 and seeded onto six-well tissue culture plates (Costar, Cambridge, MA, USA). The cells were maintained in the same culture medium in a humidified atmosphere of 5% CO_2 at 37°C and were passaged about once a week. HBSS containing 0.05% trypsin and 0.53 mM EDTA (Gibco) was used to detach cells. All RAECs used in this study were between passage numbers four and ten.

The identity of the endothelial cells was indicated by their typical cobblestone appearance (Fig. 3a) and confirmed by positive immunofluorescence using an anti-von Willebrand factor VIII antibody (Sigma) (not shown).

2.3. Determination of cell viability

HBSS, composed of (mM) 138 NaCl, 5.3 KCl, 0.8 $MgSO_4$, 1.2 $CaCl_2$, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, 5 glucose and 25 HEPES, buffered at 7.4, was used for washing and incubation. RAECs were gently washed once and cell viability was examined by the trypan blue (0.1% final concentration) exclusion test, which was performed after treatment with or without CTX for 20 min (except in Fig. 5) at room temperature. In each experiment, 100 cells in each treatment group were counted at random under a light microscope.

2.4. Fura-2 fluorescence measurement in a single RAEC

The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a single RAEC was measured using the Ca^{2+}-sensitive fluorescence dye, fura-2. HBSS, having the composition mentioned above, was used for washing and for fluorescence measurements. RAECs were grown on glass coverslips for 24–48 h, washed once with HBSS and then incubated in Medium 199 containing 2% FBS and 1 μM fura-2 acetoxy methylester for 30 min at 37°C. Thereafter, the fura-2-loaded cells were washed twice with HBSS, bathed in 500 μl of HBSS and the coverslip was mounted in a small plastic chamber. The latter was then put on the stage of an inverted microscope (Nikon, Tokyo, Japan). Single cells were selected using a rectangular diaphragm that was fitted into the emission port of the microscope, which was, in turn, coupled to a photomultiplier of the fluorescence instrument (Photon Technology International, South Brunswick, NJ, USA). The selected cell was excited alternately at 340 and 380 nm and the emitted fluorescence was detected at 510 nm. After the fluorescence signals stabilized, drugs or agonists were added in small volumes (≤ 5 μl) followed by very gentle mixing. All experiments were performed at room temperature.

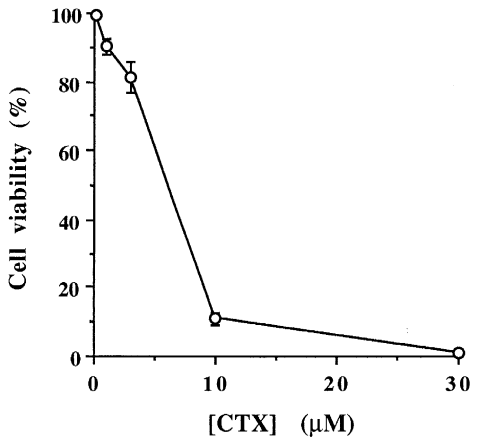

Fig. 1. CTX caused the concentration-dependent death of RAECs. Cells in HBSS containing 1.2 mM Ca^{2+} were treated with different concentrations of CTX for 20 min prior to a trypan blue exclusion test. Results are expressed as the mean ± SE of four separate experiments.

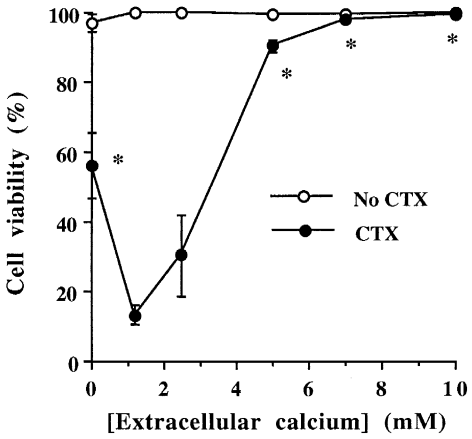

Fig. 2. Effects of extracellular Ca^{2+} concentration on CTX-induced RAEC death. Cells in HBSS containing various concentrations of Ca^{2+} were incubated with (solid circle) or without (open circle) CTX (10 μM) for 20 min and cell viability was then examined. Under Ca^{2+}-free conditions, 50 μM EGTA was added. Results are expressed as the mean ± SE of six separate experiments. An * indicates statistical significance compared to CTX-treated RAECs in HBSS containing 1.2 mM Ca^{2+} ($P < 0.05$).

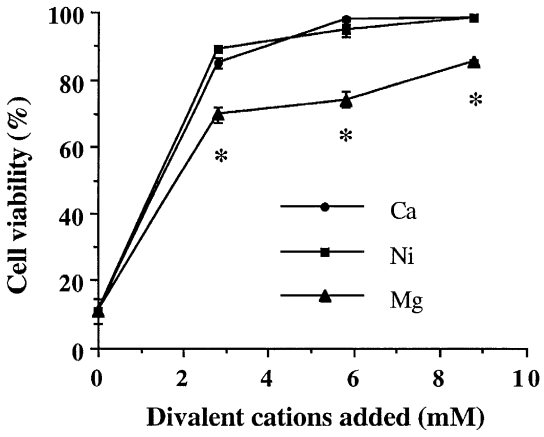

Fig. 4. The protective effects of high Ca^{2+}, Ni^{2+} and Mg^{2+} on CTX-induced cell death. Cells were initially bathed in 1.2 mM Ca^{2+}-containing HBSS. Various concentrations of Ca^{2+}, Ni^{2+} and Mg^{2+}, and CTX (10 μM) were then added for 20 min prior to the trypan blue exclusion test. Results are expressed as the mean ± SE of four separate experiments. * The protective effect of Mg^{2+} was significantly less prominent than that of Ca^{2+} or Ni^{2+} ($P < 0.05$).

2.5. Statistical analysis

The results were expressed as mean ± SEM. The Student's paired *t*-test was employed and differences were considered significant when $P < 0.05$.

3. Results

Fig. 1 shows that CTX (1–30 μM) caused cell death in 1.2 mM Ca^{2+} medium in a concentration-dependent manner. The concentration of CTX that caused 50% cell death was about 6.5 μM. As a negative control, neurotoxin, also present in snake venom and having structural similarity to CTX, did not cause cell death, even at a concentration of 50 μM (cell viability = 99.7 ± 0.3%; $n = 3$). Fig. 2 shows that extracellular Ca^{2+} exhibited a concentration-dependent biphasic effect on CTX-induced cytotoxicity. CTX-induced cytotoxicity was higher in 1.2 mM Ca^{2+}-containing HBSS than in Ca^{2+}-free HBSS. However, increasing the concentration of Ca^{2+} in

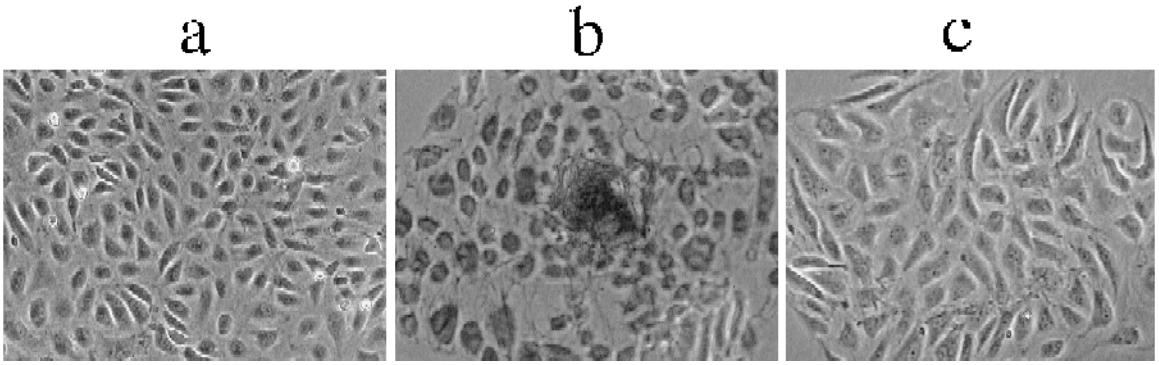

Fig. 3. Protective effect of high levels of Ca^{2+} on CTX-induced RAEC damage. (a) Untreated control cells in 1.2 mM Ca^{2+}-containing HBSS. (b) and (c) Cells were treated with 10 μM CTX in HBSS containing, 1.2 and 7 mM Ca^{2+} for 20 min, respectively. Thereafter, trypan blue (0.1% final concentration) was added. Magnification = 400 ×.

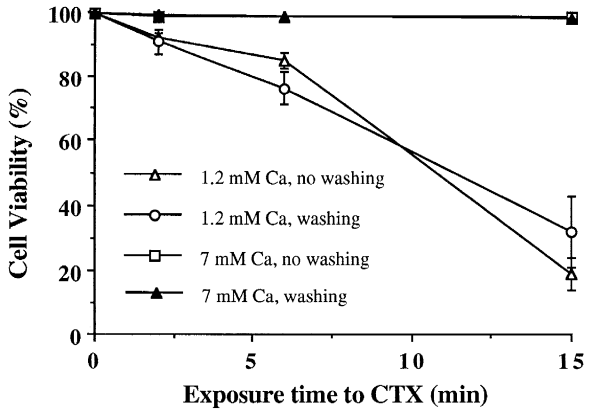

Fig. 5. The effect of the washing out of CTX on cell death during the course of CTX treatment. RAECs were initially bathed in 1.2 mM Ca^{2+} or 7 mM Ca^{2+}-containing HBSS. CTX (10 μM) was then added and cell death was determined at 2, 6 and 15 min (open triangle, square). CTX (10 μM) was also added to other groups, which were washed extensively for 30 s with 1.2 mM Ca^{2+}-containing HBSS at 2, 6 or 15 min (circle, closed triangle). These groups were then bathed in 1.2 mM Ca^{2+}-containing HBSS for a further 13, 9 or 0 min, respectively, before the trypan blue exclusion test was performed. Results are expressed as the mean ± SE of five separate experiments.

HBSS attenuated CTX-induced cytotoxicity so that in 7–10 mM Ca^{2+} HBSS, nearly all cells were protected. CTX-induced cytotoxicity and the protective effects of high Ca^{2+} were also obvious when cell morphology was examined. Fig. 3a and b show, respectively, the morphology of RAEC incubated in the absence and presence of CTX (10 μM) in 1.2 mM Ca^{2+} HBSS for 20 min. The majority of CTX-treated cells exhibited necrosis and were stained with trypan blue. However, in 7 mM Ca^{2+} HBSS, almost all the CTX-treated cells retained normal morphology (Fig. 3c).

We also tested to see if other divalent cations could prevent CTX-induced cytotoxicity in RAECs. Fig. 4 shows that high concentrations of Ni^{2+} were as effective as high concentration of Ca^{2+} in inhibiting the action of CTX. Mg^{2+} also elicited a substantial protective effect, but was significantly less effective than Ca^{2+} or Ni^{2+} ($P < 0.05$).

We next tested the reversibility of CTX action (Fig. 5). Treatment of RAECs with 10 μM CTX in 1.2 mM Ca^{2+} medium resulted in a time-dependent

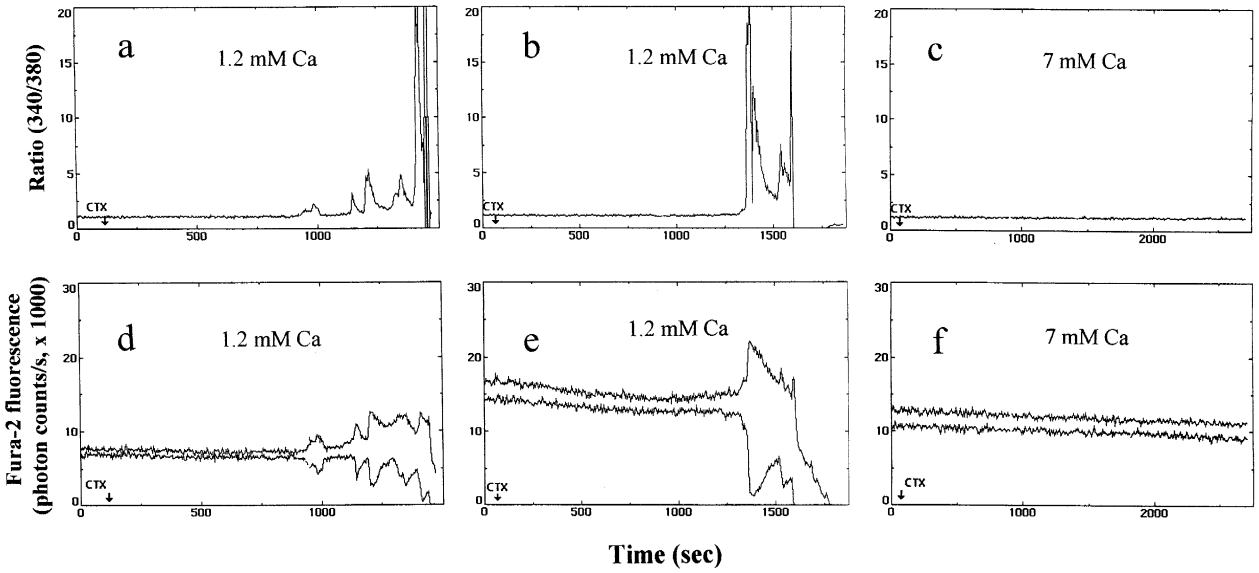

Fig. 6. The effect of CTX on $[Ca^{2+}]_i$ of a single RAEC. A fura-2-loaded RAEC in 1.2 mM Ca^{2+}-containing HBSS (a and b) or 7 mM Ca^{2+}-containing HBSS (c) was treated with 10 μM CTX (arrow). The *y*-axis represents the fura-2 fluorescence ratio of 340 nm/380 nm. Small fluctuations in $[Ca^{2+}]_i$ before the substantial $[Ca^{2+}]_i$ elevation (a) was observed in eight out of eleven cells, while the other three cells did not exhibit fluctuation before the large $[Ca^{2+}]_i$ surge (b). In (a) and (b), there was a variation in the time (7–35 min) between CTX exposure and the abrupt increase (''burst'') in the ratio value. The result shown in (c) was observed in at least three other cells tested. (d), (e) and (f) show the corresponding fluorescence at 340 nm (upper trace) and 380 nm (lower trace) of samples (a), (b) and (c), respectively.

increase in cell death. If RAECs were treated with CTX for 2 min, then washed extensively and checked for viability after 13 min, the degree of cytotoxicity was similar to that observed at the 2-min time point. This result suggests that CTX could be washed away so that there was no further cell damage during the 13 min incubation. A similar result was obtained at the 6-min time point. Ca^{2+} (7 mM) offered almost complete protection against CTX-induced cytotoxicity at all time points, regardless of whether the ''wash-out'' protocol was employed or not.

CTX has been known to activate PLA_2 and phospholipase C [3]. We investigated if the CTX-induced toxicity was due to activation of these enzymes. Mepacrine (100 μM, an inhibitor of PLA_2) did not prevent CTX-induced cytotoxicity in Ca^{2+}-containing medium (cell viability = 4.4 ± 1.0% in CTX-treated group versus 5.1 ± 1.1% in CTX- and mepacrine-treated group; $n = 8$) or Ca^{2+}-free medium (cell viability = 17.1 ± 0.7% in CTX-treated group versus 14.2 ± 1.0% in CTX- and mepacrine-treated group; $n = 8$). Neomycin (100 μM, an inhibitor of phospholipase C) also did not prevent CTX-induced cytotoxicity in Ca^{2+}-containing medium (cell viability = 5.9 ± 0.7% in CTX-treated group versus 6.6 ± 0.8% in CTX- and neomycin-treated group; $n = 8$) or Ca^{2+}-free medium (cell viability = 16.4 ± 1.0% in CTX-treated group versus 16.1 ± 1.4% in CTX- and neomycin-treated group; $n = 8$). Therefore, activation of PLA_2 and phospholipase C did not appear to mediate the cytotoxic action of CTX.

The protective effects caused by high concentrations of divalent cations on CTX-induced membrane damage were also demonstrated using the fura-2 fluorescence technique. In HBSS containing 1.2 mM Ca^{2+}, a single RAEC treated with CTX (10 μM) showed a tremendous (340 nm/380 nm fluorescence ratio > 20) $[Ca^{2+}]_i$ elevation after a variable period of 7–35 min (26.5 ± 4.2 min; $n = 11$; mean ± SEM) (Fig. 6a–b). A smaller $[Ca^{2+}]_i$ elevation was observed prior to the tremendous $[Ca^{2+}]_i$ elevation in eight out of eleven cells tested (Fig. 6a). Fig. 6d–e show the fluorescence changes (at 340 and 380 nm) of the experiment shown in Fig. 6a–b, respectively. The fluorescence changes at these two wavelengths were opposite (i.e., rise and fall at 340 and 380 nm, respectively) and, therefore, genuinely reflects $[Ca^{2+}]_i$ elevation. Note that at the very late stage, the ratio fluctuated dramatically and subsequently dropped to zero (Fig. 6a–b). The corresponding fluorescence at 340 and 380 nm also eventually dropped to zero (Fig. 6d–e), probably due to the leakage of fura-2 from the severely damaged single cell, being infinitely diluted by the large volume of the extracellular medium. The addition of 7 mM Ca^{2+} during or immediately after the large $[Ca^{2+}]_i$ elevation did not prevent the cell from disruption (not shown). However, there was no persistent elevation in $[Ca^{2+}]_i$ in CTX-treated RAECs for 40 min, if the HBSS already contained 7 mM Ca^{2+} at the beginning of the experiment (Fig. 6cFig. 6f). The protective effect of high Ca^{2+} concentrations could also be achieved by 7 mM Ni^{2+} (Fig. 7), which quenches fura-2 fluorescence at all excitation wavelengths [19]. When RAECs were treated with 10

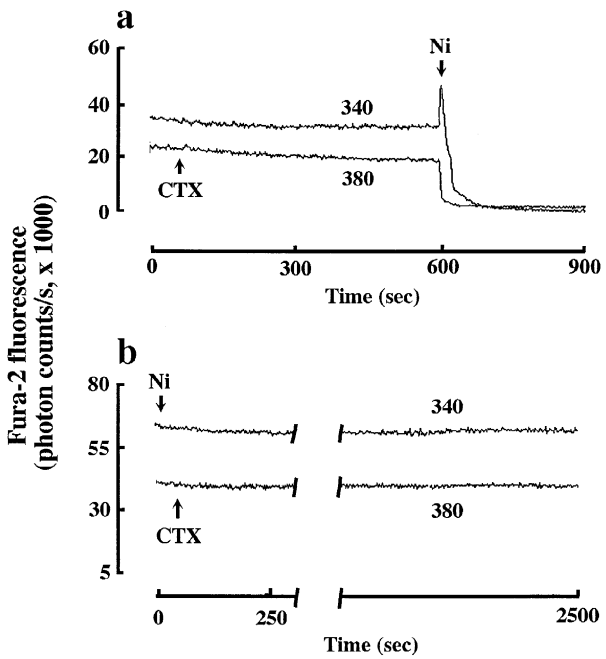

Fig. 7. High Ni^{2+} prevented CTX-induced plasma membrane leakiness in single RAECs. In (a) and (b), the upper and lower traces represent fura-2 fluorescence at 340 and 380 nm, respectively. (a) A fura-2-loaded RAEC in 1.2 mM Ca^{2+}-containing HBSS was exposed to 10 μM CTX. As soon as the fluorescence at 340 and 380 nm began to increase and decrease, respectively, 7 mM Ni^{2+} was added. (b) A fura-2-loaded RAEC in 1.2 mM Ca^{2+}-containing HBSS was treated with 7 mM Ni^{2+} before CTX (10 μM) challenge. Similar results were obtained in four other separate experiments. In (a), there was a variation in the time (7–30 min) between CTX exposure and the changes in fluorescence at both wavelengths.

μM CTX in 1.2 mM Ca^{2+} HBSS for some 3–30 min, there was an increase and decrease in fura-2 fluorescence at 340 and 380 nm, respectively, indicating Ca^{2+} influx and, hence, CTX-induced permeabilization (Fig. 7a). When Ni^{2+} was then added, the fura-2 fluorescence at both 340 and 380 nm was completely quenched, indicating the entrance of Ni^{2+} into the cytosol. However, when RAECs were preincubated with Ni^{2+} before CTX treatment, there was no change in fluorescence at both wavelengths for 40 min (Fig. 7b), suggesting that neither $[Ca^{2+}]_i$ elevation nor Ni^{2+} entry took place. The results in Figs. 6 and 7 thus suggest that high Ca^{2+}- or Ni^{2+} concentrations could prevent CTX-induced membrane damage.

The results obtained so far indicate that high concentrations of divalent cations could maintain the structural integrity of the plasma membrane of CTX-treated RAECs, thus preventing the cells from membrane leakiness and necrosis. However, whether such protected cells remain functionally intact or not is unclear. One approach to examine this is to test how well the protected RAECs respond to physiological agonists by generating Ca^{2+} signals. In cells bathed in 1.2 mM Ca^{2+}, or 7 mM Ca^{2+}, or cells bathed in 7 mM Ca^{2+} and treated with 10 μM CTX, bradykinin evoked a surge in $[Ca^{2+}]_i$, followed by a gradual decline to a plateau level that was slightly above the resting $[Ca^{2+}]_i$ (Fig. 8a–c). There is no significant difference in magnitude between the bradykinin-stimulated Ca^{2+} responses (peak and tonic) under the three different conditions used (Fig. 8d). Similar results were obtained when extracellular ATP was used as an agonist (Fig. 8e). We did not use high Mg^{2+} and Ni^{2+} in this experiment, as these two cations, besides protecting the RAECs, may exert inhibitory effects on plasmalemmal Ca^{2+} channels [20].

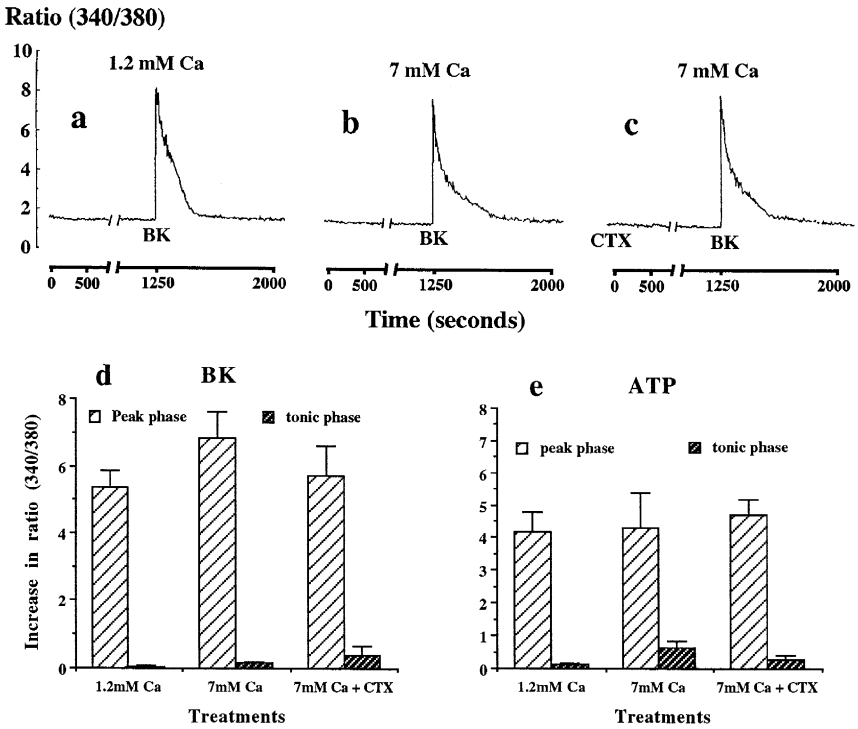

Fig. 8. Agonist-induced Ca^{2+} signaling was unaffected in high Ca^{2+}-protected, CTX-treated RAECs. Single fura-2-loaded RAECs in HBSS containing 1.2 or 7 mM Ca^{2+}, with or without CTX (10 μM) pretreatment for 20 min, were stimulated with bradykinin (BK, 10 μM) (a–c). The *y*-axis represents the fura-2 fluorescence ratio of 340 nm/380 nm. Results are quantified and shown in (d). The ratio increase at peak is taken as the difference between the pre-stimulation ratio and the highest ratio after agonist addition. The ratio increase at tonic phase is the difference between the pre-stimulation ratio and the ratio at 12.5 min after agonist stimulation. Results are expressed as the mean ± SE of eight separate experiments. Similar protocols were done using ATP (100 μM) as the agonist (e). Results are the mean ± SE of ten–thirteen separate experiments.

4. Discussion

Although there have been a number of publications demonstrating the interactions of CTX with various types of tissues [3] and its effect on the circulatory system [14,15,18,21], our report is the first to directly demonstrate the cytotoxic actions of CTX on isolated endothelial cells and the protection of these cells from CTX-induced injury by divalent cations. Our studies are of interest as endothelial cells are in immediate contact with CTX once this toxin invades the circulation. The uptake of trypan blue, membrane necrosis, and substantial leakiness to Ca^{2+} and Ni^{2+} observed in CTX-treated RAECs (Figs. 1, 3, 6 and 7), consistently suggest that CTX caused elevation of permeability to ions at the plasma membrane, eventually leading to membrane degeneration and cell death. The percentage of cell death increased with the exposure time to CTX (Fig. 5), a fact that is in concordance with the variable lag periods (7–35 min) between CTX application and the considerable rise in plasmalemmal permeability to Ca^{2+} and Ni^{2+} in a single RAEC (Figs. 6 and 7). These results suggest a heterogeneity in the sensitivity of individual RAECs to CTX. Our work (presented here) provides the first report showing the effects of CTX at the single-cell level. Tzeng and Chen [21] previously measured $[Ca^{2+}]_i$ changes induced by CTX in single neonatal rat cardiomyocytes. Since the $[Ca^{2+}]_i$ responses were averaged over those obtained from a large number of cell samples, this may account for their finding that $[Ca^{2+}]_i$ rose gradually during the 10-min CTX treatment. Such averaged results may mask the variable lag periods (between CTX application and $[Ca^{2+}]_i$ elevation) in individual cells, as we observed in single RAECs (Figs. 6 and 7). In our case, after the lag period in CTX-treated RAECs, $[Ca^{2+}]_i$ elevation was manifested as an abrupt ''burst'', with or without the preceding fluctuation in $[Ca^{2+}]_i$ (Fig. 6a–b; also, see below).

When RAECs were treated with CTX for 2 and 6 min, then washed extensively and checked for viability after 13 and 9 min, respectively, the degrees of cytotoxicity were similar to those checked immediately at the two time points (Fig. 5). These results indicate that, first, those cells that CTX had already damaged could not be recovered; second, those that had not yet been damaged by CTX could be prevented from cell death by extensive washing. Hence, there appears to be a critical stage during CTX-induced pore formation (permeabilization), after which membrane damage becomes irreversible. This proposal is further supported by the observation that the increase in plasmalemmal Ca^{2+} permeability in CTX-treated RAECs was abrupt, tremendous and was followed by severe membrane disruption (Fig. 6a–b). At the latter ''critical'' moment, the plasma membrane was damaged to an extent that Ni^{2+}, which usually does not permeate cells (also see Fig. 7b), could rapidly enter the cytosol (Fig. 7a).

High concentrations of Ca^{2+} have been known to inhibit CTX actions. For example, CTX-induced hemolysis, contracture of skeletal muscles and guinea pig papillary muscles were strongly suppressed by >10 mM Ca^{2+} [12,13,15]. In a consistent manner, such high concentrations of Ca^{2+} inhibited the binding of radiolabeled CTX to axonal membranes and chick skeletal muscles [8,13]. In the present study, when added prior to CTX, high Ca^{2+} concentrations completely suppressed CTX-induced cytotoxicity (Figs. 2 and 4). It should be emphasized that such high Ca^{2+} concentrations merely represent an experimental condition rather than a pathophysiological situation. Suppression of CTX action could be mimicked by high concentrations of two other divalent cations, Mg^{2+} and Ni^{2+} (Figs. 4 and 7). The prevention of CTX actions by high concentrations of divalent cations has been demonstrated previously in hemolysis, cytolysis and contractility studies [4,6,13,15]. All these data support the hypothesis that CTX and Ca^{2+} compete for the same anionic sites at the plasmalemma [4,13]. Such anionic sites may be located in the phospholipids [9,11]. If Ca^{2+} competes with CTX at the same sites, the activity of CTX would be expected to be highest under Ca^{2+}-free conditions. It was, in fact, observed that the rate of CTX-induced hemolysis and the binding of radiolabeled CTX to erythrocytes were both enhanced when Ca^{2+} in the physiological saline was removed by excess EGTA [7,16]. Likewise, CTX-induced contraction in the rat skeletal muscle was enhanced in Ca^{2+}-free medium [22]. However, in the same study, CTX-induced contraction in human skeletal muscle was considerably depressed in Ca^{2+}-free medium. It was also found that CTX-induced contracture in guinea pig taenia coli was strongly attenuated under

Ca^{2+}-free conditions [23]. We observed that CTX-induced cytotoxicity was much lower in Ca^{2+}-free than in 1.2 mM Ca^{2+}-containing HBSS (Fig. 2). This observation also suggests that Ca^{2+} entry leading to the elevation of $[Ca^{2+}]_i$ caused by CTX may not be a necessary condition to initiate cell damage; it is more likely that it acts by aggravating the injury process leading to cell death. It should also be noted that the concentration of CTX also profoundly affects its interaction with extracellular Ca^{2+} and the subsequent damage (eg. hemolysis) at the plasma membrane [6]. We used 10 μM CTX throughout this work, but other concentrations should be investigated in future.

The prevention of CTX-induced plasmalemmal leakiness by high Ca^{2+}- and Ni^{2+} concentrations is illustrated in Figs. 6 and 7. There was no change in basal fura-2 fluorescence at all in CTX-treated cells protected by high Ca^{2+}- or Ni^{2+} concentrations, indicating that no increases in permeability to Ca^{2+} and Ni^{2+} had taken place. This experimental protocol, while demonstrating how high concentrations of Ca^{2+} and Ni^{2+} could prevent CTX-induced cytolysis, has some advantages over previous methods, such as the hemolysis assay: (1) Small molecules (i.e., Ca^{2+} and Ni^{2+}) were used as ''leakage markers'' instead of much bigger molecules such as hemoglobin; (2) the fura-2 fluorimetric method offers a much more sensitive and real-time measurement. Thus, our data further confirm that high concentrations of divalent cations can preserve the integrity of the plasma membrane of CTX-treated cells.

CTX has been known to modulate the activities of a number of enzymes at the plasma membrane. For instance, studies using purified enzymes or isolated membranes reveal that CTX inhibits Na^+/K^+-ATPase, stimulates phosphatidylinositol 4-kinase, enhances Ca^{2+}–Mg^{2+} ATPase and adenylate cyclase [2,24–26]. Whether or not high concentrations of Ca^{2+} can prevent such modulatory activities by CTX is unknown. Therefore, although the plasmalemmal integrity of CTX-treated RAECs was preserved at high concentrations of divalent cations, it still remains unknown in such protected cells whether the plasmalemmal enzyme activities were altered by CTX or not. In line with this, there is no previous report showing if high Ca^{2+}-protected, CTX-treated cells stay functionally intact or responsive to physiological stimuli. One approach to solve this problem is to test how well CTX-treated, high Ca^{2+}-protected RAECs could generate Ca^{2+} signals in response to agonists. Endothelial cells have been shown to express functional ATP (P_{2U}- and P_{2Y}-purinoceptors) and bradykinin (B_1 and B_2) receptors [27–29]. These receptors are coupled, via a G-protein ($G_{q/11}$), to phospholipase C [30], which cleaves phosphatidyl inositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol [31]. $InsP_3$ releases Ca^{2+} from intracellular stores and, subsequently, the empty state of the stores stimulates extracellular Ca^{2+} entry [32,33]. The decline of a Ca^{2+} signal depends on the sequestration of Ca^{2+} back into the intracellular stores, and Ca^{2+} extrusion into the extracellular space achieved by the plasmalemmal Ca^{2+} pump and/or the Na^+–Ca^{2+} exchanger [34]. In high Ca^{2+} medium, the Ca^{2+} responses triggered by ATP and bradykinin in CTX-treated groups were qualitatively and quantitatively similar to those in the untreated control groups (Fig. 8), suggesting that there are intact $[Ca^{2+}]_i$ homeostatic mechanisms in CTX-treated cells. Therefore, in high Ca^{2+}-protected RAECs, CTX was unlikely to have significant effects on plasmalemmal receptors and Ca^{2+}-handling machineries, and did not seem to cause general adverse effects on plasmalemmal functions. Intracellular Ca^{2+} signalling plays an important role in endothelial cell functions. For example, an increase in endothelial $[Ca^{2+}]_i$ triggers the release of vasoactive substances, such as prostacyclin, nitric oxide and endothelin, which modulate the vascular tone [17]. The preservation of functional integrity in high Ca^{2+}-protected CTX-challenged RAECs may render the latter with the ability to control the contractility of the underlying smooth muscles.

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