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Actions of 4-chloro-3-ethyl phenol on internal Ca^{2+} stores in vascular smooth muscle and endothelial cells

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1 Recently, 4-chloro-3-ethyl phenol (CEP) has been shown to cause the release of internally stored Ca^{2+}, apparently through ryanodine-sensitive Ca^{2+} channels, in fractionated skeletal muscle terminal cisternae and in a variety of non-excitable cell types. Its action on smooth muscle is unknown. In this study, we characterized the actions of CEP on vascular contraction in endothelium-denuded dog mesenteric artery. We also determined its ability to release Ca^{2+}, by use of Ca^{2+} imaging techniques, on dog isolated mesenteric artery smooth muscle cells and on bovine cultured pulmonary artery endothelial cells.

2 After phenylephrine-(PE, 10 μM) sensitive Ca^{2+} stores were depleted by maximal PE stimulation in Ca^{2+}-free medium, the action of CEP on refilling of the emptied PE stores was tested, by first pre-incubating the endothelium-denuded artery in CEP for 15 min before Ca^{2+} was restored for a 30 min refilling period. At the end of this period, Ca^{2+} and CEP were removed, and the arterial ring was tested again with PE to assess the degree of refilling of the internal Ca^{2+} store.

3 In a concentration-dependent manner (30, 100 and 300 μM), CEP significantly reduced the size of the post-refilling PE contraction (49.4, 28.9 and 5.7% of control, respectively) in Ca^{2+}-free media. This suggests that Ca^{2+} levels are reduced in the internal stores by CEP treatment. CEP alone did not cause any contraction either in Ca^{2+}-containing or Ca^{2+}-free Krebs solution.

4 Restoring Ca^{2+} in the presence of PE caused a large contraction, which reflects PE-induced influx of extracellular Ca^{2+}. The contraction of tissues pretreated with 300 μM CEP was significantly less compared with controls. However, tissues pretreated with 30 and 100 μM CEP were unaffected. Washout of CEP over 30 min produced complete recovery of responses to PE in Ca^{2+}-free and Ca^{2+}-containing medium suggesting a rapid reversal of CEP effects.

5 Concentration-response curves were constructed for PE, 5-hydroxytryptamine (5-HT) and K^+ in the absence of and after 30 min pre-incubation with 30, 100 and 300 μM CEP. In all cases, CEP caused a concentration-dependent depression of the maximum response to PE (84.8, 43.4 and 11.6% of control), 5-HT (65.4, 25.7 and 6.9% of control) and K^+ (77.6, 41.1 and 10.8% of control).

6 Some arterial rings were pre-incubated with ryanodine (30 μM) for 30 min before the construction of PE concentration-response curves. In Ca^{2+}-free Krebs solution, ryanodine alone did not cause any contraction. However, 58% (11 out of 19) of the tissues tested with ryanodine developed contraction (6.9 ± 1.2% of 100 mM K^+ contraction, $n=11$) in the presence of external Ca^{2+}. EC_{50} values for PE in ryanodine-treated tissues (1.7 ± 0.25 μM, $n=16$) were not significantly different from controls (2.5 ± 0.41 μM, $n=22$). Maximum contractions to PE (118.5 ± 4.4% of 100 mM K^+ contraction, $n=16$) were also unaffected by ryanodine when compared to controls (129 ± 4.2%, $n=23$).

7 When fura-2 loaded smooth muscle cells ($n=13$) and endothelial cells ($n=27$) were imaged for Ca^{2+} distribution, it was observed that 100 and 300 μM CEP in Ca^{2+}-free medium caused Ca^{2+} release in both cell types. Smooth muscle cells showed a small decrease in cell length. Addition of EGTA (5 mM) reversed the effect of CEP on intracellular Ca^{2+} to control values.

8 These data show, for the first time in vascular smooth muscle and endothelial cells, that CEP releases Ca^{2+} more rapidly than ryanodine. Unlike ryanodine, CEP caused no basal contraction but depressed contractions to PE, 5-HT and K^+. The lack of basal contraction may result from altered responsiveness of the contractile system to intracellular Ca^{2+} elevation.

Keywords: 4-Chloro-3-ethyl phenol; Ca^{2+} imaging; dog mesenteric artery; bovine pulmonary artery endothelial cells; sarcoplasmic reticulum; ryanodine; Ca^{2+} release channels

Introduction

Contraction of smooth muscle depends on the mobilization of Ca^{2+} from internal storage sites, primarily sarcoplasmic reticulum (SR), and from the extracellular space (Bolton, 1979). The involvement of internal Ca^{2+} stores in muscle contraction has been identified by use of selective pharmacological tools, such as cyclopiazonic acid (CPA), thapsigargin (TSG) and ryanodine. CPA and TSG are inhibitors of the SR Ca^{2+} pump (Seidler *et al*., 1989; Thastrup, 1990). Ryanodine acts on the Ca^{2+} release channel of the SR to open it in a subconductive state or prevents Ca^{2+} release from stores (Rousseau *et al*., 1987).

Recently, 4-chloro-3-ethyl-phenol (CEP) has been shown to release internal Ca^{2+} from skeletal muscle fibres (Larini *et al*., 1995). It appeared to be a potent stimulant of Ca^{2+} release from ruthenium red-sensitive Ca^{2+} release channels in the terminal cisternae. In addition, CEP also mobilized Ca^{2+} from TSG-sensitive Ca^{2+} stores in a variety of non-excitable cell types, such as human fibroblasts, human hepatoma cell line PLC/PRF/5, Jurkat cells, PC-12 cells and HL-60 cells (Larini *et al*., 1995). The site of action of CEP on these cells was shown to be distinct from the inositol-1,4,5-trisphosphate (IP_3) receptor and had pharmacological similarities to the ryanodine Ca^{2+} release channel.

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Whether this compound is equally effective in smooth muscle is unknown. Most of the pharmacological tools which had originally been discovered to be effective, in either the SR of the skeletal muscle or the endoplasmic reticulum in non-excitable cell types, were also eventually shown to be effective in a variety of smooth muscles. For example, TSG, CPA and/or ryanodine were found to be effective in vascular smooth muscle (Deng and Kwan, 1991; Low *et al*., 1992; 1993; 1996; Darby *et al*., 1996), vas deferens (Darby *et al*., 1996), trachea (Bourreau *et al*., 1993; Janssen and Sims, 1993), ureter (Maggi *et al*., 1995; Lang *et al*., 1995) and gastrointestinal smooth muscles (Jury *et al*., 1996).

In this study, we investigated the actions of CEP on dog mesenteric artery (DMA) and endothelial cells to determine if CEP mobilized intracellular Ca^{2+}, as previously found in fractionated skeletal muscle terminal cisternae and in non-excitable cell types (Larini *et al*., 1995). We performed contractility studies on DMA, and Ca^{2+} imaging studies on freshly isolated cells of the DMA and on bovine cultured pulmonary artery endothelial cells (BPAECs). Our results show that CEP mobilized internal Ca^{2+} in vascular smooth muscle cells and endothelial cells, but depressed the contractility of intact vascular smooth muscle.

Methods

Contractility studies

By use of procedures approved by our University Animal Care Committee in keeping with the Canadian Council of Animal Care, mongrel dogs (10–30 kg) were killed with an overdose of sodium pentobarbitone (100 mg kg^{-1}, i.v.). Mesenteric arteries were dissected free, then placed in Krebs solution at pH 7.4 containing (mM): NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1 and glucose 11. After fat and connective tissues were removed under a dissecting microscope, 3–4 mm DMA rings were prepared. The endothelium was removed gently with the teeth of a pair of forceps. The rings were then mounted in a 15 ml organ bath connected to a force transducer (Grass FTO3C, Grass Instruments Co., Quincy, MA, U.S.A.) and a chart recorder (Gould 2800, Gould Inc., Cleveland, OH, U.S.A.). Because any remaining endothelium may also respond directly to CEP (Larini *et al*., 1995), we tested for complete removal by measuring any relaxation in response to 1 μM carbachol of rings precontracted with 60 mM K^+.

The organ baths and Krebs solution were bubbled continuously with 95% O_2/5%CO_2 and warmed to 37°C. The rings were equilibrated for 20 min before being stretched to the optimal resting force of around 5 g. Stimulation of the arteries with 100 mM K^+ added hypertonically was repeated every 15–20 min until reproducible contractions were obtained. For Ca^{2+}-free Krebs, $CaCl_2$ was replaced by 50 μM EGTA.

Ca^{2+} release experiments The aim of these experiments was to determine if CEP could mobilize internally-stored Ca^{2+}. Tissues initially bathed in Ca^{2+}-containing Krebs solution were rinsed 3 times with Ca^{2+}-free Krebs solution then incubated in Ca^{2+}-free Krebs solution for 5 min before the addition of 10 μM phenylephrine (PE). This PE stimulation in Ca^{2+}-free medium was used to measure the capacity of PE-sensitive store by emptying the store to produce a transient contraction. The tissue was then washed and incubated with PE-free normal Krebs solution for 30 min to allow adequate refilling of the PE-sensitive store (Low *et al*., 1992). The tissues were again washed 3 times and then incubated in Ca^{2+}-free media for 5 min before 10 μM PE stimulation again. This second PE response in Ca^{2+}-free medium was used to measure Ca^{2+} release from internal Ca^{2+} stores or the degree of refilling that had occurred. External Ca^{2+} was then restored and the magnitude of PE contraction measures the contraction due to influx of Ca^{2+} from the extracellular space. The whole procedure was repeated in the presence of different concentrations of CEP, incubated for 15 min in Ca^{2+}-free medium before restoring Ca^{2+} for refilling. The procedure was repeated again following 30 min washout of CEP.

Concentration-response curves to PE, 5-HT and potassium Increasing concentrations of PE, 5-hydroxytryptamine (5-HT) or potassium were added to the muscle baths to construct concentration-response curves to determine if CEP had any effects on these contractile responses. Rings of tissues were exposed to varying concentrations of CEP for 30 min. In some experiments, ryanodine (30 μM) was used instead of CEP for comparison.

Isolation of dog mesenteric artery

Secondary branches of the mesenteric artery were isolated and 6 mm × 5 mm rings in a nominally Ca^{2+}-free HEPES-buffered saline containing 0.2% bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) containing (in mM): NaCl 126, KC 6), N-2-hydroxyethylpiperzine-N′-2-ethanesulphonic acid (HEPES, Sigma) 10, glucose 10 and $MgCl_2$ 0.3 with pH adjusted to 7.4 with NaOH. For Ca^{2+}-containing HEPES buffer, 1.5 mM $CaCl_2$ was added. The rings were incubated at 32°C for 10–20 min and then placed in nominally Ca^{2+}-free HEPES solution with added collagenase (1.5 mg ml^{-1}; Type 1A, Sigma), elastase (0.5 mg ml^{-1}, Eisai Co. Ltd., Tokyo, Japan) and trypsin inhibitor (0.5 mg ml^{-1}, Sigma) for 20 min. Muscle pieces were then suspended in enzyme-free solution for another 10 min before they were gently triturated through the tip of a wide-bore pasteur pipette to disperse the single smooth muscle cells. Cells were allowed to settle on the bottom of the recording chamber with a glass coverslip bottom for about 1–2 h before the cells were loaded with fura-2/AM (2–4 μM, Molecular Probes, Eugene, OR, U.S.A.). Pluronic F-127 (0.02%, Molecular Probes) was used to aid loading of the Ca^{2+} fluorescent dye.

Endothelial cell culture

Bovine pulmonary artery endothelial cells (BPAECs) were obtained from an established cell line from ATCC (#CCL 209, Rockville, MD, U.S.A.). The cells were grown in MEM (Minimum essential medium, GIBCO, Grand Island, NY, U.S.A.) supplemented with 20% foetal bovine serum (GIBCO), gentamycin (0.1%, GIBCO) and fungizone (0.1%, GIBCO) in a humid atmosphere of 95% air/5% CO_2 at 36°C. For the experiments, the cells were exposed to 0.5% trypsin to dislodge cells. Cells were then centrifuged at low speed for 5 min and the supernatant discarded. Cells were plated on the recording chamber with a glass coverslip bottom and left to settle overnight before use. Cells were about 40–80% confluent at the time of use. For experiments, the recording chamber was rinsed off the culture medium with HEPES-buffered saline. Cells were bathed in Ca^{2+}-free HEPES containing 50 μM EGTA before the commencement of recording.

Ca^{2+} imaging

Experiments were conducted at room temperature (23°C) on single cells by use of a Ca^{2+} imaging system (Image-1/FL, Universal Imaging Corporation, West Chester, PA, U.S.A.) with a Zeiss lamp (HBO 100W/DC, Carl Zeiss Canada Ltd., Don Mills, ON, Canada) coupled to a Zeiss inverted microscope (Zeiss IM 35) with a 100X oil immersion lens and a numerical aperture of 1.25. Excitation by incident illumination at 340 and 380 nm produced emitted fluorescence detected at 540 nm. Images were integrated and collected by a Pulnix camera (TM-720, Pulnix America, CA, U.S.A.). Time between each ratio image was around 3 s. Background values for subtraction were obtained by defocussing. Images were analysed by assessing the fluorescence intensity of the cells for the course of the experiment by use of the computer software Image-1/

FL. Before each series of experiments, the system was calibrated with 10 μM CPA in the presence of extracellular 1.5 mM Ca^{2+} and EGTA (5 mM) for maximum and minimum fluorescence intensities. A sigmoidal curve relating the F340/380 ratio to concentration of intracellular Ca^{2+} according to the equation of Grynkiewicz *et al.* (1985) is an integrated component of Image 1/Fl imaging software.

Ca^{2+} levels were measured in the central and peripheral areas of the cell. The peripheral regions are defined as areas adjacent to and beneath the outline of the cell. There is always a distinct band showing higher ratio values around the cells than anywhere else within the lumen of the cell (central regions). Since the fura-2 method contains several intrinsic problems in the estimation of absolute cytosolic Ca^{2+} concentration (see Shin *et al.*, 1992), relative Ca^{2+} concentrations in terms of ratio are presented instead of absolute Ca^{2+} concentration. Changes in Ca^{2+} levels to CEP stimulation are expressed as a percentage of basal level. Ratio values from two regions of the cell (central and peripheral) were averaged to obtain representation of Ca^{2+} change within a cell.

Drugs

Cyclopiazonic acid (CPA, Sigma), was dissolved in dimethyl sulphoxide (DMSO) to make a stock solution of 10 mM. Phenylephrine (PE, Sigma), 5-hydroxytryptamine (5-HT, Sigma) and carbachol (Sigma) were dissolved in double distilled deionized H_2O to a stock of 10 mM. 4-Chloro-3-ethyl phenol (CEP, Anachemia Science, Mississauga, ON, Canada) was freshly dissolved in DMSO to make a stock solution of 100 mM. Further dilutions were made with H_2O. All other chemicals were of laboratory standard from various commercial sources. None of the solvents affected the tissue response when the pharmacological agents were omitted, unless otherwise stated.

Analysis of data

Where appropriate, each concentration-response curve was fitted by a sigmoidal logistic regression function (MicroCal Origin ver 3.78, Northampton, MA, U.S.A.). EC_{50} and chi squares values were estimated from these fitted curves.

Statistical analysis

Data are expressed as the mean $\pm$ s.e.mean. Differences were analysed for significance by use of two-tailed Student's *t* test or one-way analysis of variance where appropriate. When the *F* ratio was significant, the significantly differing pair was determined by Bonferroni's method. Statistical significance was accepted at $P<0.05$.

Results

Contractility experiments on DMA

Effects of CEP and ryanodine on PE-sensitive Ca^{2+} stores In Ca^{2+}-free medium containing 50 μM EGTA, PE (10 μM) was used to release Ca^{2+}, observed as a transient contraction. Figure 1a shows the traces from one such an experiment, where different concentrations of CEP (30, 100 and 300 μM) were present during the 30 min refilling period and their effects on the subsequent PE contraction in Ca^{2+}-free medium were measured. The experiment was repeated for a third time after a 30 min washout of CEP to demonstrate the reversibility of the effects of CEP.

Figure 1b summarizes the results from this experimental protocol. When the arterial rings had been pretreated with CEP, a concentration-dependent inhibition of the amplitude of PE-induced contraction in Ca^{2+}-free solution was observed, suggesting a reduced amount of Ca^{2+} available for contraction in the PE-sensitive Ca^{2+} stores. The effect of CEP was reversed upon the washout of CEP. We were concerned that the washout of 300 μM CEP was incomplete after the 5 min in Ca^{2+}-free medium and the inhibition observed could be due to residual CEP in the bathing solution. So, we also checked the effects of CEP on Ca^{2+} influx. Restoring external Ca^{2+} when

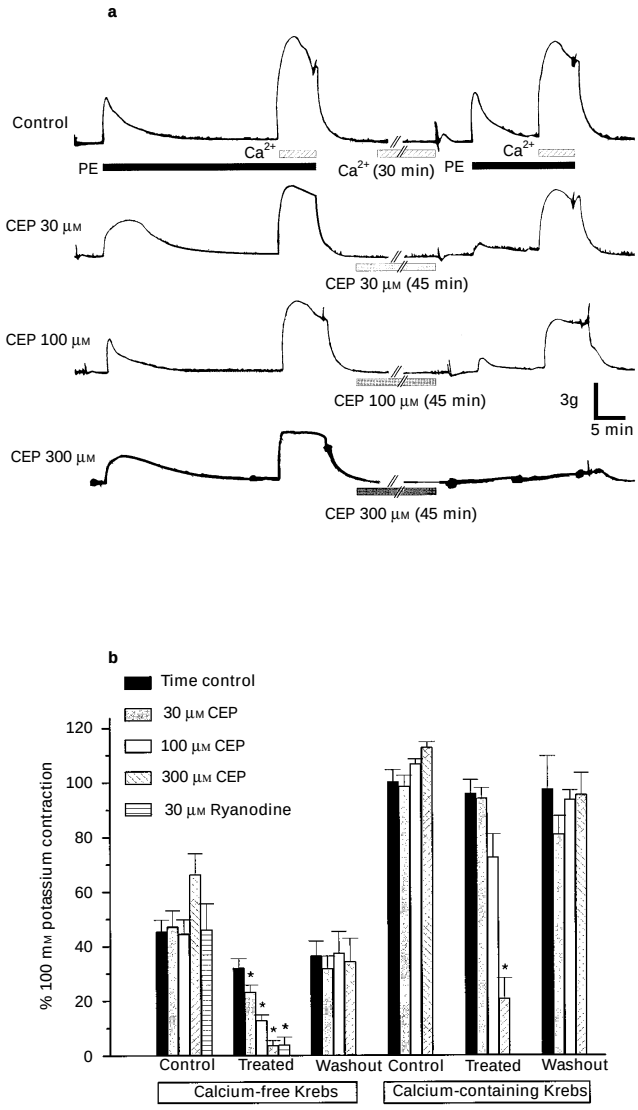

Figure 1 Effects of 4-chloro-3-ethyl phenol (CEP) on the refilling of phenylephrine (PE) sensitive Ca^{2+} stores and PE-induced Ca^{2+} entry in dog mesenteric artery. (a) Recordings from 4 arterial rings. Control tracings are shown on the left while CEP-treated responses are shown on the right. In these experiments, after emptying of PE-sensitive Ca^{2+} stores, the stores were refilled for 30 min in Ca^{2+}-containing Krebs solution. Stimulation with PE (10 μM) in Ca^{2+}-free medium to empty the Ca^{2+} internal stores produced a contraction. When the contraction returned to baseline, external Ca^{2+} was added (to determine the Ca^{2+} influx component). The experiment was repeated, but with a pre-incubation in CEP 15 min before Ca^{2+} was restored for a 30 min refilling period. Pretreatment with CEP produced concentration-dependent inhibition of PE-induced transient contractions in Ca^{2+}-free medium. indicates a break for the period as indicated. (b) Summary of the results. Significant inhibition of PE-induced transient contraction with 30, 100 and 300 μM CEP ($n=9-10$) was observed compared with controls ($n=9-10$). Effects before and after ryanodine (30 μM) pretreatment are also shown ($n=4-6$). Restoring Ca^{2+} to the medium containing PE caused large contractions attributable to Ca^{2+} influx from the exterior. These responses were not significantly affected by CEP except at 300 μM. Inhibition of the PE response in Ca^{2+}-free and Ca^{2+}-containing media was reversed following CEP washout before the refilling of PE stores. Asterisks denote statistically significant difference ($P<0.05$) compared to controls.

PE was present caused contractions which could be related to influx of Ca^{2+}. At 30 and 100 μM CEP, PE-induced contractions resulting from PE-induced Ca^{2+} influx were unaffected. However, at 300 μM CEP, the contractions were significantly inhibited. This indicated that it is likely that washout of CEP at 300 μM was incomplete after 5 min. At 30 and 100 μM CEP, a differential effect was observed such that release contracture was inhibited but not Ca^{2+} influx contracture. Furthermore, influx contracture induced by 10 μM PE was not affected by pretreatment with 100 μM CEP (Figure 1b), whereas, significant inhibition of PE contraction was observed in the presence of 100 μM CEP in the bathing medium (see Figure 2). These observations strongly suggest that inhibition of the release contracture was due to an effect on refilling, rather than a direct effect of residual CEP on contraction in general (cf. Figures 2–4).

CEP alone did not contract the rings in either the presence or absence of external Ca^{2+}.

In comparison, some experiments were carried out with ryanodine (30 μM, 30 min) in place of CEP. Tissues pretreated with ryanodine did not show any measurable contraction in Ca^{2+}-free medium. However, in tissues which had been pretreated with ryanodine, PE-induced transient contractions in Ca^{2+}-free medium were significantly lower ($4.0 \pm 2.8\%$ of 100 mM K^+ contraction, $n=6$) than those of arterial rings which were not incubated with ryanodine during the refilling period ($46.3 \pm 9.4\%$ of 100 mM K^+ response, $n=4$) (Figure 1b).

Effects of CEP and ryanodine on PE concentration-response curve Concentration-response curves to PE were constructed after pre-incubation with CEP at 30, 100 or 300 μM or 30 μM ryanodine for 30 min. In these experiments, PE concentration-response curves were constructed in the continued presence of CEP or ryanodine.

CEP depressed maximum PE contractions (Figure 2) in a concentration-dependent manner. Ryanodine-treated tissues showed concentration-response curves that were not significantly different from controls. Tissues pretreated with ryanodine also demonstrated a small basal constriction, which developed very slowly over the 30 min incubation period, in 58% of the rings tested (11 out of 19) reaching a mean basal contraction of $6.9 \pm 1.2\%$ of 100 mM K^+ contraction ($n=11$). Any contractile response to ryanodine was used to set the baseline when PE concentration-response curves of the ryanodine-treated rings were analysed. No statistically significant shifts in EC_{50} values of PE were observed in tissues treated with ryanodine (1.7 ± 0.25 μM, $n=16$) compared to controls (2.5 ± 0.41 μM, $n=22$).

Effects of CEP on 5-HT concentration-response curve Pre-incubation of the arterial rings with CEP at 30, 100 and 300 μM caused concentration-dependent inhibition of responses to 5-HT expressed as a percentage of 100 mM K^+ contraction (Figure 3). The maximum contractions to 5-HT after CEP treatments were 65.4, 25.7 and 6.9% of controls.

Effects of CEP on K^+ concentration-response curve Concentration-dependent contractions to the addition of K^+ were inhibited by increasing concentrations of CEP; i.e., 30, 100 and 300 μM (Figure 4). The maximum contractions attained after CEP-treatment were 77.6, 41.1 and 10.8% of control, respectively.

Ca^{2+} imaging experiments

Isolated smooth muscle cells All single cells tested with 100 mM K^+ stimulation shortened. In Ca^{2+}-free medium, addition of CEP (100 and 300 μM) on all of the 13 DMA cells tested showed an increase in the cytosolic concentration of Ca^{2+} ($2.4\% \pm 0.3$ ($n=4$) and $3.9\% \pm 0.3$ ($n=9$) of basal level, respectively, $P<0.05$). Ratio (F340/380) ranges of 100 μM CEP and the corresponding basal ranges were 0.9 to

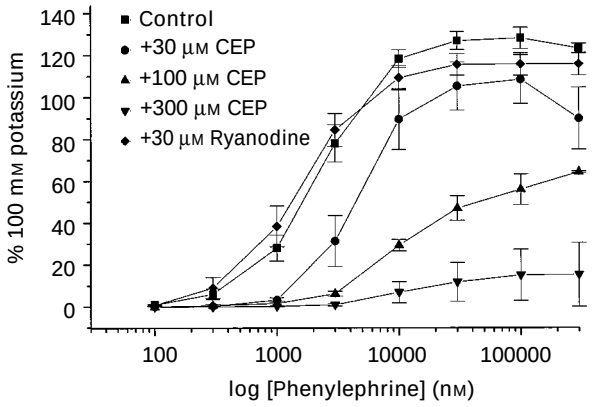

Figure 2 Concentration-dependent effects of 4-chloro-3-ethyl phenol (CEP) (30 min pre-incubation) on phenylephrine (PE) concentration-response curves. At the end of the 30 min, increasing concentrations of PE were added to the medium in the presence of CEP to construct the PE concentration-response curve. CEP at 30 ($n=4$), 100 ($n=4$) and 300 ($n=5$) μM caused a reduction in the maximum responses ($P<0.05$ for 100 and 300 μM CEP) compared to controls ($n=21$). Ryanodine (30 μM, 30 min pre-incubation, $n=13$) did not significantly affect the PE concentration response curve compared with controls.

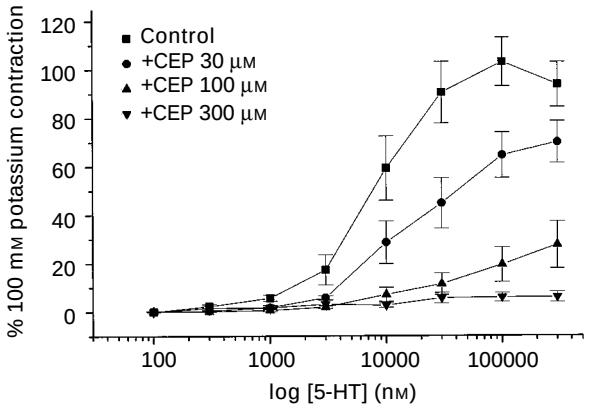

Figure 3 Concentration-dependent effects of 4-chloro-3-ethyl phenol (CEP) pre-incubated 30 min before the construction of 5-hydroxytryptamine (5-HT) concentration-response curves. Maximum contractions to 5-HT were significantly depressed ($P<0.05$) with increasing concentrations of CEP (30 μM, $n=6$; 100 μM, $n=6$; 300 μM, $n=8$) compared with controls ($n=9$).

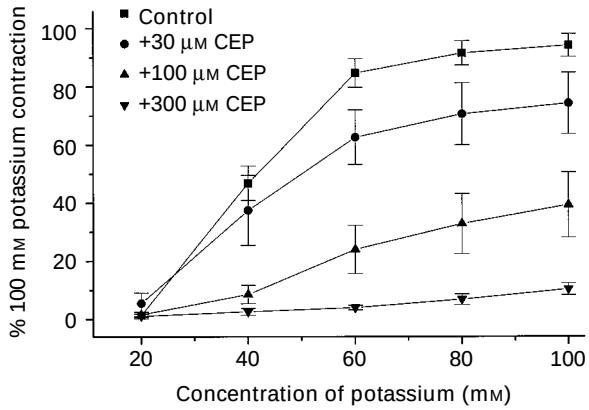

Figure 4 Maximum contractions to K^+ were significantly reduced ($P<0.05$) in a concentration-dependent manner by 4-chloro-3-ethyl phenol (CEP), 30 min pre-incubation period, (30 μM, $n=6$; 100 μM, $n=6$; 300 μM, $n=6$) compared with controls ($n=16$).

3.4 and 0.6 to 1.25, respectively, while those for 300 μM CEP and the corresponding basal values were 1.5 to 3.75 and 0.4 to 0.9, respectively. The increase in cytosolic Ca^{2+} was associated with a small decrease in the muscle length (by around 20% of control) when Ca^{2+} was restored to the extracellular medium. Addition of EGTA (5 mM) caused reduction in the cytosolic Ca^{2+} levels back to control values. Elevation in cytosolic Ca^{2+} levels first occurred at the cell periphery then spread to encompass most of the cell.

Bovine cultured pulmonary artery endothelial cells Addition of Ca^{2+} (1 mM, $n=17$) or EGTA (50 μM, $n=3$) to cells bathed in nominally Ca^{2+}-free solutions did not significantly affect the F340/380 ratio. Ca^{2+} mobilization by CEP (100 and 300 μM) in Ca^{2+}-free medium on endothelial cells was also observed in all 27 of the cells tested (5.4% $\pm$0.5 ($n=21$) and 5.7% $\pm$0.9 ($n=6$) basal levels, respectively, $P<0.05$). F340/380 ratios with 100 μM CEP were 1.25 to 4.6 and the corresponding basal ratios were 0.4 to 0.6. F340/380 ratios with 300 μM CEP were 0.9 to 1.25 and the corresponding basal ratios were 0.25 to 0.35. As in DMA cells, the increase in intracellular Ca^{2+} began in the cell periphery and spread to the central regions of the cells. Extracellular EGTA (5 mM), a Ca^{2+} chelator, created a gradient for outward movement of Ca^{2+} ions and brought values to near control levels. Figure 5 shows an example of CEP (100 μM)-induced Ca^{2+} release in endothelial cells.

Discussion

The data from this study show for the first time in smooth muscle cells and endothelial cells that CEP (100 μM) releases

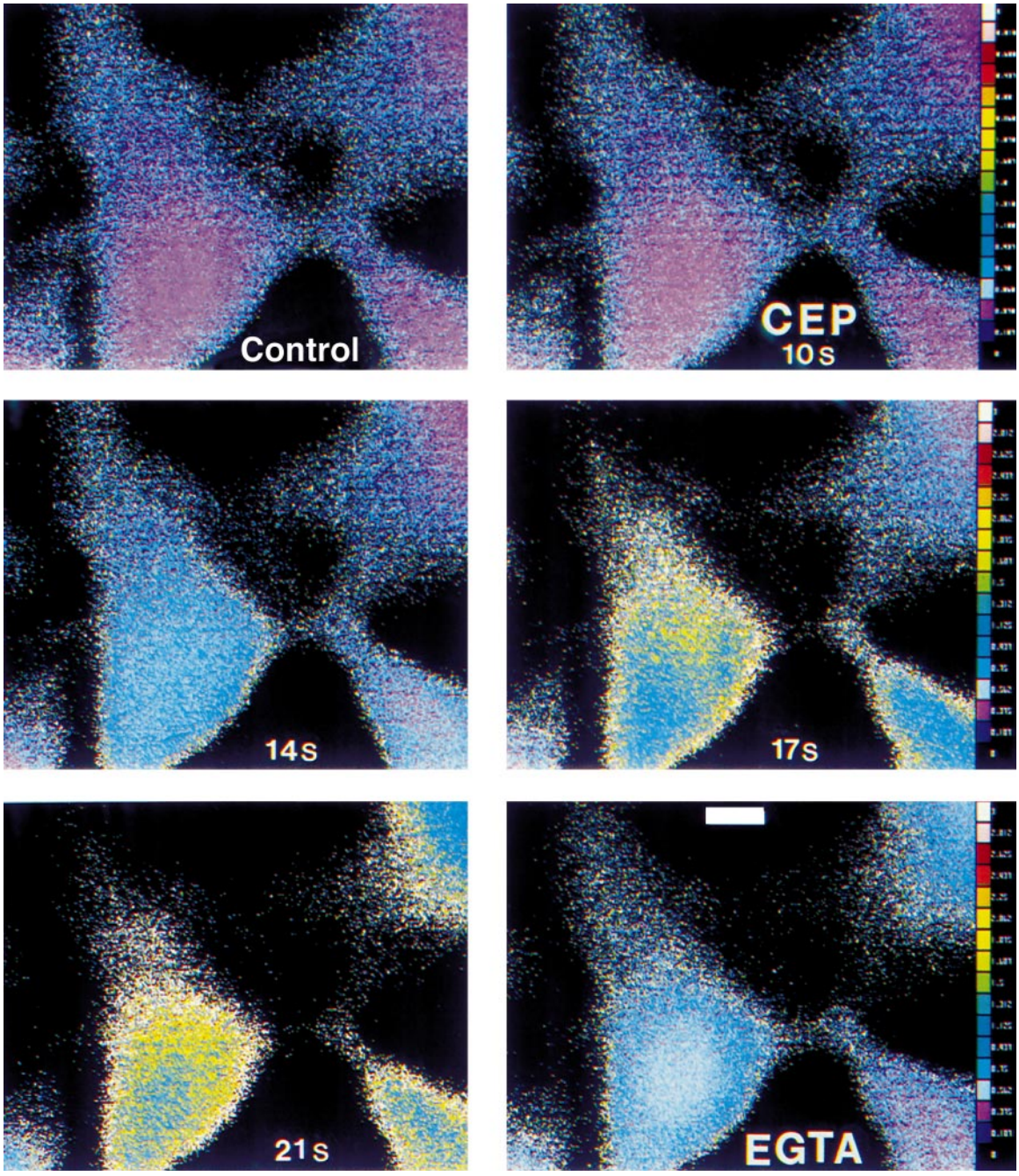

Figure 5 Endothelial cells from bovine pulmonary artery were plated and were about 40% confluent at the time of the experiment. Sequential responses to 4-chloro-3-ethyl phenol (CEP; 100 μM) are shown. After 10 s following the addition of CEP, the whole cell lightened led by the peripheral regions. Spread throughout the cell peaked at around 21 s. Addition of EGTA (5 mM) reversed Ca^{2+} signals. The pseudocolour scale shows low Ca^{2+} in the purple end and high Ca^{2+} in the red end. The corresponding numbers indicate the ratio values for the colour scale. Calibration bar represents 10 μm.

internally stored Ca^{2+}. The diminished PE contraction in Ca^{2+}-free medium of intact arterial rings suggests that CEP prevents refilling of PE-sensitive Ca^{2+} stores when present during the refilling period by causing a continuous leak of Ca^{2+}.

On its own, CEP (100 and 300 μM) caused no contraction but antagonized smooth muscle responses to several vasoconstrictors, such as, PE, 5-HT or high K^+. This non-specific inhibitory effect to agents which activate contraction through either receptors or membrane depolarization most likely results from an action downstream from the receptor or membrane ion channels, probably at the contractile apparatus. In contrast, chemically-skinned fibres from human skeletal muscle responded to CEP with tension oscillations at threshold concentrations between 0.4 to 1.6 mM (Loke *et al*., 1996). This action of CEP resembles those of caffeine on skeletal muscle fibres.

No one has yet examined the mechanism of CEP in reducing smooth muscle contractility. It could involve effects on the Ca^{2+} interaction with calmodulin, the ability of calmodulin to activate phosphorylation of myosin light chains or the interaction of myosin and actin and cross-bridge cycling (Rembold, 1992). However, our data suggest that concentrations of CEP of 30 μM have selective ability to inhibit refilling of Ca^{2+} stores without affecting the contractile apparatus in a major way (see Figure 1).

Unlike ryanodine, the effect of CEP was readily reversible after 15–20 min. While CEP could release Ca^{2+} more rapidly compared to ryanodine (Ziegelstein *et al*., 1994; L. Sormaz and A.M. Low, unpublished observations), only ryanodine caused a slowly developing basal contraction in the presence of external Ca^{2+} (Low *et al*., 1993; this study). The addition of ryanodine to tissues in Ca^{2+}-free medium did not cause contraction, but reduced the PE-induced transient contractions in Ca^{2+}-free medium, effects similar to those of CEP. It is puzzling that CEP appeared to release Ca^{2+} from stores more rapidly than ryanodine but failed to initiate contraction. This does not seem to be fully explained by the inhibitory effect of CEP on contractility. Perhaps, CEP affects yet another mechanism besides the SR calcium release channel, or perhaps ryanodine at 30 μM has actions at sites other than the calcium release channels.

Not all of the endothelial cells responded at the same time to CEP stimulation. One cell would respond first, and an adjacent cell would start when the another had reached a peak. Similar patterns were observed when cells were stimulated with either bradykinin, CPA or thapsigargin. Limited by the resolution of our camera, we cannot observe Ca^{2+} sparks. Also, at 3 s collection per frame, we cannot see a more detailed spread but a general lightening of the cells occurring from around the periphery and moving centrally, was observed. The hypothesis that Ca^{2+} stores are located close to the plasma membrane has been supported by electrophysiological data (STOCs, Benham and Bolton, 1986), occurrence of Ca^{2+} sparks beneath the plasma membrane (Nelson *et al*., 1995; Fay, 1995) and electron micrographs of SR located close to the plasma membrane (Bond *et al*., 1984). It appears that our data are consistent with this hypothesis.

In conclusion, the actions of CEP were similar to those of ryanodine in releasing internally-stored Ca^{2+} in vascular smooth muscle and endothelial cells. However, unlike ryanodine, CEP acted rapidly and its effects were reversible. Furthermore, CEP depressed the smooth muscle contractile response to a variety of vasoconstrictors, suggesting a non-specific action, probably, along the force generating process, an effect which was apparently not evident in skeletal muscle (Larini *et al*., 1995; Loke *et al*., 1996). Its reversible interference with the contractile process beyond the Ca^{2+} stores does not make it a useful pharmacological tool in contractility studies of smooth muscles, despite its inability to cause contraction in skeletal muscles. However, it is useful as a pharmacological tool in the study of cytosolic Ca^{2+} changes in non contractile cells like the endothelial cells.

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