Br. J. Pharmacol. (1993), **110**, 565–572

Cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum, increases excitability in ileal smooth muscle

Yoshiaki Uyama, [1]Yuji Imaizumi & Minoru Watanabe

Department Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, Nagoya 467, Japan

1 Effects of cyclopiazonic acid (CPA), a specific inhibitor of Ca^{2+}-ATPase in endo- and sarcoplasmic reticulum (ER/SR), on contractile responses, cytosolic Ca^{2+} concentration and spontaneous electrical activity were examined in ileal longitudinal smooth muscle strips.

2 After intracellular stored Ca^{2+} in intact ileal strips was depleted by application of 25 mM caffeine in Ca^{2+}-free solution, Ca^{2+}-loading was performed in the absence or presence of 10 μM CPA in a standard solution containing 2.2 mM Ca^{2+}. Subsequent application of caffeine in Ca^{2+}-free solution induced a phasic contraction which was significantly smaller in the strip pretreated with CPA than that in the control.

3 Spontaneous and 20 mM K^+-induced contractions in the presence of 1 μM atropine were markedly enhanced by 1–30 μM CPA, whereas that induced by 80 mM K^+ was not. The magnitude of repetitive transient elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and concomitant phasic contractions were markedly enhanced by CPA. The effects were abolished by 10 μM verapamil and restored by 10 μM Bay K 8644.

4 Application of 10 μM CPA depolarized the cell by about 5 mV, decreased the action potential (AP) afterhyperpolarization and markedly increased the frequency of spontaneous AP. These effects were mimicked by 100 nM charybdotoxin.

5 The rate of decay of $[Ca^{2+}]_i$ and tension after the bathing solution was changed from one containing 140 mM K^+ and 2.2 mM Ca^{2+} to one containing 5.9 mM K^+ and 0 mM Ca^{2+} was significantly slowed when 10 μM CPA was added to the latter solution.

6 These results indicate that CPA enhances ileal smooth muscle excitability and increases Ca^{2+}-influx through voltage-dependent Ca^{2+} channels. The effect may be consistent with the hypothesis that CPA-induced decrease in stored Ca due to Ca-pump inhibition reduces the Ca^{2+}-dependent K^+ current and indirectly enhances Ca^{2+}-influx through membrane activity resulting from the increased excitability. Direct evidence for the regulation of Ca^{2+} channel activity by intracellular Ca storage sites was not obtained in the present study.

Keywords: Cyclopiazonic acid; smooth muscle; Ca-pump; Ca^{2+}-ATPase; sarcoplasmic reticulum; Ca^{2+}-dependent K^+ current; Ca current; guinea-pig ileum; skinned fibre

Introduction

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal, cardiac and smooth muscle sarcoplasmic reticulum (SR) (Goeger & Riley, 1989; Seidler *et al.*, 1989) and inhibits Ca^{2+}-uptake of SR in skeletal muscle (Goeger *et al.*, 1988; Kurebayasi & Ogawa, 1991). Recently, we showed that CPA is a selective and reversible inhibitor of ATP-dependent Ca^{2+}-uptake of SR/ER in the skinned fibre of ileal smooth muscle (Uyama *et al.*, 1992). Moreover, it has been suggested that CPA can inhibit Ca^{2+}-ATPase in intact preparations of aorta (Deng & Kwan, 1991), trachea (Bourreau *et al.*, 1991) and mesenteric artery (Low *et al.*, 1992).

In intact smooth muscle tissue preparations, however, the effects of CPA on the contractile responses to various types of stimulation are complicated and not always consistent between different smooth muscles. This may be due to differences in characteristics of intracellular Ca-storage sites and also of Ca^{2+}-influx and -extrusion through the plasma membrane during stimulation. In addition, the function of intracellular Ca storage sites as one of the important regulatory factors of membrane ionic currents (Ohya *et al.*, 1987; Wong, 1991) has not been taken into consideration. The effects of CPA in intact preparations have been examined in electrically quiescent muscles which do not show spontaneous myogenic activity (Deng & Kwan, 1991; Bourreau *et al.*, 1991; Low *et al.*, 1992).

In a previous paper, we showed that application of CPA selectively inhibits Ca^{2+}-dependent K^+-current ($I_{K\text{-}Ca}$) in single smooth muscle cells of guinea-pig ileum and urinary bladder (Suzuki *et al.*, 1992). Activity of single large conductance Ca^{2+}-dependent K^+ channels (BK channels) are not affected by 10 μM CPA. The inhibition of $I_{K\text{-}Ca}$ may be attributable to the suppression of SR/ER Ca^{2+}-ATPase, which presumably results in a marked decrease in releasable Ca in storage sites. The large transient $I_{K\text{-}Ca}$ upon depolarization in these cells is induced by Ca-release from ryanodine-sensitive Ca storage sites (Sakai *et al.*, 1988), which is triggered by Ca^{2+}-influx through voltage-dependent Ca^{2+} channels (Ohya *et al.*, 1987; Kitamura *et al.*, 1989) via Ca^{2+}-induced Ca^{2+} release mechanisms (Suzuki *et al.*, 1992; Ganitkevich & Isenberg, 1992), which it has been suggested do not have a functional role in skinned fibres (Iino, 1989). This current may be responsible for action potential (AP) repolarization and afterhyperpolarization (AHP) (Mitra & Morad, 1985; Ohya *et al.*, 1987; Watanabe *et al.*, 1989; Wong, 1991). Moreover, $I_{K\text{-}Ca}$ significantly contributes to the regulation not only of APs, but also of the resting membrane potential (Hu *et al.*, 1989) and muscle tone (Brayden & Nelson, 1992) in some kinds of smooth muscle cells. The inhibition of $I_{K\text{-}Ca}$, therefore, may depolarize the cell membrane, increase AP

[1] Author for correspondence.

duration, decrease AHP and possibly increase spontaneous spike generation and membrane excitability of smooth muscle cells.

Based upon these results, it can be speculated that the decrease in $I_{K\text{-}Ca}$ by CPA may result in an increase in membrane excitability and, therefore, spontaneous spike generation. It is, however, not clear whether spontaneous contractile activity is increased or decreased under conditions where stored Ca is significantly decreased by CPA. The present study was undertaken to examine how CPA affects electrical activity, $[Ca^{2+}]_i$ concentration and mechanical activity in an intact tissue preparation of ileal longitudinal muscle.

Methods

Male Hartley guinea-pigs, weighing about 200 g, were killed by a blow on the head. The terminal portion of the ileum was isolated.

Intact preparation and tension measurement

Longitudinal muscle strips (2–3 mm wide and 10 mm long) were dissected from the ileum in Krebs solution (see Solutions) at room temperature. The muscle strip was mounted horizontally on a silicon rubber sheet at the bottom of an organ bath which had a volume of 0.3 ml and was filled with modified Krebs solution or HEPES buffered salt solution. The solutions were continuously bubbled with 95% O_2:5% CO_2 or 100% O_2, respectively. Temperature was maintained at 36 ± 1°C except where mentioned in figure legends. Contractile responses were measured isometrically with a strain gauge transducer and recorded on a pen-recorder.

Skinned fibre preparation

Skinning of ileal longitudinal strips was performed as described previously (Uyama *et al.*, 1992). In brief, strips were incubated with 60 μM β-escin in a solution of pCa 6.0 (Kobayashi *et al.*, 1989; Kitazawa & Somlyo, 1990). After skinning, the solution was changed to a relaxing solution containing 2 mM EGTA (R2G). Contractile responses to caffeine were tested in a relaxing solution containing 0.1 mM EGTA (R0.1G). Experiments were performed at room temperature (22 ± 1°C). Calmodulin was not added except when mentioned.

Measurement of membrane potential

The conventional glass microelectrode technique was used to measure the membrane potential of smooth muscle cells in ileal longitudinal muscle strips. A strip was prepared in the same manner as the intact preparations for tension measurement and was pinned firmly to the silicon rubber at the bottom of a chamber which had volume of about 0.3 ml. The strip was perfused with modified Krebs solution at rate of 10 ml min^{-1} and kept at 35 ± 1°C. Glass microelectrodes having a resistance of 40–60 Mohm when filled with 3 M KCl were inserted into smooth muscle cells from the outside of the strip. Electrical signals were amplified (Nihon Koden MEZ-8101), monitored on a storage oscilloscope (Nihon Koden, VC10) and recorded on a pen recorder and a video tape via a PCM recorder (Sony PCM-501ES; modified to obtain frequency response from d.c. to 20 kHz). Records on the video tape were replayed and stored on the hard disk of a computer (IBM-AT) after A-D conversion and analysed on the computer as described previously (Imaizumi *et al.*, 1990).

Simultaneous measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and contraction

Longitudinal strips of guinea-pig ileum were isolated and were treated with 5 μM acetoxymethyl ester of Fura-2 (Fura-2/AM) for 4–6 h at room temperature in 4 ml HEPES-buffer solution in a dark place. A non-cytotoxic detergent, pulronic F-127 (0.02–0.04%), was added to the loading solution to increase the solubility of Fura-2/AM. A Fura-2-loaded strip was mounted horizontally in a chamber which had volume of approximately 1.2 ml and continuously superfused at 12 ml min^{-1} with a HEPES buffered solution oxygenated by 100% O_2. The temperature of the solution was maintained at 35 ± 1°C. Fura-2-Ca^{2+} signals were measured simultaneously with muscle contractions as described by Ozaki *et al.* (1987). Muscle strips were illuminated alternately at the excitation wavelengths (340 nm and 380 nm). The amounts of 500 nm fluorescence induced by 340 nm excitation (F340) and by 380 nm excitation (F380) were measured with a fluorimeter (CAF-100, JASCO). The ratio of F340 and F380 was used as an indicator of $[Ca^{2+}]_i$, taking the ratio in resting state as 0% and that in 80 mM K^+-stimulated state as 100%.

Solutions

The composition of Krebs solution was (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 14.0. The composition of the physiological salt HEPES-buffer solution was (in mM): NaCl 137.0, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 14.0 and HEPES 10.0. The pH was adjusted to 7.4 with NaOH. A high K^+ solution was prepared by replacing NaCl with equimolar KCl. A Ca^{2+}-free solution was prepared by removing $CaCl_2$ and added 0.1 mM EGTA to the HEPES buffered solution. The composition of R2G was (in mM): K-propionate 130, $MgCl_2$ 4.0, adenosine triphosphate (ATP)-2Na 4.0, creatine phosphate 10, NaN_3 10, Tris 20.0 and EGTA 2.0. Solutions containing 0.1 or 0.5 mM EGTA (R0.1G and R0.5G) were prepared by reducing the EGTA concentration in R2G from 2.0 to 0.1 or 0.5 mM, respectively. The approximate pCa of R0.1G was calculated to be 7.2, assuming the contamination of Ca^{2+} to be 15 μM. The pH was adjusted to 6.8 with maleic acid. Solutions of pCa 6.3 and 6.0 were prepared by using a Ca^{2+}-EGTA buffer of 3 mM EGTA and corresponding Ca^{2+}.

Drugs

Cyclopiazonic acid (CPA) and β-escin was obtained from Sigma Ltd. Pulronic F-127 was from Calbiochem. Ltd. EGTA and Fura-2/AM were from Dojin. Caffeine, acetylcholine and atropine were from Wako Junyaku. Charybdotoxin was from Peptide Institute, Inc. Bay K 8644 (methyl 1,4 dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl phenyl)-pyridine-5-carboxylate) was obtained from Bayer, Japan.

Statistics

Pooled data are expressed as mean ± s.e.mean. Statistical significance was examined with a paired or unpaired Student's t test and is indicated by $*P<0.05$; $**P<0.01$ and $***P<0.001$.

Results

First, we determined whether CPA applied to intact ileal longitudinal strips can inhibit Ca^{2+}-uptake by intracellular storage sites as it does in skinned preparations (Uyama *et al.*, 1992). Figure 1 shows the effects of CPA on 25 mM caffeine-induced contraction in intact strips. To reduce spontaneous contractions, experiments were performed at room temperature (20–22°C). After the strip was loaded with Ca^{2+} in a HEPES buffered solution containing 2.2 mM Ca^{2+} (Ca^{2+}-loading or Ca^{2+}-uptake, see Methods, not shown in Figure 1a), 25 mM caffeine was applied twice in a Ca^{2+}-free solution (Figure 1a(i)). The second application of caffeine did not elicit a response, implying that Ca in storage sites may be almost depleted after the first application. The Ca^{2+}-load and

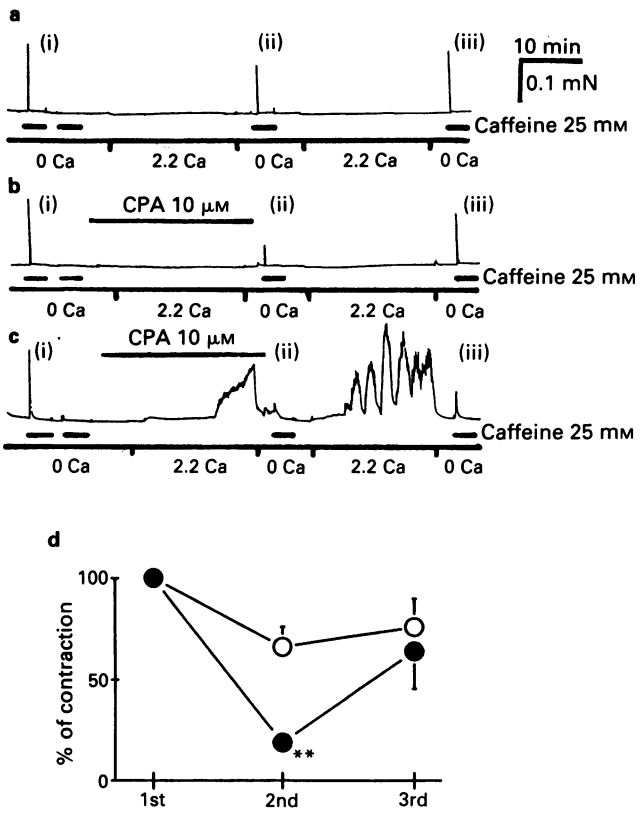

Figure 1 Effects of cyclopiazonic acid (CPA) on 25 mM caffeine-induced contraction in intact ileal smooth muscle strips. Experiments were performed at 21 ± 1°C to depress spontaneous contractions. After Ca^{2+}-loading in a HEPES buffered solution containing 2.2 mM Ca^{2+} (not shown), 25 mM caffeine was applied twice in a Ca^{2+}-free solution (i). Note that the second application of caffeine induced no response. Thereafter, Ca^{2+}-loading was performed in the absence (a) or the presence of 10 μM CPA (b,c) in a solution containing 2.2 mM Ca^{2+}. The subsequent response to caffeine was inhibited, when the Ca^{2+}-loading was performed in the presence of CPA (b(ii) and c(ii)). The contractile response to caffeine recovered substantially in the third trial as shown in (b(iii)) and (c(iii)). Application of 10 μM CPA to the solution containing 2.2 mM Ca^{2+} did not change the muscle tone in most preparations examined as shown in (b). In about 30% of preparations, however, CPA itself increased the muscle tone and induced repetitive phasic spontaneous contractions (c). This effect of CPA was occasionally irreversible. Summarized data shown in (d) are from preparations in which CPA itself did not induce contractions and indicate that 10 μM CPA significantly reduced caffeine-induced contraction in a reversible manner: (○) control response; (●) 2nd response measured after treatment with 10 μM CPA. $n = 6$.

subsequent Ca^{2+} release by caffeine was repeated three times (Figure 1(i), (ii) and (iii)) in each preparation. In the second trial, Ca^{2+}-loading was performed in the absence or presence of 10 μM CPA. Addition of 10 μM CPA in a Ca^{2+}-free solution did not change the muscle tone. The change in solution from Ca^{2+}-free solution to Ca^{2+}-containing solution in the presence of CPA did not alter muscle tone in 70% of preparations but induced tonic or phasic contraction in the other preparations. Such a contraction during Ca^{2+}-loading was not observed in the control. In strips where Ca^{2+}-loading was performed in the presence of 10 μM CPA, the subsequent contraction induced by caffeine was significantly smaller (Figure 1b, c and d). In the third trial, application of caffeine elicited substantial responses in all preparations. In strips where CPA *per se* induced contraction during Ca^{2+}-loading (Figure 1c (ii)), spontaneous phasic contractions were often observed during Ca^{2+} loading in the third trial (c).

Summarized data in Figure 1d show that the caffeine-induced contraction was significantly reduced when the preceding Ca^{2+}-loading was performed in the presence of 10 μM CPA. It is clear that 10 μM CPA reversibly reduced Ca^{2+}-uptake of storage sites in intact ileal smooth muscle strips, at least under these conditions (at room temperature and without spontaneous contractions). The results obtained from preparations which showed substantial contractions during Ca^{2+}-loading in the presence of CPA were not included in Figure 1d. When the temperature was kept at 36 ± 1°C, spontaneous contraction was observed during Ca^{2+}-loading in every preparation. Similar results with much larger variance were obtained at this temperature using the same experimental procedure (not shown). The Ca^{2+}-uptake was also significantly decreased by 10 μM CPA at 36°C ($P < 0.05$; not shown). Caffeine-induced contractions in a Ca^{2+}-free solution were, however, much smaller than the spontaneous contractions in Ca^{2+} containing solution even in the control at 36°C (not shown).

In previous work (Uyama *et al.*, 1992), caffeine- and inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release in skinned fibres were not affected by the presence of 1 μM CPA. Since higher concentrations of CPA were used in the present study, the effects of 10 μM CPA on Ca^{2+} release itself were re-examined in skinned preparations (Figure 2). After skinning, application of pCa 6.3 solution evoked a control contractile response (Figure 2a). This procedure also resulted in Ca^{2+}-loading. Subsequent application of 25 mM caffeine in a relaxing solution (R0.1G) induced a transient contraction. A second application of 25 mM caffeine in R0.1G elicited either a small or no response. The pair of contractions induced by pCa 6.3 and 25 mM caffeine was repeated three times in each preparation (three trials) at an interval of approximately 30 min. In Figure 2b, 10 μM CPA was applied about 2 min after the change in solution to R0.1G. The application of 10 μM CPA elicited a small phasic contraction in all prepara-

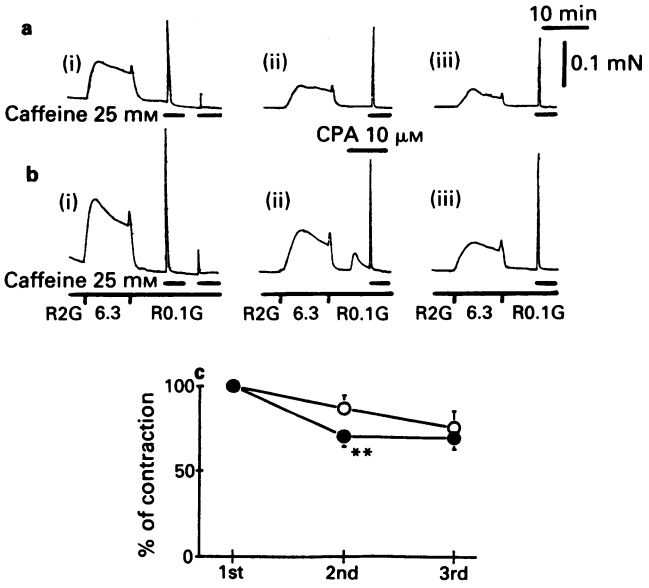

Figure 2 Effects of 10 μM cyclopiazonic acid (CPA) on 25 mM caffeine-induced contractions in skinned preparations. Skinned strips were contracted by changing R2G to a pCa 6.3 solution as indicated below (b). This was also a Ca^{2+} loading period. Caffeine (25 mM) was added twice as indicated by bars in R0.1G solution (a(i) and b(i)). Note that the second response to caffeine was very small. Ca^{2+} loading and subsequent Ca^{2+} release by caffeine were repeated three times (three trials; (i), (ii) and (iii)). The amplitude of the responses to caffeine decreased progressively even in the control (a). In b(ii) (in the second trial), 10 μM CPA was added to R0.1G solution after Ca^{2+} loading. Application of CPA induced a small phasic contraction. The subsequent caffeine-induced contraction in the presence of CPA was smaller than in the control, whereas the response recovered substantially in the third trial (b(iii)). (c) Shows the summarized data indicating that caffeine-induced Ca^{2+} release was slightly but significantly reduced in the presence of 10 μM CPA: (○) control response; (●) 2nd response measured after treatment with 10 μM CPA. $n = 4$.

tions examined ($n = 4$). When the relaxing solution containing higher concentrations of EGTA (R2G or R0.5G), CPA did not induce the small phasic contraction. The subsequent application of caffeine in R0.1G induced a response which was slightly smaller than that in the control. The effect of 10 μM CPA was removed by washout (Figure 2b). Summarized data shown in Figure 2c indicate that the caffeine-induced contraction in skinned preparations was slightly but significantly reduced in the presence of 10 μM CPA. The decrease was, however, too small to explain the large decrease in caffeine-induced Ca^{2+}-release by CPA in intact strips shown in Figure 1, implying that it was mainly due to inhibition of Ca^{2+}-uptake.

The effects of CPA on spontaneous and 20 mM K^+-induced contractions were examined at 36°C in the presence of 1 μM atropine. Figure 3 shows that CPA at concentrations higher than 1 μM enhanced the amplitude of spontaneous contraction at 36°C. High concentrations of CPA (>3 μM) also increased the muscle tone. The frequency of spontaneous contraction varied widely from preparation to preparation (0.2–5.0 contractions min^{-1}). CPA (1–30 μM) increased the frequency in all preparations examined ($n = 26$). Initial phasic and following sustained contractions were induced by 20 mM K^+ in the presence of 1 μM atropine (Figure 3a). Repetitive small phasic contractions were superimposed on the tonic contraction. A potassium concentration of 20 mM was chosen, since all the contractile responses to 20 mM K^+ were almost abolished by 1 μM nicardipine, suggesting that the responses were evoked by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. The initial phasic contractions induced by 20 mM K^+ were enhanced by CPA in a concentration range of 1–10 μM. The enhancement was rather less at higher concentrations (>10 μM) (Figure 3b). The CPA concentration giving a half maximum effect (EC_{50}) was approximately 3 μM. The effect of CPA on 80 mM K^+-induced contraction was also examined (Figure 4). Although there was a tendency for the peak amplitide of the 80 mM K^+-induced contraction to be enhanced by 10 μM CPA, the enhancement was not statistically significant (Figure 4b, $P > 0.05$). The steady phase was not affected by 10 μM CPA ($P > 0.05$).

The effects of CPA on resting membrane potential and electrical activity were examined with conventional microelectrodes in longitudinal smooth muscle strips at 35°C in the presence of 1 μM atropine (Figure 5a). Application of 10 μM CPA slightly depolarized the cell membrane from -48.6 ± 1.2 mV ($n = 34$) to -44.7 ± 1.3 mV ($n = 22$, $P < 0.05$ vs. the control). The depolarization started after a short delay of about 20–80 s, gradually developed and reached a steady level within 3 min. Although the AP frequency in the control varied widely from preparation to preparation and also depended upon how stretched the preparation was, it was in a range of 0.2–20 APs min^{-1}. A burst of several APs often occurred. After application of 10 μM CPA, the AP frequency markedly increased with the depolarization in all preparations examined ($n = 13$, $P < 0.01$) to higher than 0.5 Hz in 10 preparations out of 18, although it varied with time. Another important finding was that both peak amplitude and 50% duration of AP afterhyperpolarization (AHP) were decreased after application of 10 μM CPA (Figure 5a(ii) and (iii)). Spontaneous APs and the effects of CPA were not affected by 1 μM TTX, 0.3 μM phentolamine and 0.3 μM propranolol.

These CPA-induced effects on membrane potential were mimicked by application of 30–100 nM charybdotoxin (ChTX). The cells were depolarized by about 5 mV (-43.2 ± 1.3 mV, $n = 28$, $P < 0.05$) within 20 s of the application of 100 nM charybdotoxin (ChTX). Concomitantly, the AP fre-

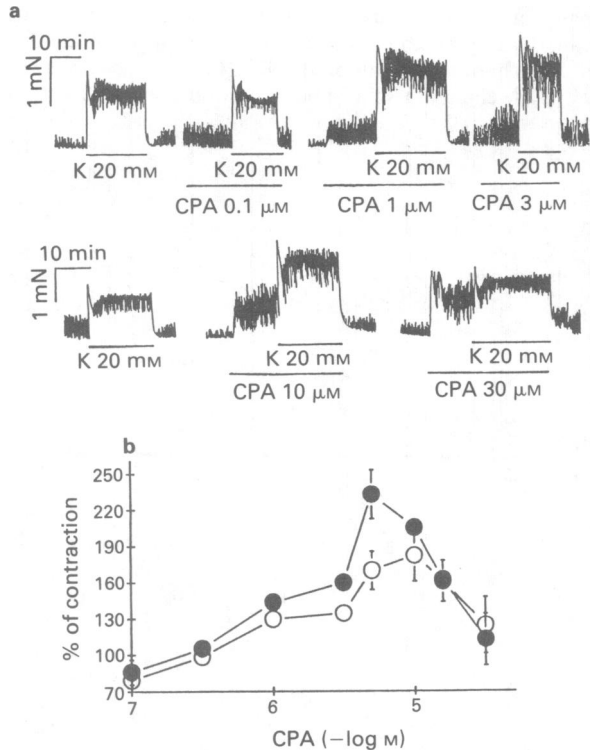

Figure 3 Effects of cyclopiazonic acid (CPA) on 20 mM K^+-induced contraction in intact ileal longitudinal smooth muscle strips at 36 ± 1°C. (a) Typical recordings of 20 mM K^+-induced contractions in the absence or presence of 0.1, 1, 3, 10 or 30 μM CPA. All experiments were performed in the presence of 1 μM atropine. CPA was added 15 min prior to the application of 20 nM K^+-solution. (b) Summarized data of the amplitude of phasic and sustained components of 20 mM K^+-induced contractions are shown by (○) and (●) respectively. Those in the control (in the absence of CPA) were taken as 100%. The amplitude of the sustained component was measured at the mean level of repetitive phasic contractions from the resting tone 10 min after the application of 20 mM K^+ solution. The number of observations was four except at 1 μM CPA where it was seven. The amplitude of both initial phasic and following sustained components was significantly larger than 100% at CPA concentrations in the range of 1 and 20 μM.

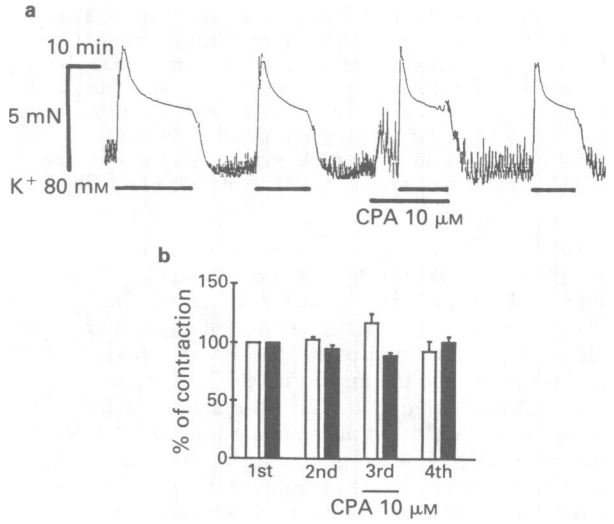

Figure 4 Effects of 10 μM cyclopiazonic acid (CPA) on 80 mM K^+-induced contractions: 80 mM K^+ solution was applied four times. In the third trial, CPA was added 10 min prior to the application of 80 mM K^+. All experiments were performed in the presence of 1 μM atropine at 36 ± 1°C. (b) Summarized data of the amplitude of 80 mM K^+-induced contractions at the peak (phasic component; open columns) and at 15 min from the application of 80 mM K^+ (sustained component; solid columns): $n = 5$. No statistically significant difference was found between the data in the first trial and those in the second, third or fourth trial for each component, respectively.

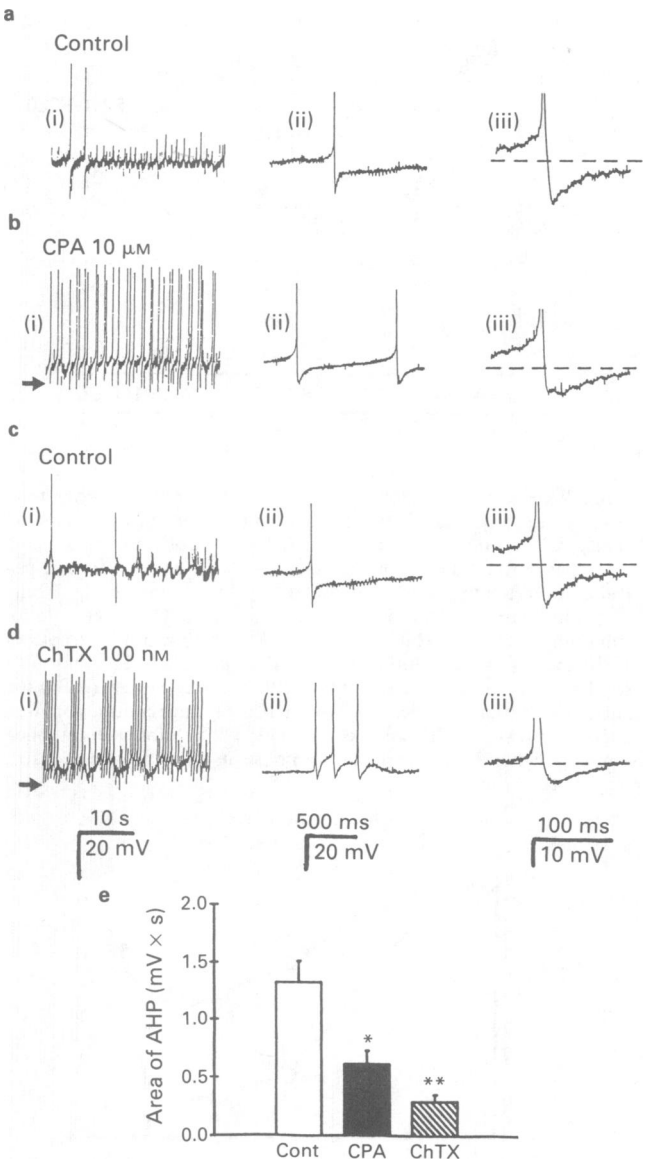

Figure 5 Effects of cyclopiazonic acid (CPA) (b) and charybdotoxin (ChTX) (d) on electrical activity in ileal longitudinal smooth muscle strips. Membrane potentials were measured with conventional glass microelectrodes in the presence of 1 μM atropine at 35°C. (a) and (b) show recordings from a smooth muscle cell before and about 5 min after the application of 10 μM CPA, respectively; (c) and (d) show recordings from a smooth muscle cell before and about 3 min after the application of 100 nM ChTX, respectively. Traces in (ii) and (iii) show faster recordings of parts of (i). The broken lines in (iii) indicate the level of the membrane potential just before the depolarization triggering an action potential. The arrows in (b(i)) and (d(i)) indicate the resting membrane potential before the application of CPA or ChTX. Note that action potential frequency was markedly increased and AHP was substantially decreased in the presence of 10 μM CPA (b) or 100 nM ChTX (d). The AHP from the level indicated by the broken line was integrated on the computer and shown as the area of AHP in (e). The AHP areas were obtained from over 10 APs in each preparation before and after application of CPA or ChTX and averaged, respectively. Shown are the average of pooled data from 4–8 preparations. The area of AHP was significantly decreased by 10 μM CPA or 100 nM ChTX.

quency and AHP amplitude markedly increased and decreased, respectively, as shown in Figure 5d. To evaluate the effects of CPA and ChTX on AHP, AHP was integrated as the area from the level of the membrane potential just before the rapid depolarization which triggered a burst of APs. The summarized data indicate that the area of AHP was significantly decreased by 10 μM CPA and more extensively by 100 nM ChTX (Figure 5e).

To confirm that the increase in spontaneous contractions and muscle tone by CPA was preceded by an increase in $[Ca^{2+}]_i$, changes in $[Ca^{2+}]_i$ were monitored at 35°C in intact strips, which were loaded with the fluorescent Ca^{2+}-indicator, Fura-2/AM. Application of 3 μM CPA, in the presence of 1 μM atropine, markedly enhanced the phasic increases in $[Ca^{2+}]_i$ (Figure 6a(i)). Concomitantly, phasic contractions were greatly enhanced (Figure 6a(ii)). The increase in $[Ca^{2+}]_i$ changes and phasic contractions by CPA were blocked by 1–10 μM verapamil (Figure 6a) or 10 mM EGTA (not shown) and recovered on further addition of 1–10 μM Bay K 8644 (Figure 6a). Similar results were obtained in five preparations out of five. When a slight increase in basal $[Ca^{2+}]_i$ and muscle tone was observed on application of 3 μM CPA in addition to the enhancement of repetitive phasic changes, it was abolished by addition of 10 μM verapamil or 10 mM EGTA ($n = 2$). Application of 100 nM ChTX showed similar effects: enhancement of spontaneous phasic increase in $[Ca^{2+}]_i$ and contractions (Figure 6b).

Although these effects of CPA on spontaneous electrical and mechanical activities may be consistent with inhibition of SR/ER Ca^{2+}-ATPase by CPA, a line of direct evidence was required to conclude that 10 μM CPA substantially reduced Ca^{2+}-uptake at 36°C. Figure 7 shows the effect of CPA on the decay time course of $[Ca^{2+}]_i$ and contraction after $[K^+]_o$ was decreased from 80 to 5.9 mM. The $[Ca^{2+}]_o$ was changed from 2.2 mM to nominally Ca-free (with 0.1 mM EGTA) at the same time. The rate of decay of $[Ca^{2+}]_i$ was markedly slowed when the Ca-free solution contained 10 μM CPA. After 5 min from the decrease in $[K^+]_o$, 100 μM acetylcholine (ACh) was applied to estimate the amount of stored Ca^{2+}.

Summarized data are shown in Figure 8. During the decay for 5 min, the relative $[Ca^{2+}]_i$ and force were significantly larger in the presence of 10 μM CPA. Since 30 μM CPA had similar potency (not shown), 10 μM CPA appeared to be almost maximally effective. It is worth noting that the increase in relative $[Ca^{2+}]_i$ and force by ACh was smaller in the presence of CPA and that the final levels of $[Ca^{2+}]_i$ in the presence of ACh were similar in the control and CPA-treated preparations.

Discussion

The present study was focused on the possibility that electrical and mechanical activities are enhanced in ileal smooth muscle, when Ca^{2+}-uptake by intracellular storage sites is significantly reduced by CPA. The results clearly show that spontaneous APs, repetitive phasic increase in $[Ca^{2+}]_i$ and concomitant phasic contractions were markedly enhanced by 1–10 μM CPA. Since the ileal longitudinal strips were prepared free from interstitial cells of Cajal and the circular muscle layer, the APs observed in the presence of atropine are myogenic from longitudinal smooth muscle cells. Phasic and tonic contractions induced by 20 mM K^+ were markedly enhanced by CPA. These results strongly suggest that Ca^{2+}-influx through voltage-dependent Ca^{2+} channels is enhanced by CPA, and may be consistent with the observation that thapsigargin, another inhibitor of SR/ER Ca^{2+}-ATPase, increases the amplitude of the spontaneous mechanical activity in rat portal vein (Mikkelsen *et al.*, 1992).

Direct evidence indicating that the increase in Ca^{2+}-influx by CPA is attributable to the inhibition of Ca^{2+}-ATPase in intracellular Ca storage sites is difficult to obtain in multicellular preparations. However, a line of indirect evidence shown in the present and previous studies strongly suggests that CPA significantly reduces $I_{K\text{-}Ca}$ by inhibition of Ca^{2+}-uptake of storage sites and consequently increases Ca^{2+}-influx in intact ileal longitudinal smooth muscle.

First, CPA slightly depolarized the cell membrane, reduced AHP and increased AP frequency in a similar manner to

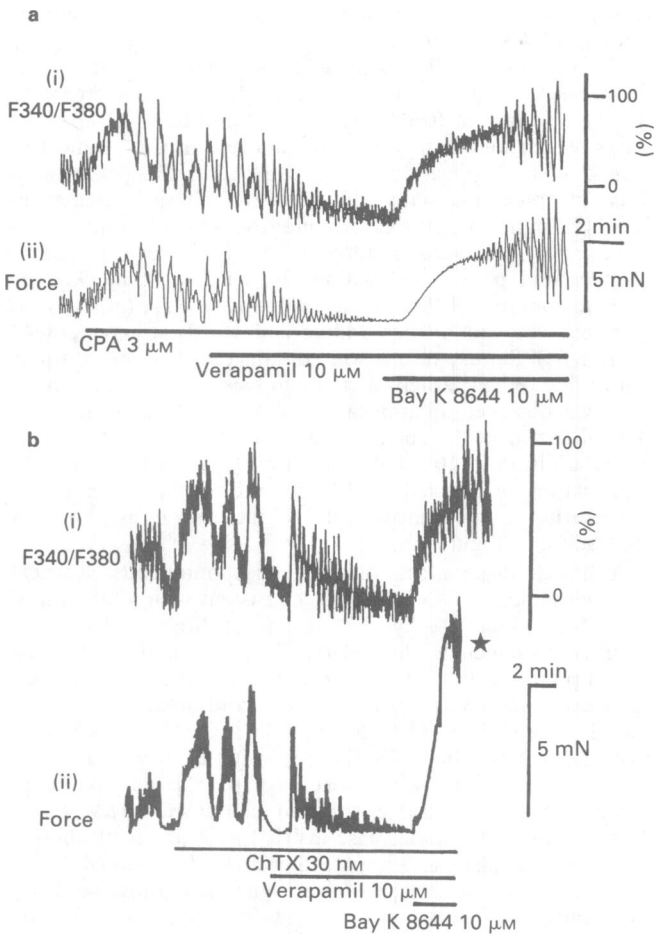

Figure 6 Effects of cyclopiazonic acid (CPA, a) and charybdotoxin (ChTX, b) on changes in $[Ca^{2+}]_i$ in a strip loaded with Fura-2/AM. $[Ca^{2+}]_i$ signals are shown as the ratio of amplitude of 500 nm fluorescence elicited by 340 and 380 nm excitation. (a) Application of 3 μM CPA induced a slow phasic increase in $[Ca^{2+}]_i$ and enhanced repetitive fast transients of $[Ca^{2+}]_i$, which corresponded to a slow phasic increase in the muscle tone and large spontaneous contractions, respectively. (b) Application of 30 nM ChTX immediately enhanced the spontaneous $[Ca^{2+}]_i$ changes and contractions. These changes were abolished by addition of 10 μM verapamil and recovered on further addition of 10 μM Bay K 8644. The asterisk in (b(ii)) indicates that the contraction after the addition of Bay K 8644 was out of scale in this preparation.

ChTX. Corresponding to the significant decrease in AHP, the $I_{K\text{-}Ca}$ activated by depolarization in single cells was very selectively blocked by 10 μM CPA (Suzuki *et al.*, 1992) or 100 nM ChTX (Uyama, unpublished observation). ChTX directly blocked single BK channel current (Brayden & Nelson, 1992) but CPA did not (Suzuki *et al.*, 1992). The same phenomena including changes in resting membrane potential, AP frequency and AHP in tissue preparations and $I_{K\text{-}Ca}$ inhibition in single cells by quinidine have been reported in ileal circular smooth muscle of the guinea-pig (Nakao *et al.*, 1986). The AP falling phase and AHP are mainly due to $I_{K\text{-}Ca}$ activation, which is susceptible to quinidine but not apamin. In rat sympathetic neurones, it has been shown that CPA reduces the ryanodine-sensitive component of AHP and facilitates repetitive AP generation (Kawai & Watanabe, 1989; Ishii *et al.*, 1992).

Second, 10 μM CPA inhibited Ca^{2+}-uptake of intracellular storage sites in intact ileal strips at 22°C. The inhibition of caffeine-induced contraction by CPA is mainly due to inhibition of Ca^{2+}-loading of the storage sites but not due to the direct change in Ca^{2+} release. Although 10 μM CPA slightly reduced caffeine-induced Ca^{2+} release in skinned preparations, it is probably due to the preceding small Ca^{2+} release

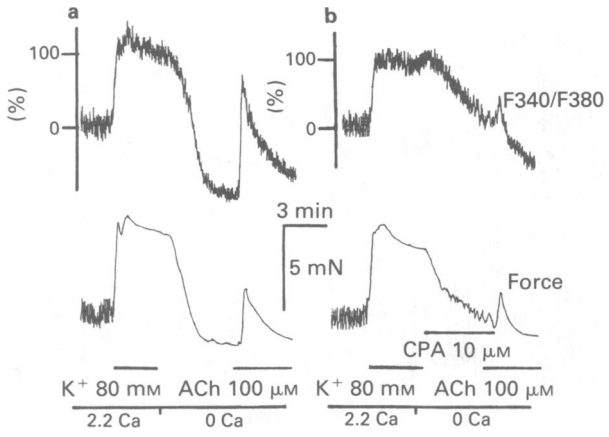

Figure 7 Effects of cyclopiazonic acid (CPA) on the decay time-course of elevated $[Ca^{2+}]_i$ and evoked contraction during washout of 80 mM K^+ solution in Fura-2 loaded ileal smooth muscle at 35°C. upper and lower traces show changes in $[Ca^{2+}]_i$ and tension, respectively. After treatment with 80 mM K^+ solution containing 2.2 mM Ca^{2+} for 4 min, the solution was changed to a Ca^{2+}-free solution containing 0.1 mM EGTA and 5.9 mM K^+ as shown at the bottom. In (b) the Ca^{2+}-free solution contained 10 μM CPA. Five min after the change to the Ca^{2+}-free solution, 100 μM actylcholine (ACh) was added. Note that the decay of both $[Ca^{2+}]_i$ and contraction were markedly slowed in the presence of 10 μM CPA. Subsequent application of ACh induced a smaller response in the CPA-treated strip than in the control.

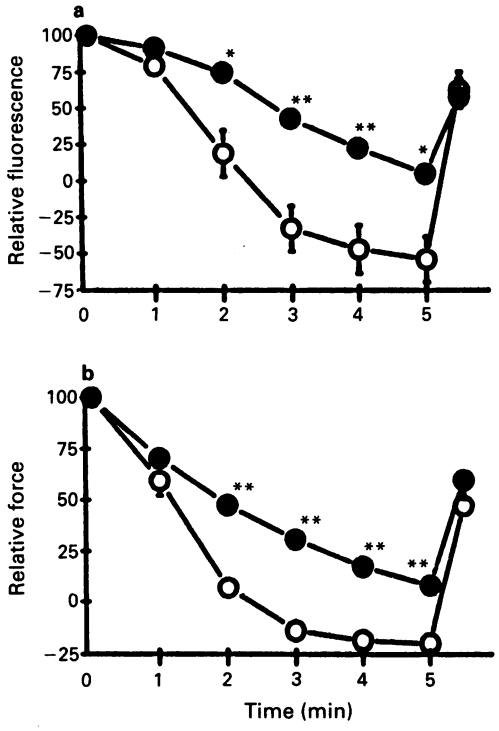

Figure 8 Summarized data of effects of 10 μM cyclopiazonic acid (CPA) on the decay time-courses of 80 mM K^+-induced high $[Ca^{2+}]_i$ and contraction. These results were obtained from experiments such as that shown in Figure 7. The time of the solution change to the Ca^{2+}-free solution was taken as 0 min. The resting and high K^+-stimulated levels of signals were taken as 0 and 100%, respectively. (a) The time-course of relative amplitude of fluorescence ($[Ca^{2+}]_i$). (b) The time-course of relative force: mean values in the absence (○) ($n = 6$) or presence (●) of 10 μM CPA ($n = 3$), are shown. In the presence of 10 μM CPA, the decay of both signals (between 2 to 5 min) was significantly slowed by CPA. Note that the peak levels of $[Ca^{2+}]_i$ and contraction induced by 100 μM acetylcholine (ACh, applied after 5 min in Ca^{2+}-free solution) in the presence of CPA were not significantly different from those in the control.

by CPA itself, which slightly reduced the amount of stored Ca. It has been suggested that Ca in storage sites is spontaneously released and is retaken up by Ca-pumps in several types of cells (Thastrup *et al.*, 1990; Stehno-Bittel & Sturek, 1992; Chen *et al.*, 1992). Ca-pump inhibition may allow accumulation of spontaneously released Ca^{2+} and result in a small contraction.

Third, the decay of high $[Ca^{2+}]_i$ and contraction induced by 80 mM K^+ after washout with Ca^{2+}-free solution containing 5.9 mM K^+ was significantly slowed by CPA. Since plasmalemmal Ca^{2+}-ATPase is not affected by CPA (Seidler *et al.*, 1989), Ca^{2+}-extrusion from the cell by the Ca-pump would not be directly affected by CPA. Effects of CPA on Ca^{2+}-extrusion by Na^+-Ca^{2+} exchange have not been clarified. Under those conditions, however, the subsequent application of ACh induces smaller Ca^{2+} release in preparations treated with CPA than in the control. A combination of these findings gives additional evidence that CPA also significantly reduced Ca^{2+}-uptake by storage sites at 35°C.

Fourth, the EC_{50} of CPA for the enhancement of 20 mM K^+-induced contraction was approximately 3 μM and close to the IC_{50} for the inhibition of $I_{K\text{-}Ca}$ (~3 μM, Suzuki *et al.*, 1992). Although the EC_{50} is about 5 times higher than the IC_{50} for the inhibition of Ca^{2+}-uptake in skinned fibres (0.6 μM; Uyama *et al.*, 1992), the difference is rather small considering the diffusion barrier of the plasma membrane.

The mechanisms of CPA-induced increase in Ca^{2+}-influx, however, may not be as simple as those of the ChTX-induced increase. The initial transient Ca release from storage sites after the application of 10 μM CPA may activate not only $I_{K\text{-}Ca}$ but also other Ca^{2+} dependent currents: Cl^- current or non-selective cationic current, as has been reported for caffeine or agonists (Amédée *et al.*, 1990; Janssen & Sims, 1992). The subsequent depolarization was small but sustained and may result from suppression of spontaneous transient outward currents (Suzuki *et al.*, 1992) which are elicited by activation of BK channels by spontaneous Ca release from local storage sites (Benham & Bolton, 1986) and possibly contribute, in part, to the resting membrane potential (Hu *et al.*, 1989; Brayden & Nelson, 1992). The CPA-induced decrease in AHP is due to the selective inhibition of $I_{K\text{-}Ca}$ (Suzuki *et al.*, 1992) which also has been reported for thapsigargin in urinary bladder smooth muscle cells (Ganitkevich & Isenberg, 1992). The decrease in resting membrane potential and AHP by CPA may be responsible for the marked increase in frequency of spontaneous APs. It is, however, likely that the increase in AP frequency caused by CPA may itself result in a further decrease in Ca store size and, thereby, AHP. Moreover, the combination of the increase in Ca^{2+}-influx and the inhibition of Ca^{2+}-uptake by CPA raised $[Ca^{2+}]_i$ and may partly compensate the decreased $I_{K\text{-}Ca}$. It may be the reason why the decrease in AHP by CPA was smaller than that induced by ChTX in tissue preparations.

It should be mentioned that the BK channel activity is regulated by $[Ca^{2+}]_i$ near the inner mouth of the channels and, therefore, may well be affected by Ca storage sites localized just beneath the cell membrane. Activation of BK channels by Ca^{2+} release from storage sites can often be observed in cell free patches (Xiong *et al.*, 1992). The activation of $I_{K\text{-}Ca}$ by Ca^{2+}-influx upon depolarization may be greatly amplified by the large increase in local $[Ca^{2+}]_i$ via Ca^{2+}-induced Ca^{2+} release from the storage sites (Suzuki *et al.*, 1992; Ganitkevich & Isenberg, 1992). On the other hand, the $[Ca^{2+}]_i$ measured by Fura-2 signals show averaged changes in $[Ca^{2+}]_i$ of the whole cytoplasm. The increase in Ca^{2+}-influx directly results in elevation of Fura-2 or Indo-1 signal (Becker *et al.*, 1989) but may not result in the increase in $I_{K\text{-}Ca}$, if releasable Ca in storage sites is substantially reduced by CPA. Such dissociation between $[Ca^{2+}]_i$ detected by Indo-1 or Fura-2 and $I_{K\text{-}Ca}$ has been suggested in smooth muscle cells (Bittel & Sturek, 1992; Ganitkevich & Isenberg, 1992).

It has been reported that Ca^{2+}-entry through the plasma membrane depends upon the filling state of the ER/SR (Putney, 1986, 1990; Missiaen *et al.*, 1990; Dolor *et al.*, 1992; Byron *et al.*, 1992). CPA and thapsigargin deplete intracellular stored Ca^{2+} and activate an influx pathway for divalent cations (Takemura *et al.*, 1989, 1991; Demaurex *et al.*, 1992). It has been suggested that some Ca^{2+}-storage sites may couple with DHP-sensitive Ca^{2+}-channels in the plasma membrane in smooth muscle cells (Bourreau *et al.*, 1991; Low *et al.*, 1992; Xuan *et al.*, 1992) as well as in other types of cells (Hoth & Penner, 1992). The present results are not opposed to this hypothesis. When $I_{K\text{-}Ca}$ is decreased and AP frequency is increased by CPA, Ca^{2+}-influx through Ca^{2+} channels must be markedly increased even when Ca^{2+} channel properties *per se* are not changed.

The tonic contraction induced by 80 mM K^+ was not significantly enhanced by CPA in the present study, while 50 mM K^+-induced contraction in aorta and mesenteric artery of the rat was enhanced by thapsigargin (Shima & Blaustein, 1992). When I_{Ca} was measured under whole-cell clamp, 10 μM CPA did not affect I_{Ca} (Suzuki *et al.*, 1992). Under these conditions, 5 mM EGTA in the pipette solution may remove stored Ca. It has been reported that a high concentration of CPA inhibits Ca^{2+}-influx pathways in rat thymic lymphocytes (Mason *et al.*, 1991). Based on these observations, it is suggested that the enhancement of Ca^{2+}-influx through voltage-dependent Ca^{2+} channels in ileal longitudinal smooth muscle is an indirect effect of CPA. The possibility that the activity of Ca^{2+} channels on the cell membrane is directly regulated by the amount of Ca in storage sites is, however, interesting and remains to be examined more exactly in smooth muscle cells.

In conclusion, CPA applied from outside the cell in intact ileal smooth muscle can reduce Ca^{2+}-uptake of intracellular Ca storage sites via the inhibition of Ca^{2+}-ATPase. The decrease in stored Ca by CPA may increase Ca^{2+}-influx. The decrease in $I_{K\text{-}Ca}$ may be one of the major causes. Of importance is the fact that inhibition of Ca^{2+}-uptake of intracellular storage sites by CPA in intact strips was partly reversible, as shown in single smooth muscle cells and skinned fibres.

Y.I. was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

References

AMÉDÉE, T., BENHAM, C.D., BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1990). Potassium, chloride and non-selective cation conductances opened by noradrenaline in rabbit ear artery cells. *J. Physiol.*, **423**, 551–568.

BECKER, P.L., SINGER, J.J., WALSH, J.V. Jr. & FAY, F.S. (1989). Regulation of calcium concentration in voltage clamped smooth muscle cells. *Science*, **244**, 211–214.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.*, **281**, 385–406.

BITTEL, L.S. & STUREK, M. (1992). Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine coronary artery smooth muscle. *J. Physiol.*, **451**, 49–78.

BOURREAU, J.P., ABELA, A.P., KWAN, C.Y. & DANIEL, E.E. (1991). Acetylcholine Ca^{2+} stores refilling directly involves a dihydropyridine-sensitive channel in dog trachea. *Am. J. Physiol.*, **261**, C497–C505.

BRAYDEN, J.E. & NELSON, M.T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science*, **256**, 532–535.

BYRON, K.L., BABNIGG, G. & VILLEREAL, M.L. (1992). Bradykinin-induced Ca^{2+} entry, release, and refilling of intracellular Ca^{2+} store; relationships revealed by image analysis of individual human fibroblasts. *Am. J. Physiol.*, **267**, 108–118.

CHEN, Q., CANNELL, M. & VAN BREEMAN, C. (1992). The superficial buffer barrier in vascular smooth muscle. *Can. J. Physiol. Pharmacol.*, **70**, 509–514.

DEMAUREX, M., LEW, D.P. & KRAUSE, K.H. (1992). Cyclopiazonic acid depletes intracellular Ca^{2+}-stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.*, **267**, 2318–2324.

DENG, H.W. & KWAN, C.Y. (1991). Cyclopiazonic acid is a sarcoplasmic reticulum Ca^{2+}-pump inhibitor of rat aortic muscle. *Acta Pharmacol. Sinica*, **12**, 53–58.

DOLOR, R.J., HURWITZ, L.M., MIRZA, Z., STRAUSS, H.C. & WHORTON, A.R. (1992). Regulation of extracelluar calcium entry in endothelial cells: role of intracellular calcium pool. *Am. J. Physiol.*, **262**, C171–C181.

GANITKEVICH, V.Y. & ISENBERG, G. (1992). Contribution of Ca^{2+}-induced Ca^{2+} release to the $[Ca^{2+}]_i$ transients in myocytes from guinea-pig urinary bladder. *J. Physiol.*, **458**, 119–137.

GOEGER, D.E. & RILEY, R.T. (1989). Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effects on Ca^{2+} binding and Ca^{2+} permeability. *Biochem. Pharmacol.*, **38**, 3995–4003.

GOEGER, D.E., RILEY, R.T., DORNER, J.W. & COLE, R.J. (1988). Cyclopiazonic acid inhibition of the Ca^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. *Biochem. Pharmacol.*, **37**, 978–981.

HOTH, M. & PENNER, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature*, **355**, 353–355.

HU, S.L., YAMAMOTO, Y. & KAO, C.Y. (1989). The Ca^{2+}-activated K^+ channel and its functional roles in smooth muscle cells of guinea pig taenia coli. *J. Gen. Physiol.*, **94**, 833–847.

ISHII, Y., KAWAI, T. & WATANBE, M. (1992). Inhibitory effects of cyclopiazonic acid on the spike after-hyperpolarization in rat sympathetic neurons. *Jpn. J. Pharmacol.*, **58**, 451–456.

IINO, M. (1989). Calcium-induced calcium release mechanism in guinea pig taenia caeci. *J. Gen. Physiol.*, **94**, 363–383.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. (1990). Characteristics of transient outward currents in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol.*, **427**, 301–324.

JANSSEN, L. & SIMS, S. (1992). Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes. *J. Physiol.*, **453**, 197–218.

KAWAI, T. & WATANABE, M. (1989). Effects of ryanodine on the spike afterhyperpolarization in sympathetic neurones of the rat superior cervical ganglion. *Pflügers Arch.*, **413**, 470–475.

KITAMURA, K., SAKAI, T., KAJIOKA, S. & KURIYAMA, H. (1989). Activation of the Ca dependent K channel by Ca released from the sarcoplasmic reticulum of mammalian smooth muscles. *Biomed. Biochim. Acta*, **48**, S364–369.

KITAZAWA, T. & SOMLYO, A.P. (1990). Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca^{2+} in smooth muscle. *Biochem. Biophys. Res. Commun.*, **172**, 1291–1297.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A.V. & SOMLYO, A.P. (1989). Cytosolic heparin inhibits muscarinic and alpha-adrenergic Ca2+-release in smooth muscle. *J. Biol. Chem.*, **264**, 17997–18004.

KUREBAYASHI, N. & OGAWA, Y. (1991). Discrimination of Ca2+-ATPase activity of sarcoplasmic reticulum from actomyosin-type ATPase activity of myofibrils in skinned mammalian skeletal muscle fibres: distinct effects of cyclopiazonic acid on the two ATPase activities. *J. Muscle Res. Cell Motil.*, **12**, 355–365.

LOW, A.M., KWAN, C.Y. & DANIEL, E.E. (1992). Evidence for two types of internal Ca^{2+}-stores in canine mesenteric artery with different refilling mechanisms. *Am. J. Physiol.*, **262**, H31–H37.

MASON, M.J., GARCIA-RODRIGUEZ, C. & GRINSTEIN, S. (1991). Coupling between intracellular Ca^{2+}-store and the Ca^{2+} permeability of plasma membrane. *J. Biol. Chem.*, **266**, 20856–20862.

MIKKELSEN, E.O., POULSEN, S.H. & CHRISTENSEN, S.B. (1992). Comparison of the effects of thapsigargin and Bay K 8644 on spontaneous mechanical activity in rat portal vein and contractile responses of rat cardiac muscle. *Pharmacol. Toxicol.*, **70**, 152–156.

MISSIAEN, L., DECLERCK, I., DROOGMANS, G., PLESSER, L., SMEDT, H.D., RAEYMAREKERS, L. & CASTEELS, R. (1990). Agonist-dependent Ca^{2+} and Mn^{2+} entry dependent on state of filling of Ca^{2+} stores in aortic smooth muscle cells of the rat. *J. Physiol.*, **427**, 171–186.

MITRA, R. & MORAD, M. (1985). Ca^{2+} and Ca^{2+}-activated K^+ currents in mammalian gastric smooth muscle cells. *Science*, **229**, 269–272.

NAKAO, K., INOUE, R., YAMANAKA, K. & KITAMURA, K. (1986). Actions of quinidine and apamin on after-hyperpolarization of the spike in circular smooth muscle cells of the guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **334**, 508–513.

OHYA, Y., KITAMURA, K. & KURIYAMA, H. (1987). Cellular calcium regulates outward currents in rabbit intestinal smooth muscle cell. *Am. J. Physiol.*, **252**, C401–C410.

OZAKI, H., SATO, K., SATO, T., & KARAKI, H. (1987). Simultaneous recording of calcium signals and mechanical activity using fluorescent dye fura-2 in isolated strips of vascular smooth muscle. *Jpn. J. Pharmacol.*, **45**, 429–433.

PUTNEY, J.W. Jr. (1986). A model for receptor-regulated calcium entry. *Cell Calcium*, **7**, 1–12.

PUTNEY, J.W. Jr. (1990). Capasitative calcium entry revisited. *Cell Calcium*, **11**, 611–624.

SAKAI, T., TERADA, K., KITAMURA, K. & KURIYAMA, H. (1988). Ryanodine inhibits the Ca-dependent K current after depletion of Ca stored in smooth muscle cells of the rabbit ileal longitudinal muscle. *Br. J. Pharmacol.*, **95**, 1089–1100.

SEIDLER, N.W., JONA, I., VEGH, M. & MARTONOSI, A. (1989). Cyclopiazonic acid is a specific inhibition of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.*, **264**, 17816–17823.

SHIMA, H. & BLAUSTEIN, M.P. (1992). Modulation of evoked contraction in rat arteries by ryanodine, thapsigargin and cyclopiazonic acid. *Circ. Res.*, **70**, 968–977.

STEHNO-BITTEL, L. & STUREK, M. (1992). Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. *J. Physiol.*, **451**, 49–78.

SUZUKI, M., MURAKI, K., IMAIZUMI, Y. & WATANABE, M. (1992). Cyclopiazonic acid, an inhibitor of sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ current in smooth muscle cells. *Br. J. Pharmacol.*, **107**, 134–140.

TAKEMURA, H., HUGHES, A.R., THATRUP, O. & PUTNEY Jr, J.W. (1989). Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.*, **264**, 12266–12271.

TAKEMURA, H., OHSHIKA, H., YOKOSAWA, N., OGUMA, K. & THASTRUP, O. (1991). The thapsigargin sensitive intracellular Ca^{2+}-pool is more important in plasma membrane Ca^{2+}-entry than the IP_3-sensitive intracellular Ca^{2+}-pool in neuronal cell lines. *Biochem. Biophys. Res. Commun.*, **180**, 1518–1526.

THASTRUP, O., CULLEN, P.J., DROBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} store by specific inhibition of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2466–2470.

UYAMA, Y., IMAIZUMI, Y. & WATANABE, M. (1992). Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile respones in skinned ileal smooth muscle. *Br. J. Pharmacol.*, **106**, 208–214.

WATANABE, M., IMAIZUMI, Y., MURAKI, K. & TAKEDA, M. (1989). A comparative study about voltage-dependent Ca currents in smooth muscle cells isolated from several tissues. In: *Calcium Protein Signaling*. ed. Hidaka, H. pp. 119–128. New York: Plenum Press.

WONG, B. (1991). Ionic conductances in dissociated smooth muscle cells. *Proc. Soc. Exp. Biol. Med.*, **197**, 125–134.

XIONG, Z., KITAMURA, K. & KURIYAMA, H. (1992). Evidence for contribution of Ca^{2+} storage sites on unitary K^+ channel currents in inside-out membrane of rabbit portal vein. *Pflügers Arch.*, **420**, 112–114.

XUAN, YU-T., WANG, OU-LI & WHORTON, A.R. (1992). Thapsigargin stimulates Ca^{2+} entry in vascular smooth muscle cells: nicardipine-sensitive and -insensitive pathways. *Am. J. Physiol.*, **262**, C1258–C1265.

(Received January 4, 1993
Revised May 25, 1993
Accepted June 1, 1993)