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Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells

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1 Effects of cyclopiazonic acid (CPA), a specific inhibitor of the Ca^{2+}-ATPase in sarcoplamic reticulum (SR), on membrane ionic currents were examined in single smooth muscle cells freshly isolated from ileal longitudinal strips and urinary bladder of the guinea-pig.

2 Under whole-cell clamp, CPA (1–10 μM) reduced peak outward current elicited by depolarization in a concentration-dependent manner. The concentration of CPA required for 50% decrease in the peak outward current was ~3 μM in ileal cells under these conditions. The current reduced by CPA recovered by more than 70% after washout.

3 The transient outward current elicited by application of 5 mM caffeine at a holding potential of −50 mV in Ca^{2+} free solution was almost abolished, when the preceding Ca^{2+}-loading of the cell in a solution containing 2.2 mM Ca^{2+} was performed in the presence of 3 μM CPA.

4 When the Ca^{2+}-dependent K^+ current (I_{K-Ca}) and Ca^{2+} current (I_{Ca}) were inhibited by addition of Ca^{2+}, the remaining delayed rectifier type K^+ current was not affected by 10 μM CPA. When outward currents were blocked by replacement of K^+ by Cs^+ in the pipette solution, the remaining I_{Ca} was not affected by 10 μM CPA.

5 CPA (10 μM) did not affect the conductance of single maxi Ca^{2+}-dependent K^+ channels or the Cd^{2+}-dependence of their open probability in both inside- and outside-out configurations.

6 These results indicate that I_{K-Ca} is selectively and strongly suppressed by CPA. Its effects may be attributed to a decrease in Ca^{2+}-uptake into SR, resulting in a decrease in Ca^{2+}-induced Ca^{2+} release which is triggered by Ca^{2+} entering through voltage-dependent Ca^{2+} channels and therefore less activation of these K channels.

7 CPA may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+}.

Keywords: cyclopiazonic acid; Ca^{2+}-dependent K^+ current; Ca^{2+}-induced Ca^{2+} release; sarcoplasmic reticulum; smooth muscle cells

Introduction

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (SR) (Goeger & Riley, 1989; Seidler *et al*., 1989). It has also been shown that Ca^{2+}-uptake by SR vesicles prepared from skeletal muscle is inhibited by CPA (Goeger *et al*., 1988). Very recently, effects of CPA on SR Ca^{2+}-ATPase activity in skinned fast-twitch skeletal muscle have been studied extensively (Kurebayashi & Ogawa, 1991). The possibility that CPA reduces Ca^{2+}-uptake by SR has also been suggested in intact aortic smooth muscle (Deng & Kwan, 1991). Direct evidence for the inhibition of ATP-dependent Ca^{2+}-uptake into SR by CPA and subsequent failure of Ca^{2+} release has also been obtained in skinned intestinal smooth muscle preparation (Uyama *et al*., 1992). The effects of CPA on electrical activity or membrane ionic currents are, however, totally unknown in any type of cell.

In several types of smooth muscle cells, the membrane ionic currents are regulated by intracellular Ca^{2+} metabolism. For instance, the Ca^{2+}-dependent K^+ current (I_{K-Ca}) in ileal smooth muscle of the rabbit is activated by an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) via influx of Ca^{2+} and subsequent Ca^{2+}-induced Ca^{2+} release from storage sites, thought to be SR (Ohya *et al*., 1987; Sakai *et al*., 1988). It is also known in various smooth muscle cells that agents (e.g. caffeine) which induce pronounced Ca^{2+} release from intracellular storage sites, first transiently enhance I_{K-Ca} and thereafter significantly reduce this current (Bolton & Lim, 1989; Cole *et al*., 1989; Imaizumi *et al*., 1989; Komori & Bolton, 1990). It is therefore expected that inhibition of the Ca^{2+}-pump in SR will change the amount of stored Ca, and result in changes in I_{K-Ca}.

Since I_{K-Ca} is the largest outward current in many types of smooth muscle cells (Watanabe *et al*., 1989), it may be useful as an assay of Ca storage available for Ca^{2+}-induced Ca^{2+} release. The possibility that the Ca^{2+}-pump activity in intracellular Ca storage sites can indirectly regulate smooth muscle membrane excitability, has not been examined previously, because a selective inhibitor of Ca-ATPase in SR was not known. The present study was undertaken to examine the effects of CPA on I_{K-Ca} and selectivity for the current against other currents.

Methods

Smooth muscle cells were isolated from ileum and urinary bladder of the guinea-pig. Cell isolation by enzymatic digestion and recording of membrane ionic currents using whole cell patch-clamp (Hamill *et al*., 1981) were performed as described previously (Imaizumi *et al*., 1989; Watanabe *et al*., 1989). The resistance of the pipette was between 2 and 5 MΩ

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for whole cell-clamp and between 7 and 15 MΩ for the patch-clamp. The seal resistance formed between cell membrane and tip under cell-attached configuration was between 10 and 50 GΩ. The composition of the solution filling the pipette (pipette solution) during recording of Ca^{2+}-dependent K^+ currents ($I_{K\text{-}Ca}$) under whole cell voltage-clamp was (in mM): K^+ 140, Na^+ 10, Mg^{2+} 4, Cl^- 148, adenosine 5′-triphosphate (ATP) 5.0, ethyleneglycol-*bis*-(β amino-ethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) 1.78, Ca^{2+} 0.5, HEPES 10. The pCa was approximately 7.5, when the pH was adjusted to 7.2 by KOH. When spontaneous or caffeine-induced transient outward currents were recorded, the concentration of EGTA and Ca^{2+} in the pipette solution was reduced to 0.05 mM and 0 mM, respectively. To record the voltage-dependent Ca current (I_{Ca}), CsCl was substituted for KCl and the EGTA concentration was increased to 5 mM in the pipette solution to block K^+ currents. The composition of the standard bathing solution was (in mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 149.7, glucose 14, HEPES 10. The pH was adjusted to 7.2 with NaOH.

Single Ca^{2+}-dependent K^+ channel currents were recorded mainly in the inside-out configuration but in a few outside-out patches as well (Hamill *et al.*, 1981). The pipette solutions used during inside-out and outside-out recordings were the standard bathing solution and the pipette solution for whole cell recordings of macroscopic $I_{K\text{-}Ca}$, respectively. The bathing solution facing the intracellular site of the excised patch membrane during inside-out recordings was the standard pipette solution in which ATP was omitted and the pCa was regulated. The pCa of these solutions was adjusted to a required value with Ca-EGTA buffer (0.5 mM Ca^{2+} and adequate concentrations of EGTA) according to the methods used by Benham *et al.* (1986). Data recording under whole cell voltage-clamp and the analysis were performed using programmes kindly provided by Dr Wayne Giles (Department of Medical Physiology, University of Calgary) in the same manner as described previously (Imaizumi *et al.*, 1990). The holding current level at −50 mV under whole cell voltage-clamp was taken as the zero current level since resting membrane potentials were around −50 mV and the resting conductance was very low (input resistance of over 2 GΩ) in these cells. In some experiments, leakage current was corrected for. A leak was extrapolated assuming a linear relationship between voltage and current obtained in the range of −80 and −50 mV and subtracted from the total current. Part of the analysis of single channel current was performed with a programme developed by Dr John Dempster (Department of Physiology and Pharmacology, University of Strathclyde). Experiments were carried out at 29 ± 1°C. Pooled data were expressed as mean ± s.e.mean. Statistical significance was examined by Student's *t* test.

Results

When single smooth muscle cells obtained from urinary bladder and ileum were depolarized for 150 ms from a holding potential of −50 to 0 mV once every 15 s, an inward current followed by transient and sustained components of outward currents were recorded (Figure 1A and B, cells from urinary bladder and ileum, respectively). The initial inward current was a time- and voltage-dependent Ca^{2+} current (I_{Ca}) as has been found in cells from rabbit intestine (Ohya *et al.*, 1986) and guinea-pig urinary bladder (Klöckner & Isenberg, 1985). The transient outward current was mainly $I_{K\text{-}Ca}$ which was activated by an increase in $[Ca^{2+}]_i$ since this current as well as I_{Ca} was suppressed by 0.3 mM Cd^{2+} (not shown, $n = 3$). As has been previously reported in various smooth muscle cells (Ohya *et al.*, 1987; Sakai *et al.*, 1988; Bolton & Lim, 1989; Cole & Sanders, 1989; Imaizumi *et al.*, 1989; Bielefeld *et al.*, 1990), the $I_{K\text{-}Ca}$ was strongly inhibited by the external application of 5 mM caffeine, 10 μM ryanodine or 2 mM tetraethylammonium (TEA), or the addition of 5 mM EGTA to the pipette solution (not shown, $n > 2$ for each).

After the application of 5 μM CPA, the peak amplitude of $I_{K\text{-}Ca}$ in urinary bladder cells decreased gradually after approximately a 1 min delay and reached a steady level within 4 min (Figure 1A). The decrease in the peak $I_{K\text{-}Ca}$ amplitude was occasionally preceded by transient enhancement of the currents by 10–30% when a low concentration (~1 μM) of CPA was applied. The decreased $I_{K\text{-}Ca}$ recovered by more than 70% after washout of CPA when concentrations lower than 10 μM were applied. In urinary bladder cells, the outward current at the end of 150 ms pulse was reduced substantially as well as the peak outward current (see Figure 1A). The decrease in $I_{K\text{-}Ca}$ was observed at potentials positive to −10 mV (Figure 1B, (a–d), ileal cells). The current-voltage relationships obtained in ileal cells showed that 3 μM CPA decreased markedly the peak outward current but had much less effect on the sustained current measured at the end of the pulse (Figure 1B). Figure 1C denotes the relationship between the CPA concentration and the peak amplitude of outward current upon depolarization to 0 mV after the application of CPA. The amplitude of the outward current is shown as a relative value (%) of that before the application of CPA. The relationship indicates that CPA reduced the peak amplitude of the outward current in a concentration-dependent manner. Maximum decrease of peak outward current was obtained by the application of 10 or 30 μM CPA. The concentration of CPA required for a 50% decrease in the peak outward current was approximately 3 μM in ileal cells under these experimental conditions. Although an approximate 60% decrease in the peak amplitude of the outward current was obtained after application of 10 or 30 μM CPA, the transient component of the outward current was almost completely abolished and only the sustained component remained as shown in the right panel in Figure 1C. A small part of the sustained component of outward current was $I_{K\text{-}Ca}$ in ileal cells since addition of 2 mM TEA reduced this current at the end of the pulse by only about 10–20% ($n = 4$). Therefore, assuming that the major component of the sustained current is not $I_{K\text{-}Ca}$, the concentration of CPA for the half maximal inhibition of the transient component of outward current is lower than 3 μM, and from Figure 1C, is approximately 0.5 μM. A similar decrease in $I_{K\text{-}Ca}$ following the application of CPA was observed in single smooth muscle cells isolated from urinary bladder (34 and 66% decrease in peak outward current by 1 and 5 μM CPA, respectively, $n = 3$ for each).

When $I_{K\text{-}Ca}$ was inhibited by addition of 0.3 mM Cd^{2+} and 5 mM EGTA to the bathing and pipette solutions, respectively, smaller outward currents which were characterized by slower onset times and a reduced TEA-sensitivity (>5 mM required to block) remained in both types of cells; these are delayed rectifier type K^+ currents ($I_{K\text{-}D}$) (Klöckner & Isenberg, 1985; Terada *et al.*, 1987; Cole & Sanders, 1989). The application of 10 μM CPA did not affect $I_{K\text{-}D}$ upon depolarization to +30 mV (Figure 2A(a), ileal cell). The peak amplitude of $I_{K\text{-}D}$ after application of 10 μM CPA was 99.5 ± 4.3% of that before application ($n = 4$, $P > 0.05$ vs. before CPA application). Figure 2A(b) shows calcium current (I_{Ca}) which was activated by depolarization from −50 to 0 mV in an ileal cell where K^+ currents were blocked using a pipette solution containing mainly CsCl. The I_{Ca} was not affected significantly by 10 μM CPA in ileal cells (93.3 ± 4.2% of that before application, $n = 3$, $P > 0.05$ vs. before application). Summarized data for the lack of effect of 10 μM CPA on $I_{K\text{-}D}$ and I_{Ca} in ileal cells at several potentials are shown as current-voltage relationships in Figure 2B. $I_{K\text{-}D}$ ($n = 3$) and I_{Ca} ($n = 2$) recorded in urinary bladder cells under the same conditions were also unaffected by 10 μM CPA. Taken together, these results indicate that CPA selectively reduces $I_{K\text{-}Ca}$.

The effects of CPA on single channel activity of Ca^{2+}-dependent K^+ channels which have a large conductance (maxi-K^+ channel) (reviewed by Latorre *et al.*, 1989) were

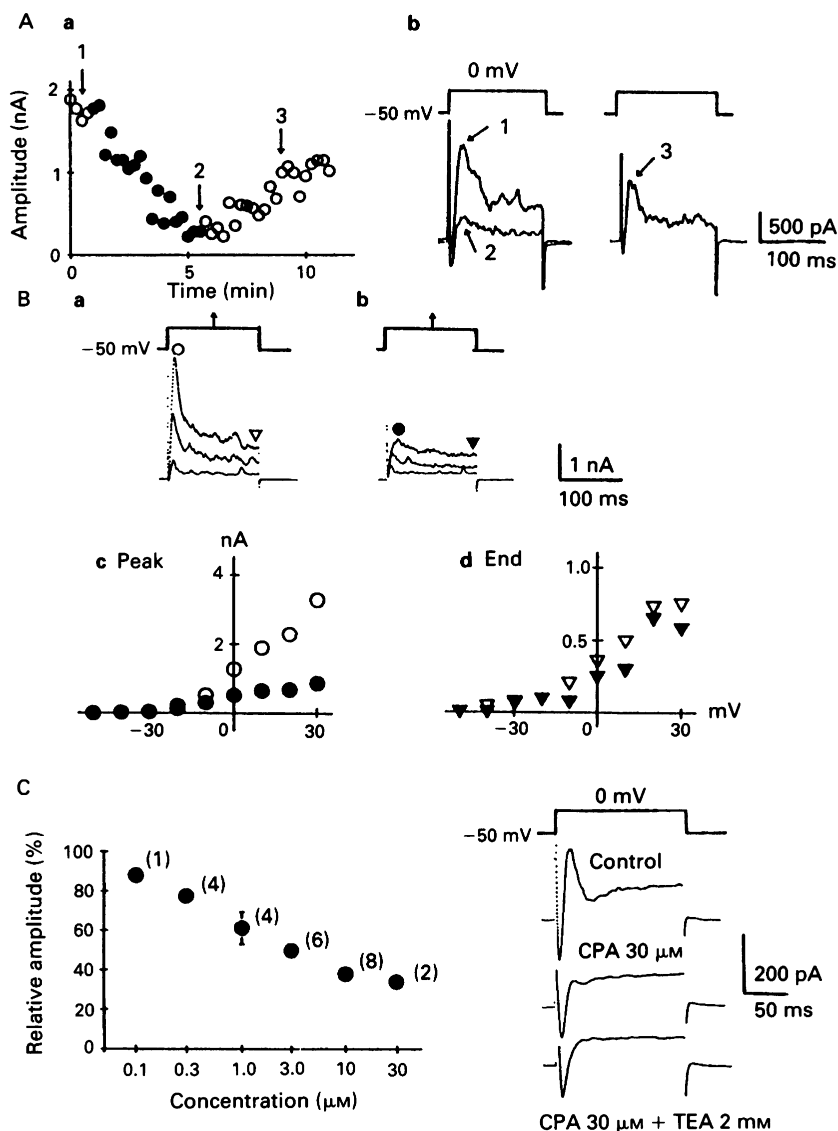

Figure 1 Effects of cyclopiazonic acid (CPA) on outward currents in smooth muscle cells from urinary bladder (A) and ileum (B and C). (A) The cell was depolarized for 150 ms from the holding potential of − 50 mV to 0 mV once every 15 s. (a) The peak amplitude of outward currents was measured and plotted against time: currents measured in the absence (○) or presence (●) of 5 μM CPA. Current recordings at times indicated by numbers in (a) correspond to those shown in (b). (1) before the application of CPA; (2) approximately 5 min after the addition of CPA; (3) 3 min after washout of CPA. Note that the effect of 5 μM CPA on the current was reversed by ~70% after washout. (B) The cell was depolarized from the holding potential of − 50 mV to potentials over the range, − 40 to + 30 mV by 10 mV steps. In (a) and (b), currents recorded at − 10, + 10 and + 30 mV are superimposed. In (a), (b) and (c) circles indicate the peak outward currents or their amplitude. Triangles in (a), (b) and (d) indicate the currents at the end of 150 ms pulse or their amplitude. Open and closed symbols indicate the currents in the absence and presence of 3 μM CPA, respectively. Note that CPA reduced the peak outward current, but not the sustained current at the end of the pulse. (C) Concentration-response curve of CPA for inhibition of $I_{K\text{-}Ca}$ in ileal cells. Experiments were performed in the same manner as shown in (A). Peak outward current upon depolarization to 0 mV was measured from the holding current level at − 50 mV and normalized as a percentage of that measured before the application of CPA. Mean values and s.e.mean are shown as circles and vertical bars, respectively. The number of observations were 1, 4, 4, 6, 8 and 2 for 0.1, 0.3, 1, 3, 10 and 30 μM CPA, respectively. The 50% decrease in peak outward current was obtained by application of approximately 3 μM CPA. The right-hand panel shows that the transient component of the outward current was almost completely abolished by application of 30 μM CPA. The sustained component of the outward current remained in the presence of 30 μM CPA and was affected little by the addition of 2 mM TEA.

examined by the inside-out patch configuration. All patches examined (ileum, $n = 25$; urinary bladder, $n = 10$) included maxi-K^+ channels and most patches contained more than three channels, as expected from the fact that $I_{K\text{-}Ca}$ is the predominant K^+ current in smooth muscle cells from ileum and urinary bladder. Figure 3A shows unitary current events and their corresponding amplitude histograms in an ileal cell due to the activity of a maxi-K channel at a holding potential of 0 mV and pCa 6.0. The open state probability of the channel was highly dependent upon pCa in the range between 7.5 and 5.0 (not shown). CPA (10 μM) applied to the bathing solution affected neither the unitary current amplitude nor the channel activity (Figure 3A(b)). For example, the open state probability of the channel under these conditions (0 mV, pCa 6.0) was not changed significantly by the application of 10 μM CPA (control: 071 ± 0.09; 10 μM CPA: 0.69 ± 0.10, $n = 5$, $P > 0.05$). Figure 3B(a) and (b) shows unitary currents at holding potentials of − 20 and 0 mV in the absence and presence of 10 μM CPA, respectively. When the pipette and bathing solutions contained 5.9 and 140 mM KCl, respectively, the averaged single channel conductance measured in the voltage-range between − 20 and + 20 mV

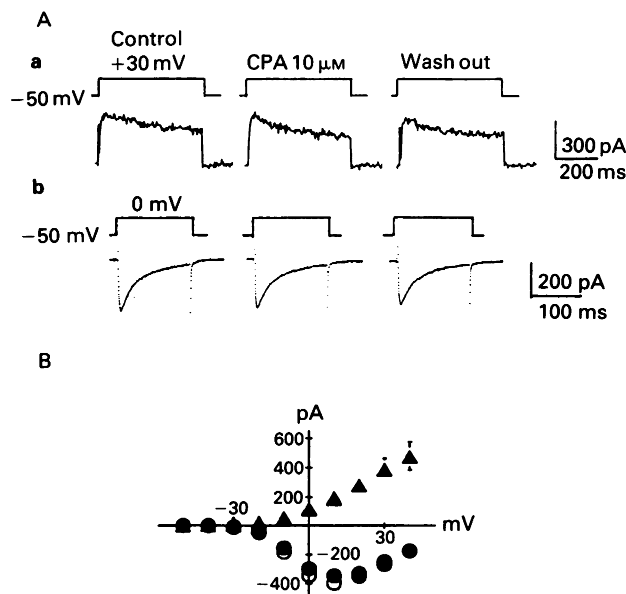

Figure 2 Effects of 10 μM cyclopiazonic acid (CPA) on the delayed rectifier K current ($I_{K\text{-}D}$) and Ca current (I_{Ca}) in ileal smooth muscle cells. (A) The three traces from the left to the right in (a) and (b) are recordings before, during the presence, and after washout of 10 μM CPA, respectively. (a) $I_{K\text{-}D}$ was elicited by depolarization from − 50 mV to + 30 mV. $I_{K\text{-}Ca}$ and I_{Ca} were blocked by simultaneous addition of 5 mM EGTA and 0.3 mM Cd^{2+} to the pipette and bathing solutions, respectively. Leak currents were subtracted on the computer. (b) I_{Ca} was elicited by depolarization from − 50 mV to 0 mV. K^+ currents were blocked by replacement of K^+ in the pipete solution with equimolar Cs^+. (B) Summarized data of the effects of 10 μM CPA on the current-voltage relationships of averaged peak $I_{K\text{-}D}$ (triangles, $n = 4$) and I_{Ca} (circles, $n = 2$). Note that 10 μM CPA affected neither $I_{K\text{-}D}$ nor I_{Ca}. (○,△) Control; (●,▲) CPA 10 μM.

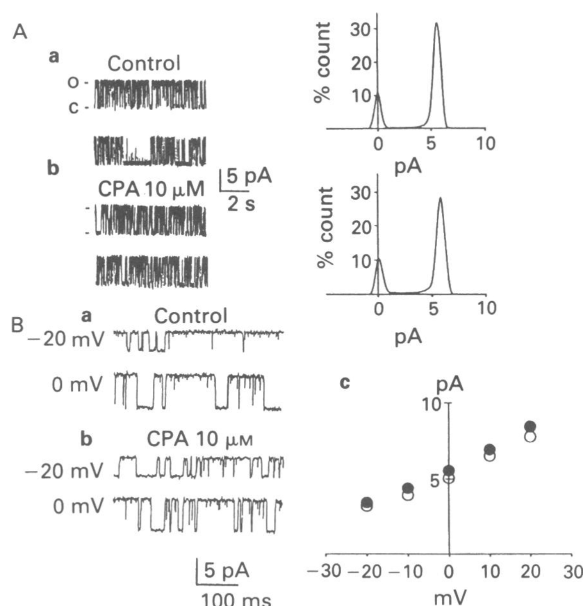

Figure 3 Effects of addition of 10 μM cyclopiazonic acid (CPA) to bathing solution on single Ca^{2+}-dependent K^+ channel current activities under the inside-out patch recording configuration in ileal cells. (A) Holding potential and pCa of the bathing solution were 0 mV and 6.0, respectively. Unitary current was 5.4 pA in the control (a) and was not affected by application of 10 μM CPA (b). Corresponding histograms of open events versus amplitude of unitary current are shown in the right panels, respectively. The ordinate scale expresses the relative time (%) spent at the corresponding amplitude in each bin (0.195 pA) versus the total recording time; 8.2 s in (a) and (b). In this particular patch, only one Ca^{2+}-dependent K^+ channel was active. The open state probability was 0.78 in (a) and 0.76 in (b), respectively. (B) Effects of 10 μM CPA on single channel conductance were examined by measuring currents at five different holding potentials (c). Recordings at − 20 and 0 mV are shown in (a) and (b). The pCa of the bathing solution was 7.5. Averaged unitary current amplitude in the absence (○) or presence of 10 μM CPA (●) ($n = 5$ for each) was plotted against potentials as current-voltage relationships in (c). S.e.means are included in the symbols. The conductance of the channel was 120 and 122 pS in the absence and presence of 10 μM CPA, respectively, and these are not significantly different ($P > 0.05$).

was 118.1 ± 3.7 pS ($n = 5$) and 121.5 ± 4.8 pS ($n = 5$, $P > 0.05$) in the absence and presence of 120 μM CPA, respectively (Figure 3B(c)). Although the effects of CPA on channel activity were not examined systematically in the outside-out patch configuration, preliminary results indicate that 10 μM CPA added to the bathing solution had no apparent effect on either unitary current amplitude or open state probability (0 mV, pCa 6.0, urinary bladder cells, $n = 2$). We concluded that under these conditions 10 μM CPA does not affect the activity of maxi-K^+ channels directly.

Caffeine enhances Ca^{2+}-release from SR and thereby increases outward currents due to activation of maxi-K^+ channel in various smooth muscle cells (Benham & Bolton, 1986; Désilets *et al.*, 1989; Imaizumi *et al.*, 1989). The Ca^{2+}-release and activation of outward currents by caffeine are transient especially in Ca^{2+}-free bathing solution, because the Ca^{2+} in the storage sites may be exhausted in a short time. Therefore, caffeine-induced transient outward currents are a convenient tool for the investigation of intracellular Ca^{2+} mobilization. The effects of CPA on Ca^{2+}-uptake by intracellular storage sites which are susceptible to caffeine were examined in these smooth muscle cells. In various types of smooth muscle cells including those of urinary bladder and ileum, caffeine-induced outward current is often observed as a transient burst of enhanced spontaneous transient outward currents (STOCs) which occur due to spontaneous Ca^{2+} release from SR in a normal solution (Benham & Bolton, 1986; Bolton & Lim, 1989; Sakei *et al.*, 1989). Therefore, effects of CPA on STOCs in the normal solution were first examined in urinary bladder cells (Figure 4A). When urinary bladder cells were clamped at − 50 mV, STOCs which had amplitude (> 25 pA) apparently larger than the noise of the recording system (~ 15 pA) were recorded in about 50% of cells examined ($n = 20$), whereas the amplitude and frequency of STOCs varied widely from cell to cell and also changed during a recording lasting for longer than several minutes. Application of 1 or 3 μM CPA for ~ 4 min did not affect STOCs (Figure 4A(a)), while higher concentrations of CPA (> 10 μM) increased the amplitude and/or frequency of STOCs for a few minutes and then tended to reduce them (Figure 4A(a)). Figure 4A(b) illustrates the summarized data regarding the effects of 3 and 10 μM CPA on STOCs. STOCs recorded for a period of 1 min after approximately 2.5 min following the application of 3 or 10 μM CPA, respectively, were integrated and expressed as a relative area versus that measured for STOCs 1 min before the application of CPA. Although 3 μM CPA did not affect STOCs significantly (109.4 ± 8.9%, $n = 5$, $P > 0.05$ vs. control), 10 μM CPA increased STOCs during this period (171.0 ± 19.6%, $n = 3$, $P < 0.05$ vs. control).

The upper trace in Figure 4B illustrates the protocol for examination of the effect of CPA on Ca^{2+}-uptake into the intracellular storage sites which are susceptible to caffeine. Caffeine was applied three times during exposure of the cell to the Ca^{2+}-free solution. Application of 5 mM caffeine induced a burst of transient outward currents for a few minutes in the Ca^{2+} free solution in cells from urinary bladder (Figure 4B(a)) and ileum (not shown) at a holding potential of − 50 mV. The caffeine-induced transient outward currents in these cells are mainly due to maxi-K^+ channel activation since 3 mM TEA almost completely

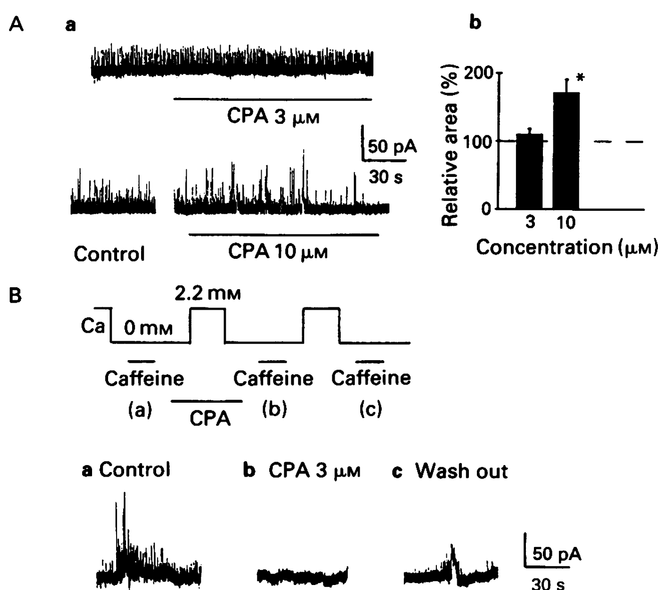

Figure 4 Effects of 3 μM cyclopiazonic acid (CPA) on spontaneous transient outward currents (STOCs) (A) and caffeine-induced transient outward currents (B) in urinary bladder cells. (A) Effects of 3 μM or 10 μM (a, upper and lower traces, respectively) CPA on STOCs were examined at holding potential of − 50 mV. Outward currents which were recorded for 1 min from just before and 2 min or 30 s after application of 3 or 10 μM CPA, respectively, were integrated from the mean level of the basal noise. Relative area (ordinate scale in (b)) indicates the integrated current after the application of CPA versus that before the application. Number of cells used was 5 and 3 for 3 and 10 μM CPA, respectively. $^*P < 0.05$ vs. 100%. (B) Upper trace; protocol of the experiment. After Ca^{2+}-loading of the cell in a normal solution containing 2.2 mM Ca^{2+}, 5 mM caffeine was applied in a Ca^{2+}-free solution. This procedure was repeated three times (a, b and c). In the second trial, Ca^{2+}-loading was performed in the presence of 3 μM CPA. CPA was withdrawn before the subsequent application of caffeine. Lower panel; (a), (b) and (c) show current recordings obtained as indicated correspondingly in the protocol. Ca^{2+}-loading was performed in the absence ((a) and (c)) and presence of 3 μM CPA (b). Note that caffeine-induced outward current was not observed in (b) but partly recovered in (c).

abolished them (not shown). This has been demonstrated in other smooth muscle cells (Benham & Bolton, 1986; Ohya et al., 1987). Small phasic inward currents occasionally remained after application of 3 mM TEA (not shown). In the second trial (Figure 4B(b)), Ca^{2+}-loading in a solution containing 2.2 mM Ca^{2+} was performed in the prescence of 3 μM CPA. Following this procedure, application of caffeine did not induce transient outward currents. In the third trial (Figure 4B(c)) which was performed under the same conditions as those used in the first trial, the caffeine-induced response recovered at least in part. Similar results were obtained in all cells examined (three urinary bladder cells and two ileal cells).

Discussion

Smooth muscle cells obtained from ileum and urinary bladder exhibited similar outward currents upon depolarization as has been reported previously (Klöckner & Isenberg, 1985; Ohya et al., 1986; Terada et al., 1987). Although the $I_{K\text{-}Ca}$ component of outward currents appeared to be larger and longer-lasting in cells from urinary bladder than those from ileum, the mechanism for activation of $I_{K\text{-}Ca}$ may be the same. The $I_{K\text{-}Ca}$ is activated upon depolarization by an increase in $[Ca^{2+}]_i$ via Ca^{2+}-induced Ca^{2+} release from SR, which is triggered by the Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Ohya et al., 1987; Sakai et al., 198; Kitamura et al., 1989; Cole & Sanders, 1989). The delayed rectifier type outward currents which remained after the inhibition of $I_{K\text{-}Ca}$ in these cells had similar characteristics; they were reduced by higher concentration of TEA (> 3 mM) but were not affected by 4-aminopyridine (Klöckner & Isenberg, 1985; Ohya et al., 1986).

The present results clearly show that CPA specifically affects $I_{K\text{-}Ca}$. The CPA concentration for the half inhibition of the peak outward current was ~3 μM. I_{Ca} and $I_{K\text{-}D}$ were not affected by 10 μM CPA. It is known that $I_{K\text{-}Ca}$ in smooth muscle cells is almost completely due to Ca^{2+}-dependent K^+ channels which have a very large conductance (so called 'maxi-K^+' or BK channel) (Inoue et al., 1985; Benham et al., 1986; Latorre et al., 1989) and are blocked by relatively low concentrations of TEA (< 2 mM) (Inoue et al., 1985; Imaizumi et al., 1990) or charybdotoxin from outside the cell membrane (Green et al., 1991). Neither the single channel conductance nor the open probability of maxi K^+ channels in smooth muscle cells from ileum and urinary bladder are affected by 10 μM CPA. The activity of at least two other membrane currents. Ca^{2+}-dependent chloride (Pacaud et al., 1989; Amédée et al., 1990) and non-specific cationic channel currents (Loirand et al., 1991), strongly depends upon intracellular Ca^{2+} concentration as well as $I_{K\text{-}Ca}$. Both currents have reversal potentials at around 0 mV under the conditions used in the present study. Therefore, the contribution of these currents to the total membrane current may be minimized by measuring currents at 0 mV. Therefore, it is concluded that CPA specifically, but indirectly reduces maxi K^+ channel activities upon depolarization in these cells under these conditions.

The suppression of $I_{K\text{-}Ca}$ by CPA indicates an additional important characteristic of CPA; this substance is effective in intact smooth muscle cells and can be washed out. Recovery of Ca^{2+}-uptake from CPA-induced inhibition has been observed in skinned smooth muscle fibres (Uyama et al., 1992) but not clearly shown in intact tissues (Deng & Kwan, 1991; Bourreau et al., 1991). In a preliminary study, such a reversible decrease in $I_{K\text{-}Ca}$ by CPA was observed also in smooth muscle cells isolated from trachea, ureter, taenia caeci and vas deferens (Suzuki, Muraki, Imaizumi & Watanabe, unpublished observation), as well as ileum and urinary bladder. Therefore, this may be a common effect of CPA on smooth muscle cells.

Since the activtion of $I_{K\text{-}Ca}$ includes Ca^{2+}-induced Ca^{2+} release processes, the amount of Ca in storage sites may affect indirectly the $I_{K\text{-}Ca}$ amplitude and/or kinetics. It is well established that $I_{K\text{-}Ca}$ is transiently potentiated and then markedly suppressed by caffeine which enhances Ca^{2+}-release and thereby depletes stored Ca in SR (Benham & Bolton, 1986; Bolton & Lim, 1989; Imaizumi et al., 1989; Bielefeld et al., 1990; Sims et al., 1990). It has also been reported that an intracellular Ca^{2+} mobilization elicited by agonists via IP_3-induced Ca^{2+} release from storage sites also increases $I_{K\text{-}Ca}$ transiently and then markedly reduces it (Cole et al., 1989; Bolton & Lim, 1989; Sims et al., 1990; Komori & Bolton, 1990). This agonist-induced suppression of $I_{K\text{-}Ca}$ can be blocked by intracellular application of low molecular weight heparin which inhibits IP_3 binding to the site on SR (Ganitkevich & Isenberg, 1990; Komori & Bolton, 1991; Takeda et al., 1991). Exhaustion of releasable Ca from storage sites by Ca^{2+}-induced or IP_3-induced Ca^{2+} release has been postulated for the suppression of $I_{K\text{-}Ca}$. The suppression of $I_{K\text{-}Ca}$ by CPA in the present study mimicked that induced by caffeine or agonists. Therefore, the mechanism of CPA-induced inhibition of $I_{K\text{-}Ca}$ may be correlated with those for caffeine or agonists. After the application of CPA at a low concentration (1−3 μM), a potentiation of $I_{K\text{-}Ca}$ upon depolarization was occasionally observed prior to the CPA-induced suppression. Since the amplitude of $I_{K\text{-}Ca}$ was also markedly reduced by an increase in frequency of the depolarizing pulse, the pulse was applied at a low rate (once

every 15 s) in order to observe stable and large $I_{K\text{-}Ca}$s. Therefore, this may be part of the reason why the potentiation of $I_{K\text{-}Ca}$ by CPA prior to the suppression was not always observed.

In the present study, it is also shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Iino, 1989) was almost completely abolished by treatment with 3 μM CPA during the preceding Ca^{2+}-load. This finding is consistent with the results we showed in skinned smooth muscle fibres of the longitudinal layer of the guinea-pig ileum, where the IC_{50} of CPA for inhibition of Ca^{2+}-uptake was approximately 0.6 μM (Uyama *et al.*, 1992). Based upon these results, it can be suggested that CPA easily penetrates into the cytoplasm through the plasma membrane, reduces Ca^{2+}-ATPase activity in SR/ER and can be washed away, at least in part. The mechanism may be the following; CPA decreases Ca^{2+}-uptake by the inhibition of Ca^{2+}-pump in the SR/ER, decreases the subsequent Ca^{2+} release from those storage sites and, thereby, reduces $I_{K\text{-}Ca}$.

The current elicited by caffeine at a holding potential of − 50 mV may include the Ca^{2+}-dependent Cl^- (Pacaud *et al.*, 1989; Amedee *et al.*, 1990) and non-specific cationic channel currents (Loirand *et al.*, 1991; Wang & Large, 1991). When the holding potential was less negative (e.g. − 30 mV), caffeine-induced outward currents were larger (not shown). The less negative holding potential, however, also enhanced STOCs and appeared to potentiate the CPA-induced effects of STOCs, resulting in difficulty in assessing effects of CPA on Ca^{2+}-uptake susceptible to caffeine. Effects of CPA on caffeine-induced Cl^- or non-specific cationic current were not examined systematically in the present study.

Since $I_{K\text{-}Ca}$ is responsible for the action potential repolarization and the afterhyperpolarization (Watanabe *et al.*, 1989), it is likely that Ca^{2+}-pump activity in SR may indirectly, but significantly, affect action potential shape and frequency of action potential firing in various types of smooth muscles. This may have an especially important role in gastro-intestinal smooth muscle, since $I_{K\text{-}Ca}$ also regulates the repolarization phase of slow wave potentials (Cole & Sanders, 1989; Carl *et al.*, 1990; Vogalis *et al.*, 1991). The effects of CPA on intestinal motor activity have not been examined systematically in the present study, although CPA does increase spontaneous contractions of intact ileal longitudinal strips (data not shown). Moreover, effects of CPA on STOCs were not examined systematically since amplitude and frequency of STOCs at the holding potential of − 50 mV varied so widely from cell to cell and also changed during a recording lasting for more than several minutes. STOCs, however, appeared to be affected at relatively higher concentrations of CPA (> 10 μM) or possibly after prolonged exposure to CPA. It may be worth examining whether Ca^{2+}-pump activity may control resting membrane potential via regulation of STOCs.

In conclusion, CPA, an inhibitor of the Ca^{2+}-pump in intracellular Ca storage sites (mainly SR), selectively and reversibly reduces $I_{K\text{-}Ca}$ without any direct block of the K^+ channel, in ileal and urinary bladder smooth muscle cells. Therefore, it is an extremely valuable pharmacological tool for investigation of the Ca^{2+}-pumping mechanism and intracellular Ca^{2+} homeostatis. This is the first report that Ca^{2+}-pump activity in SR/ER may indirectly regulate $I_{K\text{-}Ca}$ and thereby membrane excitability.

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