Effects of cyclopiazonic acid, a novel Ca^{2+} -ATPase inhibitor, on contractile responses in skinned ileal smooth muscle

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1 Effects of cyclopiazonic acid (CPA), a specific inhibitor of the Ca^{2+} -ATPase in sarcoplasmic reticulum (SR) of skeletal and cardiac muscles, on contractile responses induced by Ca^{2+} -release from intracellular storage sites were examined in the longitudinal smooth muscle strip of the guinea-pig ileum skinned with β -escin.

2 Ca^{2+} -loading of storage sites (Ca^{2+} -uptake) was performed in pCa 6.3 solution. The amount of Ca^{2+} taken up was monitored by use of the amplitude of contraction following application of 25 mM caffeine or 25 μM inositol 1,4,5-trisphosphate (IP_3).

3 Contractile responses to caffeine or IP_3 were reduced or abolished when the preceding Ca^{2+} -uptake was performed in the presence of 0.1–10 μM CPA. The dose of CPA required to inhibit the contraction induced by caffeine or IP_3 by 50% was approximately 0.6 μM . The CPA-sensitive Ca^{2+} -uptake completely depended upon the presence of ATP in the solution during Ca^{2+} -uptake.

4 When 1 μM CPA was added after Ca^{2+} -uptake, the subsequent caffeine- or IP_3 -induced contraction was not significantly affected by the presence of CPA.

5 Acetylcholine-induced contraction was also almost abolished when the preceding Ca^{2+} -uptake was performed in the presence of 10 μM CPA.

6 The relationship between pCa and contraction was not affected by the presence of 10 μM CPA in skinned fibres where Ca^{2+} storage sites had been destroyed by treatment with A23187. The enhancement of contraction in pCa 6.0 solution by calmodulin was not affected by 10 μM CPA.

7 These results suggest that CPA selectively inhibits ATP-dependent Ca^{2+} -uptake into intracellular storage sites in skinned ileal smooth muscle strips. CPA appears to be a potent, reversible, and very specific inhibitor of the Ca^{2+} -pump in the storage sites of smooth muscle, and is an extremely valuable pharmacological tool.

Keywords: Cyclopiazonic acid; skinned fibre; smooth muscle; ileum; Ca^{2+} -pump; sarcoplasmic reticulum; Ca^{2+} -ATPase; caffeine; inositol 1,4,5-trisphosphate; β -escin

Introduction

A specific inhibitor of Ca^{2+} -ATPase activity in sarcoplasmic reticulum (SR) of muscle cells is a much needed pharmacological tool for investigation of the mechanism(s) and kinetics of SR Ca^{2+} -uptake and -storage. Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+} -ATPase in skeletal muscle SR (Goeger & Riley, 1989; Seidler *et al.*, 1989). It does not affect the activities of kidney and brain Na^+ , K^+ -ATPase, gastric H^+ , K^+ -ATPase, mitochondrial F_1 -ATPase, Ca^{2+} -ATPase of erythrocytes or Mg^{2+} -activated ATPase of T-tubules and plasma membrane of rat skeletal muscle. It has also been shown that Ca^{2+} -uptake by SR vesicles prepared from skeletal muscle is inhibited by CPA (Goeger *et al.*, 1988). Recently, the effects of CPA on Ca^{2+} -ATPase in skinned fast-twitch skeletal muscle have been studied (Kurebayashi & Ogawa, 1991). Direct evidence for the inhibition of ATP-dependent Ca^{2+} -uptake into SR by CPA and subsequent failure of Ca^{2+} -release has not been obtained in any skinned muscular preparations, although this possibility has been suggested in intact aortic (Deng & Kwan, 1991) and tracheal smooth muscles (Bourreau *et al.*, 1991). Evidence should be obtained in skinned smooth muscle preparations, where the functions of the sarcolemmal Ca-pump, Na-Ca exchange system and ionic channels can be ignored and thereby Ca^{2+} -mobilization is greatly simplified. The dependence of Ca^{2+} -uptake on ATP can also be directly examined.

The present study was undertaken to evaluate the specificity of the CPA-effects on Ca^{2+} loading into storage sites in chemically skinned ileal smooth muscle fibres. The amount of Ca^{2+} -uptake into these storage sites was monitored by evoked contraction following application of caffeine (Iino, 1989) or IP_3 (Somlyo *et al.*, 1985; Suematsu *et al.*, 1984), procedures which release Ca^{2+} from SR and/or ER by separate mechanisms. The effects of CPA on Ca^{2+} -uptake into caffeine- and IP_3 -sensitive storage sites were compared, since heterogeneous Ca^{2+} storage sites with regard to sensitivity to these Ca^{2+} releasing substances have been described (Iino *et al.*, 1988). The direct effects of CPA on the Ca^{2+} -sensitivity of contractile proteins and the antagonistic effect of CPA on calmodulin have also been examined after Ca^{2+} storage function of SR had been destroyed by A23187 (Itoh *et al.*, 1991).

Methods

Male Hartley guinea-pigs weighing about 200 g were killed by a blow on the head. Thin strips 1.5–2.0 mm in length, 150–200 μm in width and less than 100 μm in thickness were prepared from the longitudinal muscle layer of ileum. Each end of the muscle strip was tied to a fine thread of about 10 μm in diameter. The strip was placed horizontally in an organ bath which had a volume of approximately 300 μl . The thread at each end of the strip was connected to a thin pin in the silicon rubber bottom of the bath and to a lever of an isometric force transducer, respectively. The contractile re-

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sponse of the intact strip to high K⁺ solution was measured two or three times before skinning. The composition of the standard physiological salt solution was: (in mM) NaCl 125.0, KCl 4.7, CaCl₂ 2.0, MgCl₂ 1.2, glucose 10.0, tris(hydroxymethyl) aminomethane (Tris) 20.0. The pH was adjusted to 7.5 with HCl. High K⁺ solution was prepared by replacing 125 mM NaCl with equimolar KCl. Before skinning, the strip was incubated for 25 min in a relaxing solution containing 2 mM ethyleneglycol-*bis*-(β -aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) (R2G, see below).

Skinning was performed by incubation in a solution of pCa 6.0 with 60 μ M β -escin (Kitazawa & Somlyo, 1990; Kobayashi *et al.*, 1989). The skinning process was monitored by a gradual increase in muscle tone, which reached a steady level after approximately 30 min. The solution was then changed to the relaxing solution (R2G). The composition of R2G was (in mM): K-propionate 130, MgCl₂ 4.0, adenosine triphosphate (ATP)-2Na 4.0, creatine phosphate 10, NaN₃ 10, Tris 20.0, EGTA 2.0. A solution containing 0.5 mM EGTA (R0.5G) was prepared by reducing the EGTA concentration in R2G from 2.0 to 0.5 mM. The contractile effects of caffeine and IP₃ were tested in R0.5G to avoid the strong Ca chelating effects of higher concentrations of EGTA. The approximate pCa of R0.5G was 7.6 ± 0.4 , assuming the contamination of Ca²⁺ to be 10–50 μ M. The pH was adjusted to 6.8 with maleic acid. Solutions of pCa 7.0, 6.0, 5.0 and 4.0 were prepared by using Ca²⁺-EGTA buffer of 3 mM EGTA and corresponding Ca²⁺. The required Ca²⁺-concentrations were calculated assuming the apparent binding constant of Ca-EGTA complex to be 1.0×10^6 M⁻¹ at pH 6.8 (Saida & Nonomura, 1978). The composition of this solution was the same as that of R2G except for the EGTA concentration. When the Ca sensitivity of the contractile proteins was studied in detail, the intracellular Ca storage sites were destroyed by treatment with 10 μ M A23187 (Itoh *et al.*, 1985) for about 20 min in R2G solution after skinning to prevent Ca²⁺-induced Ca²⁺-release. Experiments were performed at room temperature in the range between 21 and 24°C. Calmodulin was not added except when mentioned.

β -Escin (Lot. 107F0598), CPA (129F4013, 98F4037), calmodulin, and guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) were obtained from Sigma Ltd. ATP was from Oriental. Guanosine triphosphate (GTP) was from Yamasa. A23187 was from Calbiochem. Ltd. EGTA and IP₃ were from Dojin. Caffeine, Tris, Maleic acid and acetylcholine were from Wako Junyaku.

Pooled data are expressed as mean $\pm$ s.e.mean. Statistical significance was examined by Student's *t* test and was indicated by * or ** at the *P* level of <0.05 and <0.01, respectively.

Results

Figure 1 shows the effects of cyclopiazonic acid on contraction induced by 25 mM caffeine or 25 μ M IP₃ in ileal smooth muscle strips skinned with β -escin. After skinning, application of pCa 6.3 solution evoked a control contractile response (Figure 1A). The tension reached the peak within 5 min and gradually declined thereafter (Kitazawa & Somlyo, 1990). This procedure also resulted in loading of intracellular storage sites with Ca²⁺ (Ca²⁺-loading or Ca²⁺-uptake). Subsequent application of 25 mM caffeine in a relaxing solution (R0.5G) induced a transient contraction. A second application of 25 mM caffeine in R0.5G elicited either a very small or no response, confirming that most of the Ca²⁺ which had been loaded into storage sites in pCa 6.3 solution had been released by the first application of caffeine and that the released Ca²⁺ was not re-taken up into storage sites in R0.5G. Since the effect of caffeine appeared to be concentration-dependent in the range of 1 and 20 mM but varied widely between preparations, 25 mM caffeine was used to obtain a stable response. Without this Ca²⁺-loading, subse-

Figure 1 Effects of cyclopiazonic acid (CPA) on 25 mM caffeine- or 25 μ M inositol 1,4,5-trisphosphate (IP₃)-induced contraction in skinned smooth muscle strips of guinea-pig ileum. Skinned strips were contracted by changing a relaxing solution (R2G, see Methods) to pCa 6.3 solution as indicated in the line below (F). This was also a Ca²⁺ loading period. Caffeine or IP₃ was applied twice as indicated by bars in the R0.5G period. Note that the second response to caffeine or IP₃ in a paired application was very small. Ca²⁺ loading at pCa 6.3 and subsequent Ca release by caffeine or IP₃ were repeated three times (a, b and c). The amplitude of the responses decreased progressively even in the control (A and D). In (B, b) and (E, b), Ca²⁺ loading was performed in the presence of 3 μ M CPA in the second trial. Subsequent application of caffeine or IP₃ in the absence of CPA did not induce contraction, while in the third trial (B, c and E, c) the contractile responses substantially recovered. In (C, b) and (F, b), contraction induced by caffeine or IP₃ was observed in the presence of 1 μ M CPA, and not affected significantly by CPA.

quent application of caffeine did not elicit contraction. When a 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, the peak amplitude of contractions decreased progressively (Figure 1A). This may have resulted from a gradual decrease in cellular calmodulin content (Kitazawa & Somlyo, 1990) (see Figure 3). In Figure 1B, the second application of pCa 6.3 solution was performed in the presence of 3 μ M CPA. CPA *per se* did not change the muscle tone in R0.5G (Figure 1B, b). The relative peak amplitude of the second response to pCa 6.3 compared with the first one was not affected significantly by 3 μ M CPA (*P* > 0.05, *n* = 6). The subsequent application of caffeine did not induce any response (Figure 1B, b). In the third trial (Figure 1B, c), a substantial response to caffeine was

observed, indicating a recovery from CPA-induced inhibition following washout.

When 1 μM CPA was added after Ca^{2+} -loading (Figure 1C,b), the subsequent response to caffeine (in the presence of CPA) was very similar to the control (Figure 1A,b). A higher concentration of CPA ($>3 \mu\text{M}$) applied after Ca^{2+} -loading for a longer period (>10 min), caused a small and slow phasic contraction and the subsequent response to caffeine appeared to be smaller than the control (not shown). However, prolonged incubation in R0.5G after Ca^{2+} -loading *per se* progressively reduced the subsequent caffeine-induced contraction. Therefore, the effects of CPA on Ca^{2+} -release mechanisms were examined under fixed conditions: 1 or 3 μM CPA was added just after Ca^{2+} -loading was finished and 25 mM caffeine (or 25 μM IP_3) was added 5 min after the application of CPA as shown in Figure 1C. Under these conditions, CPA did not apparently affect subsequent caffeine-induced contraction (see also Figure 2).

Application of IP_3 at concentrations between 3 and 50 μM after Ca^{2+} -loading elicited contraction in a concentration-dependent manner in skinned ileal fibres. When 25 μM or higher concentrations of IP_3 were used to obtain a stable response, the results were qualitatively very similar to those obtained with 25 mM caffeine. Most of the releasable Ca^{2+} in storage sites sensitive to IP_3 was depleted by the first application of 25 μM IP_3 as shown in Figure 1D,a. The procedure of Ca loading at pCa 6.3 and subsequent application of 25 μM IP_3 in R0.5G was repeated three times in each preparation. The contraction induced by IP_3 was also transient but the decline of tension from the peak was slightly slower than that induced by caffeine. IP_3 -induced contraction was almost completely blocked when the preceded Ca loading was performed in the presence of 3 μM CPA (Figure 1E,b) but not when 1 μM CPA was added after the Ca^{2+} -loading (Figure 1F,b).

The responses induced by the third application of caffeine or IP_3 in the control procedure shown in Figure 1A,c and 1D,c were 61.1 ± 6.3 and $56.1 \pm 1.6\%$ ($n = 3$ for each) of corresponding first responses (Figure 1A,a and 1D,a), respectively. When 3 μM CPA was added to the Ca-loading solution in the second trial (Figure 1B,b and 1E,b), the responses in the third trial (Figure 1B,c and 1E,c) were 48.4 ± 5.2 and $56.3 \pm 7.7\%$ ($n = 3$ for each) which was not significantly different from those in the control ($P > 0.05$ vs. 61.1 and 56.1%, respectively), respectively. Therefore, the inhibitory effect of 3 μM CPA can be almost completely removed by washout.

Summarized data are shown in Figure 2. The peak amplitude of contraction induced by the first application of 25 mM caffeine or 25 μM IP_3 was taken as 100%, respectively. The

Figure 2 Summarized data shown in Figure 1; dose-response relationships of cyclopiazonic acid (CPA) for caffeine- (open symbols) or inositol 1,4,5-trisphosphate (IP_3)- (closed symbols) induced responses. No CPA on abscissa scale indicates the responses in the second trial in the control (Figure 1A,b and D,b) as a percentage of the first corresponding responses. The number of observations was 3 for each symbol except at CPA dose 0.3 μM where four observations were obtained for each. Triangles indicate the results obtained when caffeine or IP_3 were applied in the presence of 1 μM CPA in the second trial as shown in Figure 1 (C,b) and (F,b).

second responses to caffeine and IP_3 in the control were 70.3 ± 5.6 ($n = 3$) and $73.3 \pm 1.8\%$ ($n = 3$) indicated by open and closed circles with 'no CPA', respectively. The second application of caffeine or IP_3 preceded by Ca-loading in the presence of CPA yield dose-dependent decreases in contraction between 0.1 and 3 μM of CPA. Triangles indicate the data obtained when CPA was added after Ca^{2+} -loading (Figure 1C and F). The values shown by open ($63.8 \pm 2.4\%$, $n = 3$) and closed triangles ($81.0 \pm 4.2\%$, $n = 3$) are not statistically different from those of the open and closed circles with 'no CPA', respectively ($P > 0.05$), demonstrating that 1 μM CPA does not directly reduce Ca release induced by caffeine and IP_3 . The approximate half-inhibition dose of CPA for Ca release by caffeine and IP_3 was the same, in spite of the different Ca releasing mechanisms ($\sim 0.6 \mu\text{M}$).

To examine whether CPA inhibits ATP-dependent Ca^{2+} -uptake into storage sites, the effects of CPA were compared in the presence and absence of ATP and creatine-phosphate (Figure 3). After a standard contraction was obtained in a pCa 6.3 solution, stored Ca^{2+} was depleted by application of 25 mM caffeine. Subsequent removal of ATP and creatine-phosphate induced rigor contraction in R2G solution. Thereafter, a change in bathing solution from R2G solution to pCa 6.3 solution (ATP and creatine-phosphate-free) either did not alter the tone significantly or slightly increased it, suggesting that the tone may not be Ca^{2+} -dependent. During the incubation in pCa 6.3 solution for 15 min, preparations were treated in three different ways; 4.0 mM ATP was added for 10 min (Figure 3a), nothing was added (Figure 3b), or 10 μM CPA and 4.0 mM ATP were added (Figure 3c). The application of ATP induced transient relaxation and subsequent contraction, regardless of the presence of CPA (indicated by arrows in Figure 3a and c). Thereafter, the bathing solution was returned to ATP- and creatine-phosphate-free R2G solution for 1 min and then changed to the standard R2G solution. Finally, the response to 25 mM caffeine was examined in R0.5G solution. A caffeine-induced contraction was observed only in a preparation to which ATP but not CPA was applied during the preceding incubation in pCa 6.3 solution (Figure 3a, $44.1 \pm 3.8\%$ of the first contraction induced by caffeine, $n = 3$). These results demonstrate that the Ca^{2+} released by the application of caffeine was previously sequestered into storage sites by an ATP-dependent mechanism and that 10 μM CPA completely inhibited this uptake in a reversible manner.

Although these results strongly suggest that CPA acts on ATP-dependent Ca-uptake into intracellular storage sites, two other mechanisms might be involved; CPA may act as a calmodulin inhibitor, or directly decrease the activity of the contractile apparatus. These possibilities were also examined. To prevent any contribution of Ca^{2+} -induced Ca^{2+} -release, the ability of intracellular sites to store Ca^{2+} was destroyed by treatment with 10 μM A23187 for about 20 min after skinning. Under these conditions, responses to caffeine and IP_3 were abolished.

As mentioned previously, contractile responses in pCa 6.3 solution could be elicited repetitively but the amplitude decreased. Gradual loss of calmodulin from cytoplasm may be, in part, responsible for this phenomenon. In Figure 4a, the control responses to pCa 6.0 solution were observed three times. The fourth application of pCa 6.0 solution, which was performed after 2 μM calmodulin had been added, induced a larger response than the third one (Figure 4a). Summarized data (Figure 4b) show that the fourth response recovered significantly ($P < 0.05$ vs the third one) in the presence of calmodulin. This recovery with calmodulin did not change significantly in the presence of 10 μM CPA ($P > 0.05$) which was added about 5 min before the fourth response.

To test whether CPA could directly affect the contractile proteins, pCa-tension relationships were obtained by stepwise increases in Ca^{2+} -concentration as shown in Figure 5. The maximum response was obtained at pCa 5.0. Further increase to pCa 4.0 or 3.0 did not increase tension significantly, imply-

Figure 3 Dependence of cyclopiazonic acid (CPA)-sensitive Ca²⁺-loading on adenosine 5'-triphosphate (ATP). After loading in pCa 6.3 solution, Ca²⁺ was released by two applications of 25 mM caffeine. Subsequent change to R2G solution which did not contain ATP and creatine-phosphate induced rigor contraction. NaN₃ was present throughout the experiment (see Methods). When rigor contraction reached a steady level, the solution was changed to ATP- and creatine-phosphate-free pCa 6.3 solution. During application of pCa 6.3 solution, the strips were treated in three different ways. (a) ATP (4 mM) was added. (b) Nothing was added. (c) CPA (10 μM) was first added and then 4 mM ATP was added. Addition of ATP-induced relaxation in (a) and (c) as indicated by arrows. Thereafter, the fibre was returned to ATP- and creatine-phosphate-free R2G solution. The solution was then changed to one containing ATP and creatine-phosphate, resulting in a large relaxation from rigor contracture in (b). Subsequent application of 25 mM caffeine was performed in R0.5G solution. Note that a contractile response to caffeine was observed in (a) but not in (b) and (c). After subsequent Ca²⁺-loading in pCa 6.3 solution, the responses to 25 mM caffeine were observed in (b) and (c), as well as (a).

ing that the contribution of non-skinned cells in the fibre, if any, to the contractile response was negligible. Moreover, maximum contraction in skinned fibres was comparable to or slightly larger than that in 130 mM K solution in intact preparations before skinning ($n > 20$, see Figure 5A). The pCa-tension relationships were examined twice in each preparation. The relative amplitude of contraction at each pCa in the second trial was calculated versus the first maximum response. When the second trial was performed in the presence of 10 μM CPA, the response was not affected significantly, as summarized in Figure 6 ($P > 0.05$; vs. corresponding control at each pCa). Treatment with GTPγS, which is known as a substance increasing the Ca²⁺ sensitivity of contractile proteins (Kitazawa *et al.*, 1991), significantly potentiated the contraction.

A great advantage of skinning with β-escin is that the functions of receptors on the cell membrane and the related intracellular signal transduction are preserved after skinning

(Kobayashi *et al.*, 1989; Itoh *et al.*, 1991). In the present study, the response to acetylcholine (ACh, Figure 7) was observed in the presence of 50 μM GTP after skinning. The response to ACh appeared to be dose-dependent in the range of 0.3 to 100 μM. To evaluate the effects of CPA, Ca²⁺-loading in pCa 6.3 and the subsequent application of 100 μM ACh was repeated three times in each preparation in the same manner shown in Figure 1 for caffeine and IP₃. The response to 100 μM ACh was transient as was that to IP₃. The amplitude of the response to ACh in the control (Figure 7A) was decreased progressively during three trials but not as much as that to pCa 6.3 (see also Figures 1 and 4). When Ca²⁺-loading was performed in the presence of 10 μM CPA in the second trial, the subsequent response to ACh was almost completely abolished. In the third trial (Figure 7B), CPA was absent during Ca²⁺-loading and the subsequent response to ACh substantially recovered. The second and third responses to 100 μM ACh were 82.8 ± 7.8 and

Figure 4 Effect of cyclopiazonic acid (CPA) on calmodulin (CaM) action in the skinned ileal smooth muscle. Intracellular Ca storage sites were destroyed by treatment with 10 μ M A23187 for 20 min after skinning. (a) Contraction induced in pCa 6.0 solution was observed four times; in the absence of calmodulin three times and in the presence of 2 μ M calmodulin the last time (4th). The amplitude of the response decreased gradually with time but recovered markedly following treatment with calmodulin prior to the fourth contraction. (b) Summarized data for contractions in pCa 6.0 solution. Open and solid columns indicate the data obtained in the absence of CPA (control). The first response was taken as 100%. The hatched column indicates the relative amplitude of the fourth contraction which was induced in the presence of 10 μ M CPA as well as 2 μ M calmodulin (+ CaM) in the same preparations shown by the closed column. The number of observations was 6, 5 and 5 for open, solid and hatched columns, respectively. Note that the contraction decreased with time and recovered significantly when 2 μ M calmodulin was added. This recovery was not affected in the presence of 10 μ M CPA.

$65.7 \pm 8.8\%$ ($n = 3$), respectively, in the control and 2.10 ± 1.09 ($P < 0.01$ vs 82.8) and $39.1 \pm 8.7\%$ ($n = 3$, $P < 0.01$ vs. 2.10), respectively, in the CPA-treated preparations when the first response to ACh was taken as 100% in each preparation. It is clearly shown that Ca^{2+} -uptake by storage sites which is released by application of ACh was also inhibited by CPA. Qualitatively the same results were obtained when 3 μ M substance P (SP) was used as an agonist ($n = 2$).

Discussion

Our results show that CPA in a concentration-range between 0.1 and 10 μ M decreases the amplitude of contractions induced by caffeine or IP_3 in skinned ileal smooth muscle strips. This effect was observed only when CPA was applied during the preceding Ca^{2+} -loading into the intracellular storage sites. Our results also suggest that 1 μ M CPA does not affect the Ca^{2+} -release mechanism itself, whereas inhibition of Ca^{2+} -release by CPA has been reported in SR vesicle of skeletal muscle (Goeger *et al.*, 1988). The CPA-sensitive Ca^{2+} -uptake was due to an ATP-dependent mechanism which is likely to be the SR/ER Ca^{2+} -pump. The Ca sensitivity and

Figure 5 Effects of 10 μ M cyclopiazonic acid (CPA) and 100 μ M guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) on pCa-tension relationship in skinned fibre pretreated with 10 μ M A23187. The contractile response to 130 mM K^+ solution was examined in each preparation before skinning as shown in (A). The Ca^{2+} concentration was increased stepwise from pCa 7.0 to 6.5, 6.0, 5.0 and 4.0, twice in each preparation. In (C) and (D) the second trial was performed in the presence of 10 μ M CPA or 100 μ M GTP γ S, respectively. Note that GTP γ S did but CPA did not affect the pCa-tension relationship. Calmodulin was not added to the solutions.

Figure 6 Summarized data shown in Figure 5. Open circles and triangles denote the mean values of the relative amplitude of the contractile response in the control, in the presence of 10 μ M cyclopiazonic acid (CPA) or 100 μ M guanosine 5'-O-(3-thiotriphosphate) (GTP γ S), respectively. The number of observations was 8, 5 and 3 in the control, in the presence of CPA or GTP γ S, respectively. Abscissa scale denotes pCa in the solutions. The maximum response in the first trial (see Figure 5) in each preparation was taken as 100%.

the maximum contractile ability of preparations with Ca-storage sites destroyed by A23187 (Itoh *et al.*, 1985) were not affected significantly by 10 μ M CPA. CPA did not inhibit the enhanced contraction due to addition of calmodulin. Together, our data indicate that the effect of CPA is quite selective for the ATP-dependent Ca^{2+} -uptake mechanism of intracellular storage sites. This is consistent with previous results showing that CPA is a selective inhibitor of Ca^{2+} -ATPase in skeletal and cardiac muscle SR (Goeger *et al.*, 1988; Goeger & Riley, 1989; Kurebayashi & Ogawa, 1991). The effective concentration-range of CPA for inhibition of caffeine- or IP_3 -induced contraction in skinned smooth muscle ($\text{IC}_{50} = 0.6 \mu\text{M}$) is also comparable to that reported for

Figure 7 Effects of cyclopiazonic acid (CPA) on acetylcholine (ACh)-induced contraction in skinned ileal strip. The response to 100 μ M ACh subsequent to Ca²⁺-loading in pCa 6.3 solution was observed three times in each preparation. ACh was applied in the presence of 50 μ M guanosine triphosphate (GTP) in relaxing solution (R0.5G). Ca²⁺-uptake in the second trial was performed in the absence or presence of 10 μ M CPA in (A) and (B), respectively.

skeletal muscle ($\sim 0.2 \mu$ M) (Kurebayashi & Ogawa, 1991).

In skinned smooth muscle fibres which showed rigor type contracture in ATP- and creatine-phosphate-free solution, addition of ATP induced transient relaxation followed by contraction at pCa 6.3. The relaxation may be explained by detachment of rigor cross-bridges by ATP which occurs much faster than phosphorylation of the myosin light chain (Somlyo *et al.*, 1988). Although the rate of detachment is much faster than the relaxation, the diffusion rate of ATP into the fibre might be responsible for the slow relaxation and following contraction, since the relaxation is much more transient when ATP is released by laser-flash photolysis (Somlyo *et al.*, 1988).

In most smooth muscle cells, two kinds of intracellular Ca²⁺-releasing mechanism have been identified; a Ca²⁺-induced Ca²⁺-release mechanism which is affected by caffeine or ryanodine and a separate IP₃-induced Ca²⁺-release mechanism. Two corresponding physically separated storage sites have been postulated; those which are sensitive to IP₃ only and those sensitive to both ryanodine and IP₃ (Iino *et al.*, 1988). Since 10 μ M CPA almost completely inhibited both caffeine- and IP₃-induced contractions, the Ca²⁺-ATPase which is inhibited by CPA may be involved in Ca²⁺-uptake into both types of storage sites. Thapsigargin which is also known as an inhibitor of Ca²⁺-ATPase in SR/ER, decreases Ca²⁺-uptake into IP₃-sensitive storage sites more effectively than that into IP₃-insensitive ones in cultured smooth muscle cells (Bian *et al.*, 1991; Missiaen *et al.*, 1991).

It is well established that ACh activates phospholipase C, increases formation of IP₃ and thereby induces Ca²⁺-release from SR/ER (Abdel-Latif, 1986). Therefore, the result that Ca²⁺-uptake into storage sites sensitive to ACh as well as those sensitive to IP₃ were inhibited by CPA is consistent with the proposed mechanism. The possibility that the potency of CPA for inhibiting contraction induced by ACh is different from that by IP₃ has not been examined carefully in the present study. It might be possible since sensitization of

contractile proteins to Ca²⁺ by ACh via GTP binding protein has been reported (Kitazawa & Somlyo, 1990).

A series of Ca²⁺-ATPase isoforms in SR and ER of muscle and non-muscle tissues has been reported. Molecular cloning of smooth muscle SR/ER Ca²⁺-ATPase has demonstrated that smooth muscle expresses the same gene as slow skeletal muscle, cardiac muscle and non-muscle tissues (Gunter-Hamblin *et al.*, 1988; Lytton *et al.*, 1989). From this gene, three different mRNAs are transcribed by alternative processing (Eggermont *et al.*, 1990). Recent work has shown that the sensitivity to CPA (and thapsigargin as well) is identical in three types of avian ER/SR Ca²⁺-ATPase encoded by three distinct cDNAs. These three cDNA sequences have high homology to SERCA1 in fast skeletal muscle, SERCA2a in slow skeletal and cardiac muscles, and SERCA2b in brain and smooth muscle in mammals, respectively (Cambell *et al.*, 1991). The effective concentrations of CPA in our preparation are similar to those required for inhibition of Ca²⁺-ATPase in skinned fibre of fast skeletal muscle. These results may be consistent with the prediction based upon the molecular biological knowledge that CPA shows similar potency inhibiting Ca²⁺-uptake into Ca²⁺-storage sites in skeletal and smooth muscles.

Our results are consistent with the proposal that CPA decreases Ca²⁺-uptake by inhibition of the Ca²⁺-pump in the SR/ER, thereby decreasing subsequent Ca²⁺-release from those storage sites. In the present study, however, Ca²⁺-ATPase activity *per se* was not measured. Ca²⁺-ATPase activity in SR of cardiac and smooth muscle is regulated by factors such as phospholamban (Tada *et al.*, 1975; Raeymaekers & Jones, 1986). Moreover, it has been reported that CPA inhibits Ca²⁺-release from vesicles prepared from skeletal SR as well as Ca²⁺-uptake. Although phenomena suggesting such kind of action were not found using relatively low concentrations of CPA in the present study, the possibility that higher concentration of CPA ($> 3 \mu$ M) affects Ca²⁺-releasing mechanisms remains to be examined. It might be possible that CPA has alternative or additional effects on regulation of the Ca²⁺-ATPase activity.

One of the most important findings in the present study is that the inhibitory effect of CPA at concentrations less than 3 μ M was almost completely reversible by washout in skinned smooth muscle strips. The velocity of Ca²⁺-uptake which may directly correlate to Ca²⁺-ATPase activity was not, however, measured in our experiments. Therefore, it is possible that the total amount of Ca²⁺ taken up over 10 min in pCa 6.3 solution was not affected even if the Ca²⁺-ATPase activity was still partly suppressed by CPA remaining after washout. This has been found in skinned skeletal muscle preparations where inhibition of Ca²⁺-ATPase activity by CPA was only partly removed by washout (Kurebayashi & Ogawa, 1991). Although the effects of CPA in intact smooth muscle preparations reported so far (Deng & Kwan, 1991; Bourreau *et al.*, 1991) appear to be consistent with our results in skinned fibres, it is not clear whether the effects of CPA are reversible in intact cells. Our preliminary results suggest that the effects of CPA in intact smooth muscle are removed by washout at least in part. Since it has been suggested that another SR/ER Ca²⁺-ATPase inhibitor, thapsigargin, irreversibly affects Ca²⁺-uptake in intact smooth muscle (Low *et al.*, 1991), removal of CPA-induced inhibition, even if it is not complete, may be pharmacologically important.

We conclude that CPA is a selective and reversible inhibitor of the Ca²⁺-pump of intracellular Ca storage sites, mainly SR, in skinned ileal smooth muscle. It therefore is an extremely valuable pharmacological tool for investigation of Ca²⁺-pumping mechanisms and intracellular Ca²⁺ homeostasis.

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