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Effects of cyclopiazonic acid on rhythmic contractions in uterine smooth muscle bundles of the rat

*†Yasuyo Kasai, *[1]Masamitsu Iino, †Osamu Tsutsumi, †Yuji Taketani & *Makoto Endo

*Department of Pharmacology and †Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

1 We studied the effects of cyclopiazonic acid (CPA) on rhythmic contractions and on Ca^{2+} uptake by the intracellular stores in longitudinal muscle strips of the rat uterus at 30°C.

2 Oxytocin (1 μM) in Ca^{2+}-free solution induced a transient rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contraction after Ca^{2+} loading of the stores in high-K^+- and Ca^{2+}-containing solution. CPA inhibited oxytocin-induced Ca^{2+} release and contraction, the half and full inhibitory concentrations of CPA being 0.3 and 10 μM, respectively. In contrast, addition of CPA after Ca^{2+} loading exerted no significant inhibitory effects.

3 Oxytocin (10 nM) applied in Ca^{2+}-containing solution induced rhythmic increases in both force and $[Ca^{2+}]_i$. CPA (10 μM) had no effect on oxytocin-induced rhythmic contractions.

4 At a high concentration (300 μM), CPA inhibited the rhythmic contractions induced by 10 nM oxytocin; the frequency and the peak height were decreased, and in many bundles contractions were completely abolished. These inhibitory effects were reversed after CPA washout.

5 CPA (300 μM) inhibited the rate of rise of $[Ca^{2+}]_i$ due to depolarization induced by high-K^+-containing solution.

6 These results suggest that low concentrations of CPA inhibit the loading of Ca^{2+} into intracellular stores in intact tissue strips, and that the Ca^{2+} stores are not directly involved in the uterine rhythmic contractions. It is also suggested that a high concentration of CPA inhibits the mechanism that is responsible for the generation of rhythmic contractions as well as voltage-dependent Ca^{2+} channels.

Keywords: Cyclopiazonic acid; Ca^{2+}; uterine smooth muscle; oxytocin

Introduction

Uterine smooth muscles undergo spontaneous rhythmic contractions without neuronal or hormonal stimulation *in vitro*. Changes in the membrane potentials were recorded from the myometrial cells during these contractions, and the rhythmic contractions were found to be accompanied by groups of action potentials (Kleinhaus & Kao, 1969). The inward currents mediating the upstroke of the action potentials are due mainly to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mironneau, 1973; Kao & McCullough, 1975). L-type Ca^{2+} channels were also shown to be responsible for the inward currents which were blocked by a dihydropyridine Ca^{2+} channel antagonist (Jmari *et al.*, 1986; Honore *et al.*, 1989). Agonists like oxytocin and prostaglandins can increase the force and frequency of rhythmic contractions (Mironneau, 1976), which are also suppressed by L-type Ca^{2+} channel blockers or by removal of extracellular Ca^{2+} (Edwards *et al.*, 1986; Kawarabayashi *et al.*, 1986; Tasaka *et al.*, 1991). Therefore, Ca^{2+} influx through L-type Ca^{2+} channels is important in Ca^{2+} regulation in both spontaneous and agonist-stimulated rhythmic contractions of uterine smooth muscle cells.

Recently, the primary structure of the human oxytocin receptor was determined by molecular cloning of its complementary DNA and the receptor molecule was predicted to have seven transmembrane stretches, typical of G-protein-coupled receptors (Kimura *et al.*, 1992). Indeed, oxytocin has been shown to activate phospholipase C in the myometrium through a G-protein-mediated process. This results in the rapid formation of inositol 1,4,5-trisphosphate (IP_3) (Marc *et al.*, 1986; 1988), which releases Ca^{2+} from the sarcoplasmic reticulum (SR) vesicles of the uterine smooth muscle (Carsten & Miller, 1985). Coleman and colleagues (1988) also showed that oxytocin causes contractions in the myometrium in the absence of extracellular Ca^{2+} due to Ca^{2+}-release from the intracellular stores. These results suggest that oxytocin can increase $[Ca^{2+}]_i$ both by enhancing Ca^{2+} influx through L-type Ca^{2+} channels and by causing IP_3-induced Ca^{2+} release from the intracellular Ca^{2+} stores. However, the relative roles of extracellular and intracellular sources of Ca^{2+} to the contractile actions of oxytocin are unclear.

CPA is one of the mycotoxins and was first reported as a potent inhibitor of the Ca^{2+} uptake and ATPase activity of rat skeletal muscle SR vesicles by Goeger *et al.* (1988). Subsequently, CPA was shown to be a specific inhibitor of the Ca^{2+}-ATPase of skinned skeletal muscle SR (Kurebayashi & Ogawa, 1991) and skinned smooth muscle bundles (Uyama *et al.*, 1992). These workers showed that smooth muscle 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, whereas the addition of CPA after Ca^{2+} uptake had no significant effects (Uyama *et al.*, 1992). Therefore, CPA may be used as a pharmacological agent to inhibit the function of Ca^{2+} stores of smooth muscle cells. To determine the role of the intracellular Ca^{2+} stores in the oxytocin-induced rhythmic contractions, we have now studied the effect of CPA on contractions of uterine smooth muscle cells.

Methods

Non-pregnant female Wistar rats, weighing 200–300 g, were stunned and exsanguinated. A vaginal smear test was performed, and only rats in oestrus were used. Uteri were excised, and longitudinal muscle bundles (1.5 mm in length, 150–200 μm in width, and 150 μm in thickness) were carefully dissected in physiological salt solution (PSS) with the composition given below. The dissection was carried out at room temperature, and the experiments were performed at 30°C.

[1] Author for correspondence.

Measurement of tension and fluorescence intensity

To load the smooth muscle cells with a fluorescent Ca^{2+} indicator, Fura-2, muscle bundles were incubated in 20 μM Fura-2AM in PSS with 0.005% Pluronic F-127 for 3–5 h at 30°C. Then each end of the muscle bundle was tied with silk filament and the bundle was attached to a pair of stainless steel hooks, with one end connected to an isometric tension transducer (AE801, Akers, Norway) and the other to a micromanipulator for the adjustment of muscle length. The muscle strips were placed in an experimental trough (capacity 300 μl) mounted on a fluorometer (CAF-100, JASCO, Japan), and were illuminated alternately by 340 and 380 nm lights at 50 Hz. Fluorescence signals from the muscle strips were collected by a photomultiplier tube through a 500 nm interference filter. When $[Ca^{2+}]_i$ rose, the changes in the fluorescence intensity (photomultiplier current) at 340 and 380 nm excitations showed mirror-image features. Therefore, the ratio of the two fluorescence intensities (F340/F380) was used as an indicator of $[Ca^{2+}]_i$. To change solutions, the solution in the trough was first aspirated and a new solution was then pumped in. The procedure was controlled by a personal computer (PC9801, NEC, Japan) and was accomplished within 5 s. The two fluorescence intensity signals as well as the isometric tension signal were collected using an analog-to-digital converter board in the computer and stored on floppy disks for later analysis and plotting using another computer (Quadra 800, Apple, U.S.A.). The ratio of the fluorescence intensities and the tension record were also displayed on a pen recorder (SR6221, Graphtec, Japan).

When the rate of rise of $[Ca^{2+}]_i$ induced by a high concentration of K^+ solution was to be compared, the muscle bundle was attached to a stainless steel wire (100 μm in diameter) and was placed in a capillary cuvette with an internal diameter of 400 μm. The solution was changed within 1 s by a rapid flow of solutions through the cuvette. F340/F380 of the muscle bundles was measured with a fluorometer (CAF-110, JASCO, Japan).

The solutions used were as follows (in mM). Physiological salt solution (PSS): NaCl 150, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid) 5, glucose 5.6. High-K^+ solution: KCl, 154, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, glucose 5.6, sucrose 44. High-K^+ Ca^{2+}-free solution: KCl 154, $MgCl_2$ 3, HEPES 5, EGTA 5, glucose 5.6, sucrose 44. The pH of all solutions was adjusted to 7.4 with NaOH.

Materials

Chemicals used were: Fura-2AM from Dojin (Japan), Pluronic F-127 from BASF (U.S.A.), CPA, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), carbachol and nicardipine from Sigma (U.S.A.) and oxytocin from Peptide Institute, Inc. (Japan).

Control study and statistical analysis

CPA was prepared in 10 mM and 100 mM stock solutions in dimethyl sulphoxide (DMSO), which at the highest concentration used (0.3%) had no effect on oxytocin-induced Ca^{2+} release, rhythmic contractions and high-K^+ induced contractions in the time-match control experiments; also had no effects on fluorescence signals of Fura-2 in cuvette calibration.

All values are expressed by means ± s.e.mean, and n is the number of observations. We obtained only one preparation from one animal.

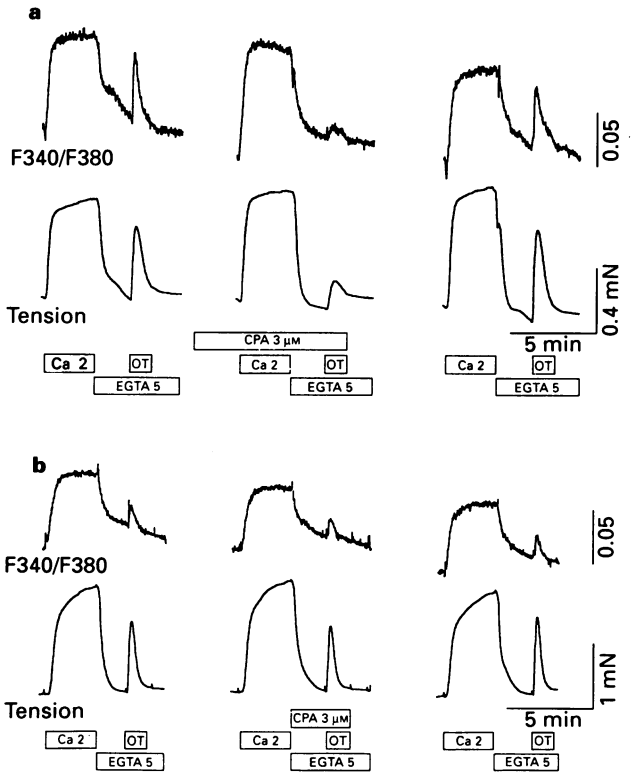

Figure 1 Effects of CPA on Ca^{2+} release from intracellular Ca^{2+} stores. Ca^{2+} was loaded into the stores during high-K^+ (154 mM K^+, 2 mM Ca^{2+})-induced contraction for 3 min, then oxytocin (OT, 1 μM) was applied in the absence of extracellular Ca^{2+} (EGTA 5 mM). Changes in $[Ca^{2+}]_i$ (upper traces) and isometric tension (lower traces) were simultaneously measured. (a) Three consecutive runs obtained from the same bundle ($n = 6$). CPA (3 μM) was applied 3 min before Ca^{2+} loading until the end of oxytocin application in the second run as shown by the box below the traces. In the presence of CPA, the response to oxytocin was markedly reduced (middle figure). The effect of CPA was reversible and the response induced by oxytocin recovered in the subsequent control run. (b) Similar to (a), but 3 μM CPA was added after Ca^{2+} loading in the second run. There was no significant effect of CPA with this protocol ($n = 3$).

Results

Effects of CPA on Ca^{2+} release from intracellular Ca^{2+} stores

CPA inhibits Ca^{2+} uptake by the sarcoplasmic reticulum in the skinned fibres of both skeletal muscle and smooth muscle (Kurebayashi & Ogawa, 1991; Uyama *et al.*, 1992). We studied whether CPA could also inhibit Ca^{2+} uptake by the stores in intact myometrial muscle strips. As shown in Figure 1, Ca^{2+} was loaded into the stores during high-K^+ (154 mM K^+, 2 mM Ca^{2+})-induced contraction for 3 min, then extracellular Ca^{2+} was removed with 5 mM EGTA-containing solution in the continued presence of a high concentration of K^+. After 2 min in the Ca^{2+}-free solution, oxytocin (1 nM–1 μM) was applied and it induced transient $[Ca^{2+}]_i$ increase and contraction (Figure 1a, left). This response was due to Ca^{2+} release from the stores since extracellular Ca^{2+} was absent. We were able to obtain the same response repeatedly from one strip when oxytocin was applied every 11–12 min after the same Ca^{2+} loading procedure. The peak size of the transient contraction was dependent on the concentration of oxytocin; 14.6 ± 10.4% (1 nM), 57.1 ± 8.7% (10 nM) and 81.5 ± 7.0% (100 nM) of that for 1 μM oxytocin, and the rise of $[Ca^{2+}]_i$ was 8.3 ± 10.2% (1 nM), 48.4 ± 22.1% (10 nM), 68.8 ± 13.7% (100 nM) of that for 1 μM (average ± s.e.mean, $n = 3$; all the concentrations of oxytocin were tested in each muscle). In subsequent experiments, we used 1 μM oxytocin as the standard stimulus to examine the effect of CPA on the function of the Ca^{2+} stores.

If 3 μM CPA was applied 3 min before the Ca^{2+} loading and remained until the end of oxytocin application, the response to oxytocin was markedly reduced (Figure 1a, mid-

dle). However, if CPA was added after Ca^{2+} loading, no significant suppression was observed (Figure 1b, middle). The effect of CPA was reversible and the response induced by oxytocin recovered in the following control run (Figure 1a and b, right). These results show that CPA inhibits Ca^{2+} uptake, thereby reducing subsequent Ca^{2+} release from the Ca^{2+} stores.

The open symbols in Figure 2 show concentration-dependent effects of CPA on the Ca^{2+} release. Peak responses obtained in a test run with CPA application during the Ca^{2+} loading were normalized by the average of the control runs obtained before and after the test run. In the presence of 10 μM CPA during the Ca^{2+} loading, the rise of $[Ca^{2+}]_i$ due to oxytocin application was completely inhibited (open triangle). The relative value of the corresponding contractile response was also reduced to 0.09 ± 0.02 (open circle). The remaining contraction was slow in onset and was very probably due to the increase in Ca^{2+} sensitivity of the contractile proteins induced by agonists (see Karaki, 1989).

Rhythmic contractions of uterine smooth muscle bundles

Figure 3 shows the response of a uterine smooth muscle bundle to 10 nM oxytocin in the presence of 2 mM Ca^{2+}. A rapid rise in tension which lasted for approximately 2 min occurred, followed by a spontaneous partial relaxation and subsequent rapid contraction. This partial relaxation and contraction cycle went on a few times until constant rhythmic contractions from the baseline tension developed (Figure 3, lower trace). The frequency of the rhythmic contractions was $0.75 \pm 0.03\ min^{-1}$ (mean ± s.e.mean, $n = 21$). These changes in the tension synchronized with the rise of $[Ca^{2+}]_i$ (Figure 3, upper trace). After drug washout, both tension and $[Ca^{2+}]_i$ signals returned to basal levels. Similar rhythmic contractions were sometimes seen spontaneously and were observed when activated by 1 μM $PGF_{2\alpha}$ ($0.75 \pm 0.09\ min^{-1}$, $n = 3$) or 10 μM carbachol ($0.70 \pm 0.16\ min^{-1}$, $n = 3$).

Effect of CPA on rhythmic contractions

We then examined the effects of 10 μM CPA, which is enough to inhibit the function of Ca^{2+} stores, on the rhythmic contractions induced by 10 nM oxytocin. Figure 4a illustrates

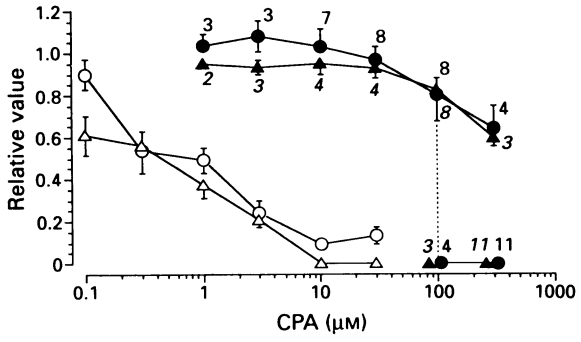

Figure 2 Concentration-response relationship of the effect of CPA on Ca^{2+} release induced by 1 μM oxytocin and rhythmic contractions by 10 nM oxytocin. Shown here are peak sizes of oxytocin-induced contraction (○) and $[Ca^{2+}]_i$ rise (△) following Ca^{2+} loading in the presence of CPA at the concentrations shown on the abscissa scale (see Figure 1). Results were expressed relative to the average of the corresponding values of control runs obtained before and after the test run (mean ± s.e.mean, $n = 7-8$ for ≤ 10 μM, $n = 3$ for 30 μM). Peak sizes of the rhythmic contractions (●) or $[Ca^{2+}]_i$ change (▲) from 10 min to 15 min after the application of CPA were normalized by the corresponding peak values prior to the drug application (see Figure 4, mean ± s.e.mean). At 100 and 300 μM CPA, data from the muscle bundles with complete loss of rhythmic contraction were pooled separately to obtain averages. Numbers beside symbols indicate the number of observations. In some bundles $[Ca^{2+}]_i$ was not measured.

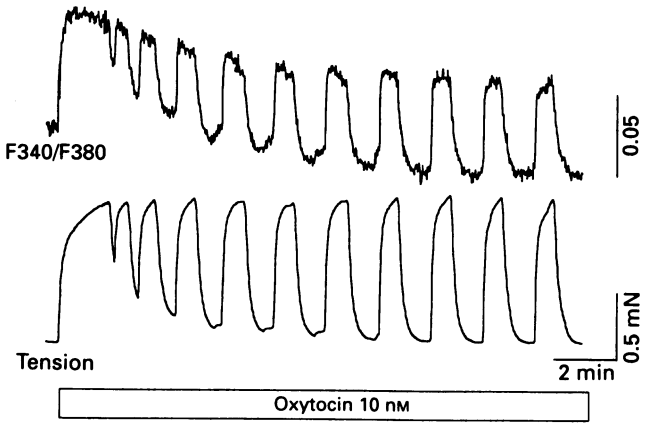

Figure 3 Rhythmic contractions of a uterine smooth muscle bundle induced by 10 nM oxytocin in the presence of 2 mM Ca^{2+}. The resting tension was 0.5 mN, and both tension and $[Ca^{2+}]_i$ remained quiescent until application of 10 nM oxytocin, upon which rhythmic contractions started. The change in the tension (lower trace) synchronized with the rise of $[Ca^{2+}]_i$ expressed by F340/F380 (upper trace).

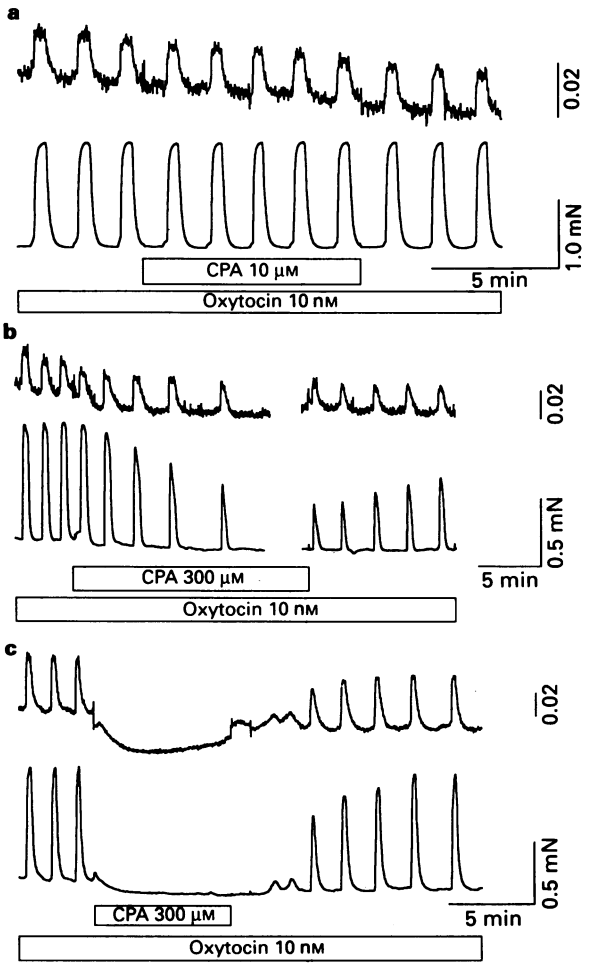

Figure 4 Effect of CPA on the rhythmic contractions induced by 10 nM oxytocin. In each panel, upper and lower traces show F340/F380 and tension, respectively. The gradual decrease in F340/F380 was probably due to dye bleaching. (a) CPA (10 μM) was added during the rhythmic contractions induced by 10 nM oxytocin ($n = 7$). There were two types of inhibition by 300 μM CPA. (b) In some preparations the frequency and the peak of rhythmic contractions were gradually decreased ($n = 4$). The break in the traces was due to a temporary halt of data acquisition necessary for the transfer of accumulated data to the floppy disk. Both traces showed no change in the chart record of the pen recorder. (c) In other muscle bundles, the rhythmic contractions were inhibited immediately after the drug application ($n = 11$). The suppression was reversed after the washing out of CPA.

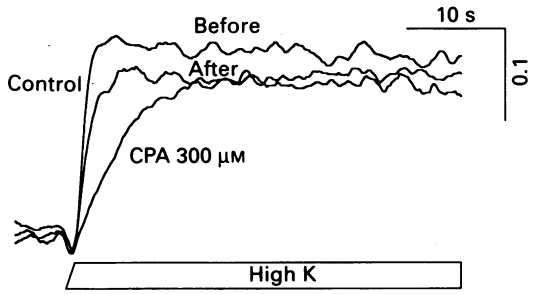

Figure 5 Effect of 300 μM CPA on the depolarization-induced $[Ca^{2+}]_i$ rise. The change in $[Ca^{2+}]_i$ (F340/F380) induced by high-K^+ solution was compared in the presence and absence of 300 μM CPA. CPA was added 3 min before the application of high-K^+ solution. CPA decreased the rate of initial rise of $[Ca^{2+}]_i$. As the CPA induced a slight rise in the resting level of $[Ca^{2+}]_i$ in PSS under these conditions, curves were shifted vertically to facilitate comparison of the rate of rise of $[Ca^{2+}]_i$. The dips in the traces before the onset of $[Ca^{2+}]_i$ rise were because of movement due to flushing of solution. These results are representative of 5 muscles.

that 10 μM CPA had no effect either on the amplitude or frequency of the rhythmic contractions. However, when 300 μM CPA was added during the rhythmic contractions induced by oxytocin, we observed clear inhibitory effects, which were of two types. Sometimes, both the amplitude and frequency of $[Ca^{2+}]_i$ and tension transients gradually decreased (Figure 4b, $n = 4$). Alternatively, both responses were suppressed immediately after the CPA application (Figure 4c, $n = 11$). All inhibitory effects of CPA were reversed after washing out of the drug.

The solid symbols in Figure 2 show the concentration-dependent inhibitory effect of CPA on the rhythmic contractions induced by 10 nM oxytocin. The peak size of the rhythmic rise in tension (circle) or $[Ca^{2+}]_i$ (triangle) 10 min to 15 min after the application of CPA was normalized by the respective peak values prior to the drug application. CPA had no effect on the rhythmic contractions at or below 30 μM where significant inhibition of Ca^{2+} stores was observed. At 100 μM CPA, we observed complete inhibition in 4 samples as in Figure 4c, and in 8 muscles the peak values of tension and $[Ca^{2+}]_i$ change as well as their frequency were decreaed (Figure 4b). When there was no effect of CPA on the peak tension, we did not observe a significant effect on the frequency of rhythmic contractions. These results suggest that the Ca^{2+} stores are not directly involved in the rhythmic tension changes induced by oxytocin. At higher concentrations above 100 μM, CPA inhibited the rhythmic contractions.

Effect of CPA on high-K^+-induced contractions

We then examined whether the high concentration of CPA has any effect on the depolarization-induced $[Ca^{2+}]_i$ rise. To change solutions as quickly as possible, we placed the muscle strip in a glass capillary cuvette (see Methods). Therefore, in this type of experiment, we were not able to measure isometric tension simultaneously, but we could observe rapid change in $[Ca^{2+}]_i$ more closely. We examined the high-K induced $[Ca^{2+}]_i$ change first during a control run and then when CPA was added 3 min before the second high-K-induced depolarization. We then performed a second control run. As shown in Figure 5, the extent of the $[Ca^{2+}]_i$ change due to high-K^+-induced depolarization was almost identical with or without application of 300 μM CPA. The relative value of the response with CPA normalized by the average of the control runs obtained before and after the test run was 0.97 ± 0.06 (mean ± s.e.mean, $n = 3$). The most conspicuous effect of CPA was on the rate of rise of $[Ca^{2+}]_i$, which was reduced in the presence of 300 μM CPA. The relative value of the half time of $[Ca^{2+}]_i$ rise to the peak was 3.91 ± 0.80 ($n = 3$). This inhibition was reversed after wash out of CPA. These data suggest that high concentrations of CPA have a partial inhibitory effect on voltage-dependent Ca^{2+} channels.

Discussion

In this paper, we examined the role of Ca^{2+} stores in oxytocin-induced rhythmic contractions using CPA as a pharmacological agent to inhibit the function of Ca^{2+} stores. In the presence of 10 μM CPA during the Ca^{2+} loading, the rise of $[Ca^{2+}]_i$ due to subsequent oxytocin application was completely inhibited, but not when CPA was added after Ca^{2+} loading. Therefore, CPA ($\leqslant 10$ μM) has rather specific effects on Ca^{2+} uptake and almost no direct effect on the Ca^{2+} release mechanism *per se* or on the Ca^{2+}-induced contractile reaction. However, CPA at 10 μM did not change the oxytocin-induced rhythmic contractions (Figure 4a). These results clearly suggest that there is little contribution of Ca^{2+} stores in the rhythmic contractions of uterine smooth muscle cells induced by oxytocin.

Thapsigargin, a tumour-promoting sesquiterpene lactose, was found to discharge intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase in rat hepatocytes (Thastrup *et al.*, 1990). We also found that 10 μM thapsigargin inhibited Ca^{2+} uptake by the Ca^{2+} stores of uterine smooth muscle cells and that it did not affect the rhythmic contraction in accordance with the results obtained with CPA (data not shown). Because it was difficult to remove the effect of thapsigargin by washout, we did not carry out a systematic study using this agent.

At higher concentrations ($\geqslant 100$ μM), CPA decreased the frequency of the rhythm and reversibly inhibited the rhythmic contractions (Figures 2 and 4b). This inhibitory effect of CPA on uterine rhythmic contraction is not related to its inhibitory effect on the Ca^{2+} uptake by the stores, because the required concentration was 10 times greater than that for complete inhibition of Ca^{2+} uptake (present study). We studied the effect of CPA on the high-K^+-induced increase in $[Ca^{2+}]_i$ in order to examine any possible action on voltage-dependent Ca^{2+} channels. The rate of rise of $[Ca^{2+}]_i$ induced by high-K^+ solution was inhibited by 300 μM CPA (Figure 5). Therefore, high concentrations of CPA seem to have inhibitory effects on the voltage-dependent Ca^{2+} channels. Further studies are required to clarify whether the inhibition of the rhythmic contractions by CPA is entirely due to the inhibition of voltage-dependent Ca^{2+} channels or whether other mechanisms are involved.

Oxytocin activates phospholipase C which cleaves phosphatidyl inositol 4,5-bisphosphate into IP_3 and diacylglycerol (Marc *et al.*, 1986; 1988). Our results indicate that one of the two branches of the phosphatidyl inositol pathway, i.e. IP_3-induced Ca^{2+} release, is not directly related to the oxytocin-induced rhythmic contraction. Therefore, future studies should examine the role of either the diacylglycerol-protein kinase C pathway (Nishizuka, 1984) or of other receptor-coupled mechanisms.

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