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Characteristics of Ca^{2+} Oscillations in Ileal Longitudinal Muscle Cells of Guinea Pig

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ABSTRACT—We studied the mechanisms and characteristics of the spontaneously evoked intracellular Ca^{2+} changes (Ca^{2+} oscillations) in ileal longitudinal smooth muscle from guinea pig. Two-dimensional images of Ca^{2+} oscillations were obtained at 33-ms intervals with a Ca^{2+}-sensitive fluorescence probe, fluo-3 using the intensified CCD camera. Nicardipine (10^{-7} M) significantly decreased the maximum level of fluorescence intensity of the Ca^{2+} oscillations, inhibited the frequency of the oscillations and tended to decrease the basal level of fluorescence intensity. However, tetrodotoxin (3×10^{-7} M) did not affect these oscillations. Phorbol 12,13-dibutyrate (10^{-7} M) significantly increased the maximum level of fluorescence intensity and the frequency of Ca^{2+} oscillations, and it changed them to steady and chronometric Ca^{2+} oscillations. Cyclopiazonic acid (3×10^{-5} M) also significantly increased the frequency of Ca^{2+} oscillations. Acetylcholine (10^{-8} M) increased the basal and maximum level of fluorescence intensity and the frequency of Ca^{2+} oscillations, and accelerated their onset. The increase of basal level of fluorescence intensity was then decreased by cyclopiazonic acid treatment. These results suggest that the augmentation of Ca^{2+} oscillations is mainly due to the activation of L-type Ca^{2+} channels, which is modulated by protein kinase C, and that the emptying of intracellular Ca^{2+} stores may activate the Ca^{2+} oscillations mediated through the increase of Ca^{2+} influx in ileal smooth muscle of guinea pig.

Keywords: Ca^{2+} oscillation, Ca^{2+} channel, Protein kinase C, Ca^{2+} store, Ileal smooth muscle

Recently it has been reported that a large number of cells, including smooth muscle cells (1, 2), have Ca^{2+} oscillations or Ca^{2+} waves that are temporally and spatially organized variations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Ca^{2+} oscillations arise from the entry of Ca^{2+} across the plasma membrane through voltage-dependent Ca^{2+} channels associated with membrane depolarization or, alternatively, from the release and reuptake of Ca^{2+} in intracellular stores (3 – 5). In smooth muscle, intracellular Ca^{2+} increase is an important physiological factor for muscle contraction. In Ca^{2+}-indicator fura-2-loaded smooth muscle, some agonists induce intracellular Ca^{2+} increase, induce muscle contraction (6 – 9) and produce intracellular signaling substances. In isolated intestinal cells, stimulation of muscarinic receptors by acetylcholine (ACh) and carbachol (CCh) causes activation of non-selective cation channels, leading to membrane depolarization; this then increases the discharge rate of action potentials, and when its extent exceeds a critical level, the discharge of action potentials ceases (10, 11). Muscarinic receptor stimulation produces synchronous oscillations of $[Ca^{2+}]_i$ and membrane potential in intestinal smooth muscle cells (12, 13) and also causes Ca^{2+} release from internal stores by inositol 1,4,5-trisphosphate formed through phosphatidylinositol breakdown (14, 15). Ryanodine, which inhibits ryanodine-sensitive Ca^{2+} channels in the sarcoplasmic reticulum, and thapsigargin, which indirectly inhibits ryanodine-sensitive Ca^{2+} channels by blocking the sarcoplasmic reticulum Ca^{2+}-ATPase, inhibit Ca^{2+} sparks in cerebral arteries (16). Voltage-dependent Ca^{2+} channels also play an important role in the increase of $[Ca^{2+}]_i$ in smooth muscle cells. Protein kinase C controls the activity of L-type Ca^{2+} channels directly or indirectly (17 – 20). These events in the plasma membrane and cytoplasm would account, at least in part, for oscillatory, transient and/or sustained rises in intracellular Ca^{2+}.

In the present experiment, we observed spontaneously-producing Ca^{2+} oscillations in fluo-3-loaded longitudinal smooth muscle cells and studied the characteristics of the oscillations using pharmacological methods. Our results

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suggest that plasma membrane Ca^{2+} entry through L-type Ca^{2+} channels and activity of these channels have a critical role to play in regulating the discharge frequency as well and that the oscillations are augmented by the functions of intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Tissue preparation

Male guinea pigs, weighing 250 – 350 g, were anesthetized with an intravenous injection of pentobarbital sodium (50 mg/kg) and killed by bleeding from the carotid arteries. A longitudinal muscle strip was isolated by carefully slipping an ileal segment over a tapered glass rod. The longitudinal strips were incubated with normal physiological saline solution (PSS) gassed with 100% O_2, which contained: 145 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 10 mM *N,N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), 11 mM glucose and 10 μM ethylenediamine *N,N'*-tetraacetic acid (EDTA·2Na) (pH = 7.4 at 37°C). Ca^{2+}-free PSS was prepared without $CaCl_2$ and with 2 mM ethyleneglycol bis (β-aminoethylether) *N,N'*-tetraacetic acid (EGTA). The strips were incubated with 10 mM fluo-3/acetoxymethylester (fluo-3/AM) in normal PSS for 3 – 4 h at room temperature in the presence of 0.2% Cremophor EL and then rinsed with the solution for 15 min. They were connected to a rectangular glass rod (6-mm-wide, 5-mm-thick, 15-mm-long). Then each strip was mounted in a trough machined from a Perspex plate, containing PSS with a coverslip at the bottom. To suppress the movement of the smooth muscle cells, we added to the PSS 10 μM cytochalasin D, a capping agent of actin filaments, which inhibits smooth muscle contraction without changing $[Ca^{2+}]_i$, through uncoupling of the force generation from the activated actomyosin Mg^{2+}-ATPase (21).

Ca^{2+} imaging

Changes in fluorescence intensity were observed on the surface of longitudinal smooth muscle. The tissues were illuminated by ultraviolet light via an epifluorescence illuminator from a Xenon lamp equipped with an interference filter. Fluorescence of cells was imaged using a Nikon fluo (×20 objective). Video images were obtained using a silicon-intensified target camera (Hamamatsu Photonics, Hamamatsu), with the output digitized to a resolution of 512 × 512 pixels by Argus (Hamamatsu Photonics). Fluorescence intensities were also obtained by dividing, pixel by pixel, the 530-nm image after background subtraction. In order to minimize photobleaching, exposure to excitation light was limited during data collection (0.27 s/each collection) by an electrically controlled shutter, and neutral density filters were placed in the exciting light paths to prevent photobleaching.

Statistics

Numerical results are expressed as means ± S.E.M., and statistical significance was calculated with Duncan's new multiple range test. A P value less than 0.05 was considered to indicate a significant difference.

Drugs

The following drugs were used: fluo-3/AM, EGTA, EDTA·2Na, TPEN and HEPES (Dojindo Laboratories, Kumamoto); Cremophor EL and ACh chloride (Nacalai Tesque, Kyoto); cytochalasin D, cyclopiazonic acid, nicardipine hydrochloride, phorbol 12,13-dibutyrate (PDBu) and tetrodotoxin (Sigma Chemical Co., St. Louis, MO, USA); all in powder form. Other chemicals used were of analytical grade.

RESULTS

Ca^{2+} oscillations were spontaneously produced. Figure 1 shows typical results of an experiment; the Ca^{2+} fluorescence images of fluo-3-loaded longitudinal smooth muscle cell layers are shown in Fig. 1A. These oscillations were frequently initiated at a specific cell in a fixed location in the tissues and propagated cell-to-cell along the short axes of cells in a reproducible spatial pattern. In this area of the tissue, the increase of $[Ca^{2+}]_i$ occurred at the site indicated by colored numbers (1 or 2) and was propagated in a specific direction at a velocity of 0.2 – 0.8 $\mu m \cdot ms^{-1}$. The maximum level of fluorescence intensity from basal level in one cycle of the oscillation was obtained within 0.1 s and returned to the basal level of fluorescence intensity within 1.5 – 2.0 s. The basal and maximum level of fluorescence intensity were rather constant and remained almost unchanged in the tissue throughout the experiment. The oscillations occurred with a frequency of approximately 10 – 30 times·min^{-1}. For each of the regions (No. 1 – 6) in Fig. 1A, a graph of the changes in Ca^{2+} oscillation for 8 s is shown in Fig. 1B. These Ca^{2+} oscillations were eliminated by the application of an L-type Ca^{2+} channel blocker nicardipine (10^{-7} M) (Fig. 2); however, the sodium channel blocker tetrodotoxin (3×10^{-7} M) did not affect these oscillations (Fig. 3). Protein kinase C activator PDBu (10^{-7} M) increased the maximum level of fluorescence intensity and the frequency of Ca^{2+} oscillations, changing them to steady and chronometric Ca^{2+} oscillations (Fig. 4). The maximum level of fluorescence intensity of the Ca^{2+} oscillations increased 30% and the frequency of occurrence of the oscillations also increased approximately 30%. These Ca^{2+} oscillations were completely eliminated by the application of nicardipine (10^{-7} M). Cyclopiazonic acid

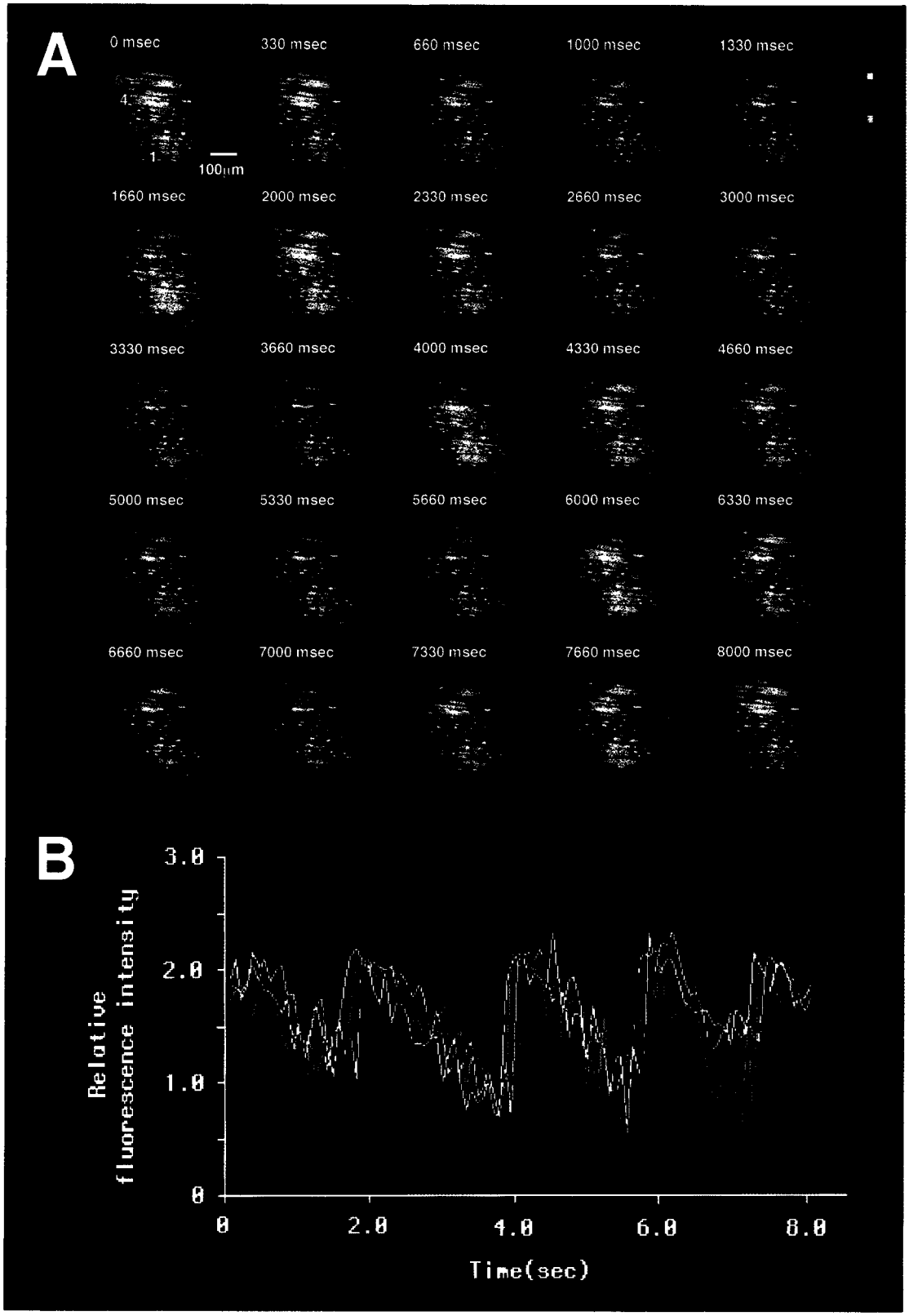

Fig. 1. Two-dimensional fluorescence images of Ca^{2+} oscillations (A) and changes in fluorescence intensity of different regions obtained every 33 ms (B) in ileal longitudinal muscle of guinea pig. The smooth muscle was loaded with fluo-3. In panel A, the color scale on the right indicates fluorescence intensity expressed as a ratio to the basal intensity of muscle cells (pseudocolor ratio image). In panel B, time course of the fluorescence changes at the sites indicated by colored numbers (1, 2, 3, 4, 5, 6) in panel A.

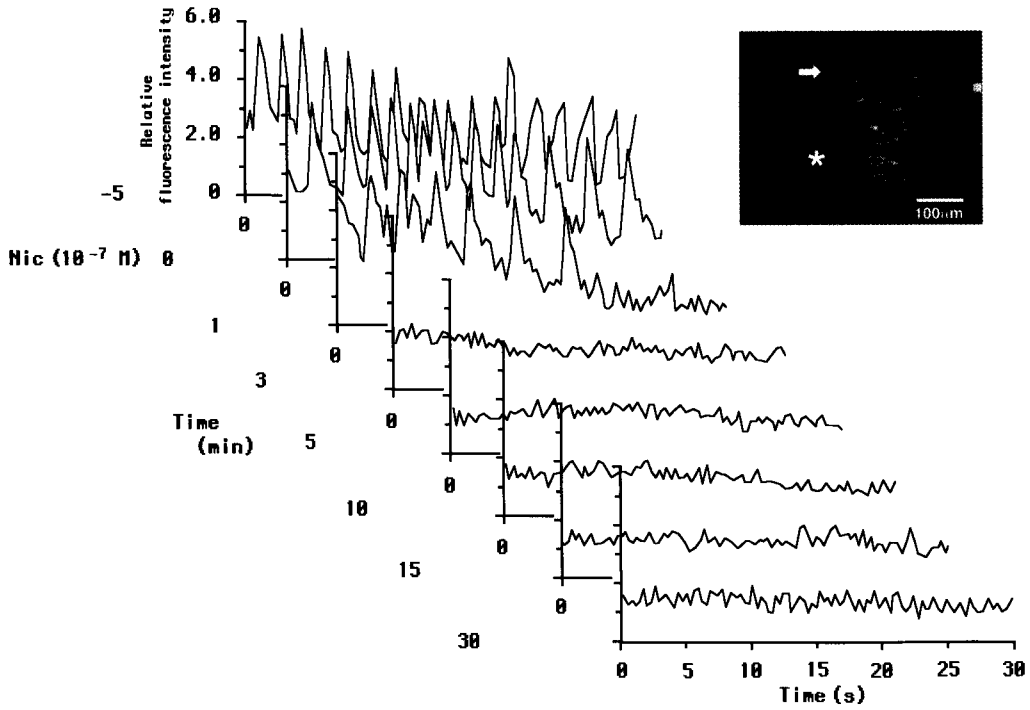

Fig. 2. Inhibitory effect of nicardipine (Nic, 10^{-7} M) on Ca^{2+} oscillations in ileal longitudinal muscle of guinea pig. Fluorescence intensity was quantified every 330 ms. Inset, the asterisk indicates the pacemaker site of Ca^{2+} oscillations. The arrow indicates the detective cells of Ca^{2+} oscillations.

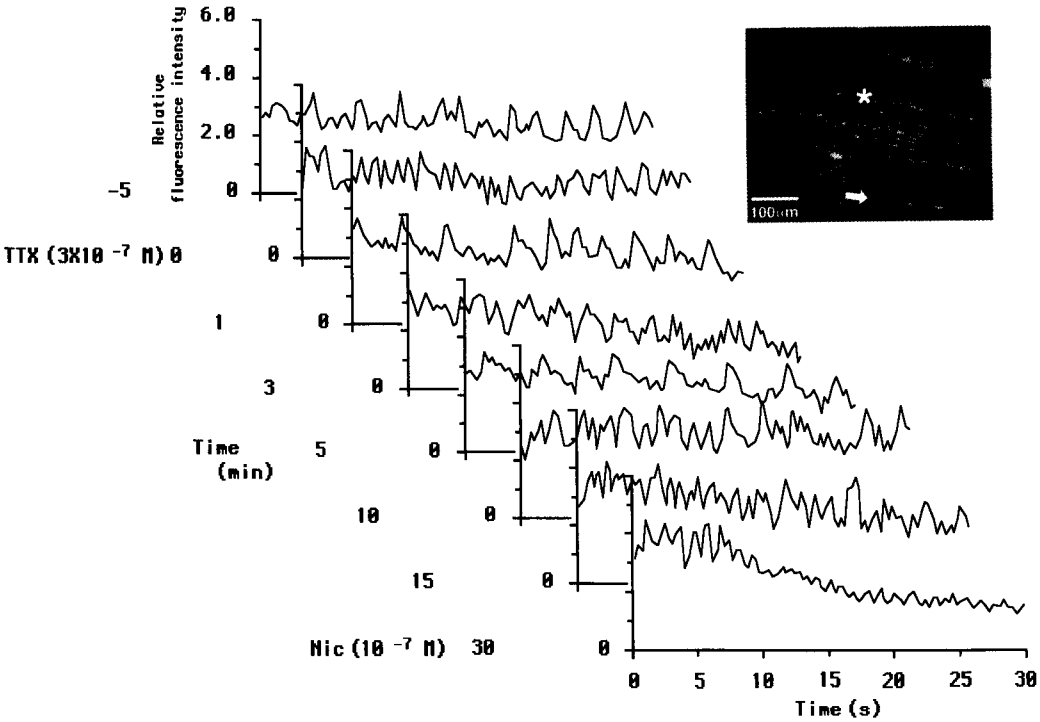

Fig. 3. Influence of tetrodotoxin (TTX, 3×10^{-7} M) on Ca^{2+} oscillations in ileal longitudinal muscle of guinea pig. Fluorescence intensity was quantified every 330 ms. Nicardipine (Nic, 10^{-7} M) was added at 30 min. Inset, the asterisk indicates the pacemaker site of Ca^{2+} oscillations. The arrow indicates the detective cells of Ca^{2+} oscillations.

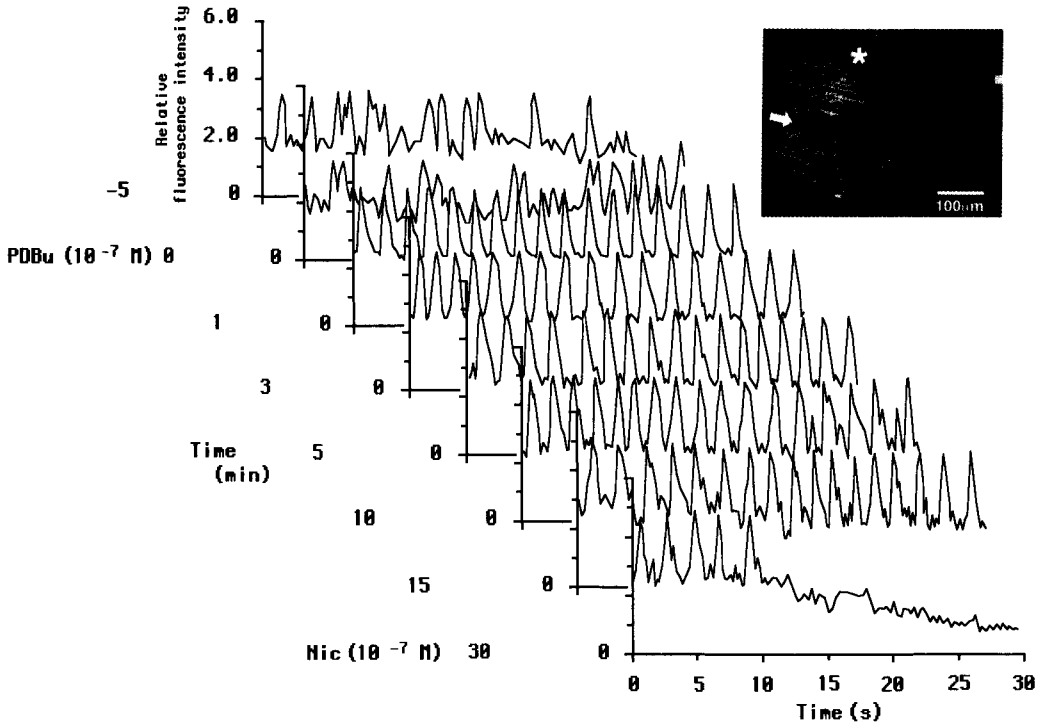

Fig. 4. Effect of phorbol 12,13-dibutyrate (PDBu, 10^{-7} M) on Ca^{2+} oscillations in ileal longitudinal muscle of guinea pig. Fluorescence intensity was quantified every 330 ms. Nicardipine (Nic, 10^{-7} M) was added at 30 min. Inset, the asterisk indicates the pacemaker site of Ca^{2+} oscillations. The arrow indicates the detective cells of Ca^{2+} oscillations.

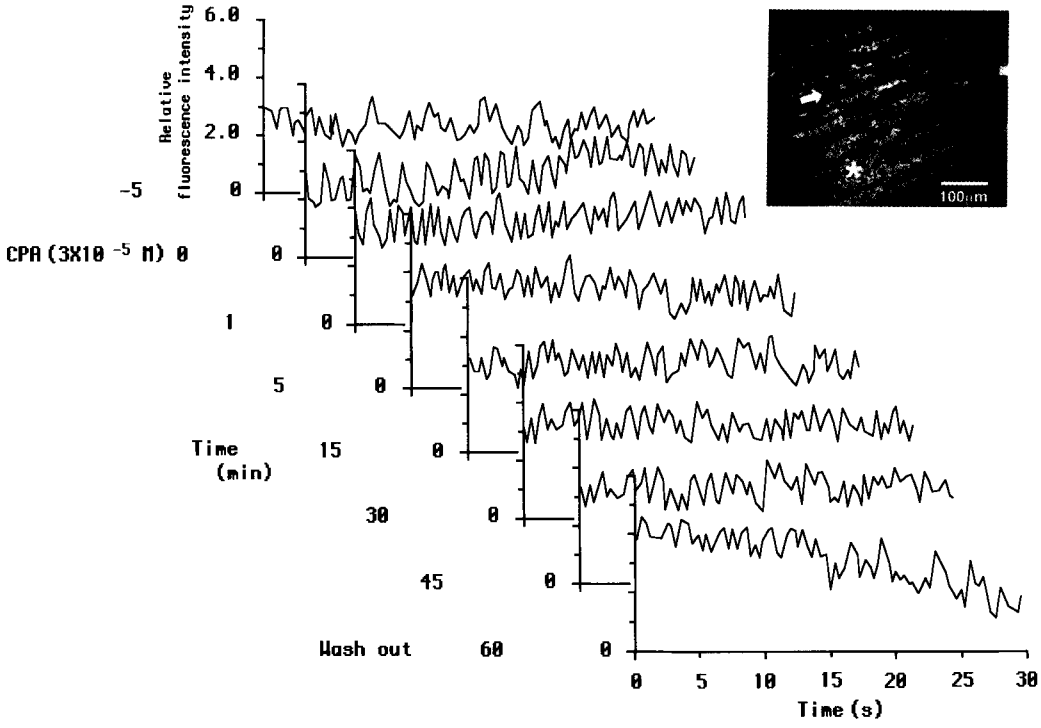

Fig. 5. Effect of cyclopiazonic acid (CPA, 3×10^{-5} M) on Ca^{2+} oscillations in ileal longitudinal muscle of guinea pig. Fluorescence intensity was quantified every 330 ms. The preparation was washed out at 45 min. Inset, the asterisk indicates the pacemaker site of Ca^{2+} oscillations. The arrow indicates the detective cells of Ca^{2+} oscillations.

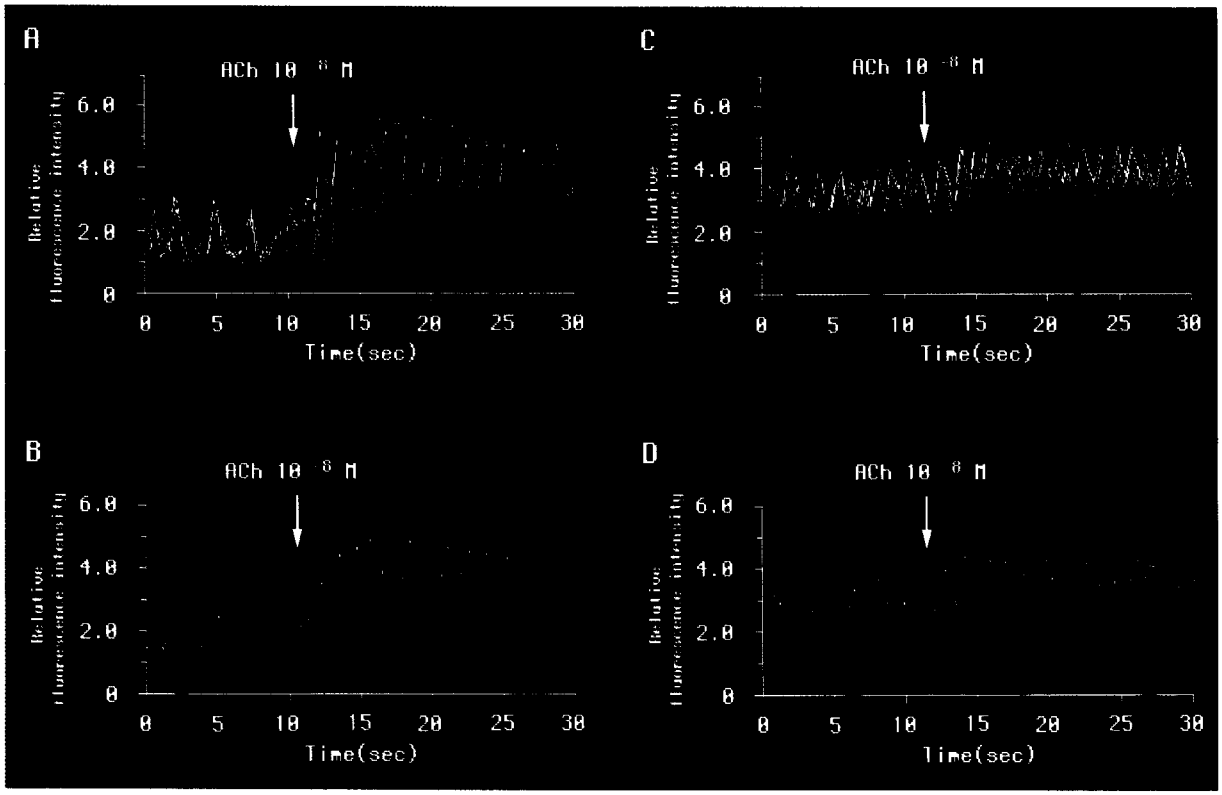

Fig. 6. Effect of acetylcholine (ACh, 10^{-8} M) (A and B) on Ca^{2+} oscillations and inhibitory effect of cyclopiazonic acid (CPA, 3×10^{-5} M) (C and D) on ACh-induced augmentation of Ca^{2+} oscillations in ileal longitudinal muscle of guinea pig. Fluorescence intensities in seven different regions were quantified every 330 ms, and each color indicates different region (A and C). The intensities (B and D) were averaged over all in the imaging field obtained from panels A and C, respectively. CPA was added 30 min before the application of ACh.

Table 1. Effects of nicardipine, tetrodotoxin, phorbol 12,13-dibutyrate, cyclopiazonic acid and acetylcholine on basal and maximum level of fluorescence intensity, amplitude and frequency of Ca^{2+} oscillation in ileal longitudinal muscle cells of guinea pig

	n	Fluorescence intensity		Amplitude	Frequency (Hz)
		Basal	Maximum		
Untreated	4	1.39 ± 0.30	3.51 ± 0.77	2.03 ± 0.47	0.45 ± 0.07
Nic	4	1.07 ± 0.29	1.33 ± 0.33*	0.27 ± 0.15*	—
TTX	4	1.57 ± 0.34	3.51 ± 0.75	1.93 ± 0.38	0.43 ± 0.05
PDBu	4	2.33 ± 0.33	4.73 ± 0.37*	2.40 ± 0.35	0.58 ± 0.04*
CPA	4	2.38 ± 0.11	3.93 ± 0.18	1.55 ± 0.18	0.71 ± 0.09*
ACh	4	3.10 ± 0.38*	4.93 ± 0.52*	1.80 ± 0.44	0.72 ± 0.03*

Amplitude: [Maximum – Basal] of fluorescence intensity. Values are means ± S.E.M. Nic, 10^{-7} M nicardipine; TTX, 3×10^{-7} M tetrodotoxin; PDBu, 10^{-7} M phorbol 12,13-dibutyrate; CPA, 3×10^{-5} M cyclopiazonic acid; ACh, 10^{-8} M acetylcholine. *$P<0.05$, compared with untreated preparation (Duncan's new multiple range test).

(3×10^{-5} M), which interferes with sarcoplasmic reticulum function, increased the frequency of Ca^{2+} oscillations (Fig. 5). A muscarinic cholinoceptor agonist, ACh (10^{-8} M) significantly increased the basal level of fluorescence intensity and increased the frequency of Ca^{2+} oscillations (Fig. 6: A and B). As shown in Fig. 6, C and D, with pretreatment by cyclopiazonic acid, both the basal increases and augmentation of Ca^{2+} oscillations were decreased. Basal and maximum level of fluorescence intensity, amplitude and frequency of Ca^{2+} oscillation are summarized in Table 1.

DISCUSSION

In the present study, the characteristics of Ca^{2+} oscillation were investigated in isolated longitudinal smooth muscle of the guinea pig ileum using a Ca^{2+}-sensitive fluorescent dye, fluo-3. The Ca^{2+} oscillations produced in smooth muscle were spontaneously made up of rapid brief increases in $[Ca^{2+}]_i$. The oscillations were frequently initiated at a specific cell in a fixed location in the tissue and propagated cell-to-cell along the short axes of cells in a reproducible spatial pattern. The results in this study provide evidence that the production of Ca^{2+} oscillations can be affected by membrane Ca^{2+} entry in guinea pig ileal longitudinal smooth muscle. In guinea pig ileal longitudinal muscle, protein kinase C plays an important role in the increase of Ca^{2+} sensitivity (22) and the positive regulation of Ca^{2+} channel activation (23) in muscle contraction. As shown in Fig. 4, a protein kinase C activator PDBu (10^{-7} M) strongly enhanced the Ca^{2+} oscillations, which were inhibited by nicardipine. A number of researchers reported that in many tissues, protein kinase C controls the activity of L-type Ca^{2+} channels (17–20). In vascular cells, Ca^{2+} current through L-type Ca^{2+} channels was increased by the activation of protein kinase C (24), and this was blocked by the selective protein kinase C inhibitor staurosporine or chelerythrine (19), suggesting that protein kinase C activates L-type Ca^{2+} channels directly or indirectly. In pig detrusor smooth muscle, Uchida et al. (20) reported that tonic contractile response is mediated mainly by activation of protein kinase C and their contractions are abolished by pretreatment with H-7; these observations suggested that protein kinase C opens voltage-dependent Ca^{2+} channels, in part through inactivation of ATP-sensitive potassium channels and depolarization of the smooth muscle cell membrane. Also, Bonev and Nelson (25) reported that a potassium channel opener did not affect the KCl-induced contraction and that a muscarinic receptor stimulator inhibited ATP-sensitive potassium channels through activation of protein kinase C using an electrophysiological method. In the present study, PDBu produced potentiation of Ca^{2+} oscillations. This effect seems to be caused by the opening of L-type Ca^{2+} channels activated by protein kinase C.

In addition to a Ca^{2+} increasing mechanism mediated through L-type Ca^{2+} channels, the application of drugs such as cyclopiazonic acid that inhibit the endoplasmic reticulum Ca^{2+} pump also increases $[Ca^{2+}]_i$ by the acceleration of Ca^{2+} influx via channels that are activated by the depletion of intracellular Ca^{2+} stores (26–28). And as shown in Fig. 5, the application of cyclopiazonic acid (3×10^{-5} M) produced augmentation of Ca^{2+} oscillations. On the other hand, the pretreatment with cyclopiazonic acid decreased ACh-induced increase of the basal level of fluorescence intensity (Fig. 6), indicating that Ca^{2+} in intracellular Ca^{2+} stores of smooth muscle cells is depleted by cyclopiazonic acid treatment (3×10^{-5} M). Observations in mouse anococcygeous (26, 29), rat aorta (30) and several types of cells (27, 31) indicate that depletion of intracellular Ca^{2+} stores using cyclopiazonic acid, thapsigargin and ryanodine increases Ca^{2+} entry from extracellular sources via voltage-insensitive cation channels, that is to say, through capacitative Ca^{2+} entry. The presence of a Ca^{2+}-permeant channel that is activated by depletion of intracellular Ca^{2+} stores by CCh or caffeine has also been suggested in guinea pig jejunal cells (32) and rat ileal cells (33). In our preliminary experiment, the capacitative Ca^{2+} entry blocker SK&F96365 (10^{-5} M) inhibited the cyclopiazonic acid-induced augmentation of Ca^{2+} oscillations (data not shown). These findings lead us to assume that the increase of Ca^{2+} oscillations produced by cyclopiazonic acid might be due to this capacitative Ca^{2+} entry and to assume that the augmentation of the Ca^{2+} entry induced by Ca^{2+} depletion in Ca^{2+} stores is involved in the modulation of the frequency of Ca^{2+} oscillations.

As we have mentioned before, intracellular Ca^{2+} changes were produced in smooth muscle tissue spontaneously. The oscillations seem to be frequently initiated at a cell in a fixed location in the tissue and propagated to adjacent cells. Although oscillation of $[Ca^{2+}]_i$ is similar to intercellular Ca^{2+} oscillations in secretory cells such as salivary gland reported by Zimmermann and Walz (34), generating mechanisms of Ca^{2+} oscillation in ileal smooth muscle may be different from those of other cells such as secretory cells and endothelium (35, 36). This is because Ca^{2+} oscillations observed in the present study seem to be divided into two steps: the first step is initiation of Ca^{2+} oscillation, and the second is propagation of intracellular Ca^{2+} increases to other cells. In intestinal tissues, the origin of Ca^{2+} oscillation may be pacemaker cells (Cajal cells) that produce membrane depolarization by Ca^{2+} release from the sarcoplasmic reticulum, including inositol 1,4,5-trisphosphate- and Ca^{2+}-induced Ca^{2+} release (37). Spontaneous activation of each pacemaker cell stimulates the adjacent smooth muscle cells and intracellular Ca^{2+} increases propagate to other cells. Since Ca^{2+} oscillations travel over a distance >400 μm with no decrease in velocity or amplitude, propagation of Ca^{2+} oscillations involves the regulation of the messenger and is clearly not a result of passive diffusion. As these longitudinal smooth muscle cells were tightly connected by gap junctions to behave as a functional continuum, Ca^{2+} may diffuse through gap junctions to change $[Ca^{2+}]_i$ and membrane potentials. Wang and Thompson (38) suggested that rapid Ca^{2+} diffusion is a key process in the propagation of Ca^{2+} oscillations and proposed that the close apposition of gap junctions and Ca^{2+} release sites in neighboring cells is important. In

cardiac single cells, Ca^{2+} sparks, which are microscopic elevations in $[Ca^{2+}]_i$, are triggered from the activation of sarcoplasmic reticulum Ca^{2+} release channels (39). They are evoked by the activation of L-type Ca^{2+} channels in the t-tubular membrane and are inhibited by L-type Ca^{2+} channel blockers (40, 41). In our study, the Ca^{2+} oscillations did not propagate to other cells in the presence of nicardipine. These findings suggest the possibilities that L-type Ca^{2+} channels are related to the production of Ca^{2+} oscillations in pacemaker cells and/or the propagation of Ca^{2+} oscillations in smooth muscle cells. Further studies are obviously needed, however, to substantiate the oscillatory mechanisms of $[Ca^{2+}]_i$ in pacemaker cells and the propagating mechanisms in smooth muscle cells.

In summary, the mechanisms underlying Ca^{2+} oscillations are not due to the depolarization induced by activation of sodium channels, but the activation of L-type Ca^{2+} channels regulated by protein kinase C is important for propagation of Ca^{2+} oscillations in guinea pig ileum longitudinal muscle cells. Our present data suggest the possibility of an additional mechanism in which the entry of extracellular Ca^{2+} via voltage-insensitive cation channels, which is induced by the depletion of intracellular Ca^{2+} stores (capacitative Ca^{2+} entry), underlies the production of Ca^{2+} oscillations.

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