Carbachol-induced $[Ca^{2+}]_i$ oscillations in single smooth muscle cells of guinea-pig ileum

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1. Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by carbachol (CCh) were measured in single smooth muscle cells of guinea-pig ileum using a Ca^{2+}-sensitive fluorescent dye, fura-2, to clarify the underlying mechanisms of muscarinic $[Ca^{2+}]_i$ oscillations.

2. Half of the cells, when exposed to 0·2 μM CCh, exhibited repeated changes in $[Ca^{2+}]_i$ giving a serrated appearance. The oscillatory changes in $[Ca^{2+}]_i$ were very similar to those evoked by increasing extracellular K^+ concentration ($[K^+]_o$) to 30 mM, which were abolished by removal of extracellular Ca^{2+}, nifedipine and La^{3+}, but remained unchanged after depletion of internal Ca^{2+} stores with cyclopiazonic acid, thapsigargin and ryanodine.

3. Every individual $[Ca^{2+}]_i$ oscillation was just like a $[Ca^{2+}]_i$ increase generated spontaneously in about 8% of cells or triggered by an action potential evoked by a current pulse in current-clamped cells.

4. In the remaining half of the cells exposed to 0·2 μM CCh, slower $[Ca^{2+}]_i$ oscillations were elicited and every individual $[Ca^{2+}]_i$ oscillation was always preceded by the fast brief increase in $[Ca^{2+}]_i$.

5. $[Ca^{2+}]_i$ oscillations elicited by 2 μM CCh were temporally and functionally distinct from those induced by high $[K^+]_o$. They were more or less regular in the periodicity and pattern, comprised pacemaker potential-like $[Ca^{2+}]_i$ increases or sinusoidal types of $[Ca^{2+}]_i$ increases, and could be elicited even in 100 mM K_o^+.

6. Removal of extracellular Ca^{2+} or application of nifedipine, methoxyverapamil (D600), diltiazem or La^{3+} during CCh (2 μM)-induced $[Ca^{2+}]_i$ oscillations caused them to disappear. In cells in which internal Ca^{2+} stores were depleted, 2 μM CCh did not evoke $[Ca^{2+}]_i$ oscillations but occasionally induced single or repeated generation of the increase in $[Ca^{2+}]_i$ with a serrated appearance.

7. The results indicate that CCh can induce two types of $[Ca^{2+}]_i$ oscillation in guinea-pig ileal smooth muscle cells; one arises from Ca^{2+} influx associated with action potential discharges and the other from periodic release of Ca^{2+} from internal stores. The latter $[Ca^{2+}]_i$ oscillation requires extracellular Ca^{2+} to sustain it.

Many different kinds of cell exhibit oscillating changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) which usually occur in response to hormones and neurotransmitters, and sometimes spontaneously. Because of the ubiquitous occurrence of $[Ca^{2+}]_i$ oscillations and the existence of cellular functions which are mediated by an increase in $[Ca^{2+}]_i$, possible roles for the $[Ca^{2+}]_i$ oscillations have been suggested (Tsien & Tsien, 1990). $[Ca^{2+}]_i$ oscillations can arise from entry of Ca^{2+} across the plasma membrane through voltage-gated Ca^{2+} channels associated with membrane depolarization or, alternatively, from release and reuptake of Ca^{2+} in intracellular stores (Berridge & Galione, 1988; Berridge, 1990). The explanation for the underlying mechanisms of the latter type of $[Ca^{2+}]_i$ oscillation is different among different cell types, but a common feature is the involvement of inositol 1,4,5-trisphosphate (IP_3) as a

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Ca^{2+}-releasing second messenger (for reviews see Tsien & Tsien, 1990; Meyer & Stryer, 1991; Fewtrell, 1993).

Single isolated smooth muscle cells from the intestine discharge action potentials spontaneously or in response to depolarizing pulses and stimulatory substances (Benham, Bolton & Lang, 1985; Inoue & Isenberg, 1990*a*, *b*). The upstroke of the action potentials is known to be due to an explosive activation of voltage-gated Ca^{2+} channels in the plasma membrane (Bolton, 1979; Tomita, 1981). Thus, in such cells, the discharge pattern of action potentials could determine $[Ca^{2+}]_i$ oscillations. 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 (Benham *et al.* 1985; Inoue & Isenberg, 1990*a*). Furthermore, ACh and CCh suppress the inward Ca^{2+} current evoked by stepping the membrane potential to a depolarized level (Russell & Aaronson, 1990; Unno, Komori & Ohashi, 1995). These muscarinic receptor-mediated electrical events in the plasma membrane would account, at least in part, for oscillatory and/or sustained rises in $[Ca^{2+}]_i$.

Stimulation of muscarinic receptors also causes Ca^{2+} release from internal stores by IP_3 formed through phosphatidylinositol breakdown (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989; Komori & Bolton, 1990, 1991). Our recent studies on the changes in $[Ca^{2+}]_i$ in single smooth muscle cells of guinea-pig ileum (Komori, Kawai, Pacaud, Ohashi & Bolton, 1993; Zholos, Komori, Ohashi & Bolton, 1994) showed that CCh induces oscillatory changes in $[Ca^{2+}]_i$ brought about by periodic release of Ca^{2+} stores via an IP_3-dependent mechanism under voltage-clamp conditions.

Now, questions arise as to whether or not such Ca^{2+} store-dependent $[Ca^{2+}]_i$ oscillations can be demonstrated in a more physiological setting and how they contribute to the overall muscarinic receptor-mediated Ca^{2+} signalling in intestinal smooth muscle cells.

In this paper, we estimated $[Ca^{2+}]_i$ after application of CCh, electrical stimuli and introduction of high $[K^+]_o$ by measuring fluorescence emission from fura-2, a Ca^{2+}-sensitive dye, in single cells from ileal muscle of the guinea-pig, and characterized the change of $[Ca^{2+}]_i$ produced by stimulation of muscarinic receptors. The results show that muscarinic receptor activation induces $[Ca^{2+}]_i$ oscillations in two different ways: one originating in the plasma membrane and associated with action potential discharge due to depolarization, the other originating in internal Ca^{2+} stores, in which IP_3 plays an essential role, suggesting the existence of a population of Ca^{2+} channels with different properties from those responsible for action potentials, to sustain the Ca^{2+} store-dependent $[Ca^{2+}]_i$ oscillations.

METHODS

Preparation of cells

Male guinea-pigs, weighing 350–450 g, were stunned and killed by exsanguination. A 15 cm length of the ileum was removed and divided into three 5 cm segments. The longitudinal muscle layer of the intestinal segments was peeled from the underlying circular muscle and washed in physiological salt solution (PSS; composition given below). The three muscle layers were cut into ~0·5 cm^2 pieces, placed in a test-tube containing 3 ml Ca^{2+}-free PSS and allowed to equilibrate for 10 min at 37 °C. The Ca^{2+}-free PSS in the test-tube was replaced with 1 ml PSS containing 30 μM Ca^{2+} (low-Ca^{2+} PSS). Two successive 15 min incubations at 37 °C were carried out in fresh low-Ca^{2+} PSS containing collagenase (0·2–0·6 mg ml^{-1}), papain (0·3–0·6 mg ml^{-1}) and bovine serum albumin (5 mg ml^{-1}). After this enzyme digestion, the enzyme solution was removed. Tissue pieces were then placed in 2 ml PSS containing 120 μM Ca^{2+}, and agitated by drawing in and out of a blunt glass pipette 40–50 times. The solution in which isolated cells were suspended was removed and retained. The procedure was repeated several times at room temperature (22–25 °C). The cell-rich solutions obtained were combined and centrifuged at ~700 r.p.m. for 2 min. The cells were suspended in PSS containing 0·5 mM Ca^{2+}, and 0·1 ml aliquots were placed on the central part of glass coverslips (20 mm diameter), and allowed to settle at room temperature for 20–30 min in a moist atmosphere.

Fura-2 loading of cells

Fura-2 loading of cells was achieved by placing the coverslips in a darkroom kept at 25 °C for 30 min after replacing the medium with a Ca^{2+} (0·5 mM)- and fura-2 acetoxymethyl ester (fura-2 AM; 2 μM)-containing PSS (~0·1 ml volume). Fura-2-loaded cells were stored in a refrigerator at 4 °C in a moist atmosphere and were used for experiments within 8 h after fura-2 loading.

Measurement of $[Ca^{2+}]_i$

A shallow chamber (0·5 ml volume) for viewing the fura-2-loaded cells was formed by placing a hole (1·8 cm diameter), which was drilled in the centre of the base of a plastic dish (11 cm diameter; 2 mm wall thickness), over one of the prepared coverslips (see above), and sealed by means of silicon grease between the base of the dish and the coverslip. The dish was then mounted on the stage of an inverted fluorescence microscope (Olympus IMT-2, Tokyo). For chamber medium exchange, two fine polyethylene inlet and outlet tubes were fixed to the chamber and each connected to a syringe. The chamber was perfused with 5–10 ml PSS to wash away fura-2 AM and contaminants in the cell suspension, then filled with fresh PSS and left for about 10 min to equilibrate before starting experiments. A new coverslip was used for each experiment. $[Ca^{2+}]_i$ was estimated from fura-2 fluorescence by the ratio method using dual-wavelength excitation (340 and 380 nm) and single-wavelength emission (510 nm) (Grynkiewicz, Poenie & Tsien, 1985). With the two excitation wavelengths alternating at 100 Hz, fluorescence was measured at room temperature (22–25 °C) using a Ca^{2+} microspectrometric system (model OSP-3; Olympus, Tokyo) with a ×40 objective lens. Fluorescent light was collected from the whole area of a single observed cell and counted by a photomultiplier tube through a bandpass filter (510 ± 30 nm). Counts of the light at 340 nm (F_{340}) and 380 nm excitation (F_{380}) were sampled at 20 Hz and the F_{340}/F_{380} ratio was calculated after background subtraction at each wavelength. $[Ca^{2+}]_i$ was calculated from this ratio according to the

following formula (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R).$$

The maximum fluorescence ratio (R_{max}) was obtained by exposing cells to a 50 μM concentration of a Ca^{2+} ionophore, ionomycin, in PSS (containing 2 mM Ca^{2+}). Immediately after determining R_{max}, the solution was replaced with a Ca^{2+}-free solution containing 50 μM ionomycin and 5 mM EGTA, and the minimum fluorescence ratio (R_{min}) was determined. The fluorescence ratio of F_{380} in Ca^{2+}-free solution to that in PSS (β) was determined in the experiments mentioned above. Mean values for R_{min}, R_{max} and β were $0{\cdot}34 \pm 0{\cdot}02$, $4{\cdot}28 \pm 0{\cdot}21$ and $2{\cdot}94 \pm 0{\cdot}33$ ($n = 6$), respectively. These mean values and the dissociation constant (K_d) for the Ca^{2+}–fura-2 complex, taken to be 224 nM (Grynkiewicz *et al.* 1985), were used for calculation of $[Ca^{2+}]_i$.

Simultaneous measurements of $[Ca^{2+}]_i$ and membrane potentials

For simultaneous measurements of $[Ca^{2+}]_i$ and membrane potentials, fura-2-loaded cells were held under current clamp by using the nystatin perforated-patch technique (Wakamori, Hidaka & Akaike, 1993). In brief, a cell-attached patch configuration was established using a patch pipette with a resistance of 5 MΩ and filled with a KCl-based solution (composition given below) containing nystatin (0·2 mg ml^{-1}), and then a 200 ms hyperpolarizing step pulse (10 mV in amplitude) was applied every 1 s under the voltage-clamp conditions with a holding potential of −40 mV. After capacitative current, in response to the voltage step pulse, reached a maximum level over a period of 10–20 min, the voltage-clamp mode was replaced with the current-clamp mode to record changes in membrane potential. Measurements of the membrane potential via a patch-clamp amplifier (CEZ-2400; Nihon Kohden, Tokyo), were stored on a PCM data recorder (RD-111T; TEAC, Musashino City, Tokyo) and illustrated using a thermal array recorder (RTA-1100M; Nihon Kohden) for analysis and illustration.

Values in text are given as means ± S.E.M. Statistical significance was tested using Student's unpaired t test and differences were considered significant when $P < 0{\cdot}05$.

Application of drugs

Drugs were applied to cells by replacing the chamber medium with the drug-containing solution 5–7 times during a period of less than 10 s.

For brief exposure to high $[K^+]_o$, a micropipette with a tip diameter of 2·5–5·0 μm was filled with high-K^+ (100 mM) solution (see below) and placed within 10 μm of the cell of interest. The pipette was connected to a nitrogen cylinder by a polyethylene tube via a three-way solenoid valve (UMGI-T1; CKD Corp., Nagoya, Japan), so that the high-K^+ solution was ejected by pressure pulses (1·5–2·0 kg cm^{-2}) of 50–100 ms duration. The pulse duration and interpulse interval were controlled by a stimulator (SEN-3013; Nihon Kohden).

Solutions and drugs

The normal PSS used in the experiments had the following composition (mM): NaCl, 134; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1·2; glucose, 14; and Hepes, 10·5 (adjusted to pH 7·2 with NaOH). Solutions with increased K^+ concentration (20–100 mM; high-K^+ solutions) were obtained by replacing Na^+ in PSS with an equivalent amount of K^+. Ca^{2+}-free solutions, except for those used for cell dispersion (see above), always contained 2 mM EGTA and no added Ca^{2+}. The KCl-based solution used as pipette solution for current-clamp experiments had the following composition (mM): KCl, 134; Hepes, 10·5 (adjusted to pH 7·2 with KOH), to which nystatin dissolved in methanol (5 mg ml^{-1}) was added to give a final concentration of 0·2 mg ml^{-1}.

Drugs used were diltiazem, methoxyverapamil (D600), cyclopiazonic acid, thapsigargin, papain, collagenase (Type XI), nystatin and ionomycin, all of which were purchased from Sigma; caffeine, EGTA, ryanodine and bovine serum albumin (Wako, Osaka, Japan); carbachol chloride and nifedipine (Tokyo Kasei, Tokyo); and fura-2 AM (Dojin Kagaku, Kumamoto, Japan). All other reagents were of the highest grade commercially available.

RESULTS

Most cells (92%) showed a steady level of $[Ca^{2+}]_i$ in PSS. The steady level of $[Ca^{2+}]_i$ varied among cells from 20 to 70 nM with a mean of $31{\cdot}0 \pm 0{\cdot}7$ nM ($n = 200$), which was a few times lower than most of the corresponding values measured in other smooth muscle cells (e.g. 82 ± 8 nM in rat aorta, Hassid, 1986; 144 ± 28 nM in canine stomach, Vogalis, Publicover, Hume & Sanders, 1991).

A small fraction of cells (8%) repeated an increase in $[Ca^{2+}]_i$ with a serrated appearance (hereafter referred to as a serrated increase in $[Ca^{2+}]_i$) at varied intervals ranging from a few seconds to 60 s, as shown in Fig. 1*A*. The serrated increases in $[Ca^{2+}]_i$ occurred as a sudden transition from a basal level of $36{\cdot}2 \pm 2{\cdot}8$ nM ($n = 14$), reached a peak in 0·1–0·3 s (time to peak) and then declined to the basal level in 3–5 s. Occasionally a subsequent rise in $[Ca^{2+}]_i$ occurred during the falling phase of the preceding rise. The maximum rate of rise of $[Ca^{2+}]_i$ was $645{\cdot}9 \pm 60{\cdot}7$ nM s^{-1} ($n = 14$) and the amplitude, measured as the difference in $[Ca^{2+}]_i$ between the basal level and the peak, varied among cells from 20 to 100 nM with a mean of $44{\cdot}1 \pm 4{\cdot}7$ nM ($n = 14$). However, the amplitude was rather constant and remained almost unchanged throughout the experiment in one cell. When two or more serrated $[Ca^{2+}]_i$ increases occurred in a shorter period than the duration of a single serrated $[Ca^{2+}]_i$ increase (3–5 s), these summated to produce a larger stepwise increase in $[Ca^{2+}]_i$. It seems likely that the spontaneously generated $[Ca^{2+}]_i$ increases are caused by action potentials.

$[Ca^{2+}]_i$ oscillations induced by high levels of extracellular K^+

To see how $[Ca^{2+}]_i$ is affected by prolonged membrane depolarization which evokes action potentials or increases their discharge rate, the medium (normal PSS, 6 mM K^+) bathing cells was replaced with a high-K^+ solution containing 30 mM KCl (see Methods).

When a cell showing a steady level of $[Ca^{2+}]_i$ was exposed to high-K^+ (30 mM) solution, it exhibited repetitive discharge of a rapid brief increase in $[Ca^{2+}]_i$ superimposed on sustained

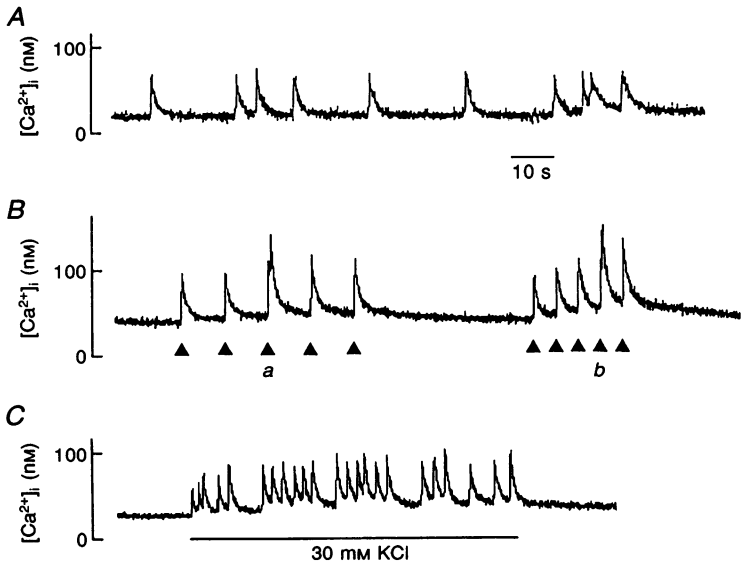

Figure 1. Spontaneously generated and high $[K^+]_o$-induced increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single smooth muscle cells from the longitudinal muscle layer of guinea-pig ileum

A, serrated increases in $[Ca^{2+}]_i$ generated spontaneously, with a rapid rising phase, a slow falling phase and a stable amplitude. *B*, $[Ca^{2+}]_i$ increases in response to brief high $[K^+]_o$ exposures which were achieved by ejection of 100 mM K^+ solution from a micropipette by pressure pulses of 50 ms duration (▲). In response to brief high $[K^+]_o$ exposures indicated by *a* and *b*, a brief $[Ca^{2+}]_i$ increase was superimposed by the next one occurring immediately after it reached a peak, which gave rise to a compound $[Ca^{2+}]_i$ response. *C*, brief $[Ca^{2+}]_i$ increases induced by bath application of 30 mM K^+. Time calibration in *A* is also applicable to *B* and *C*. *A–C* are from three different cells.

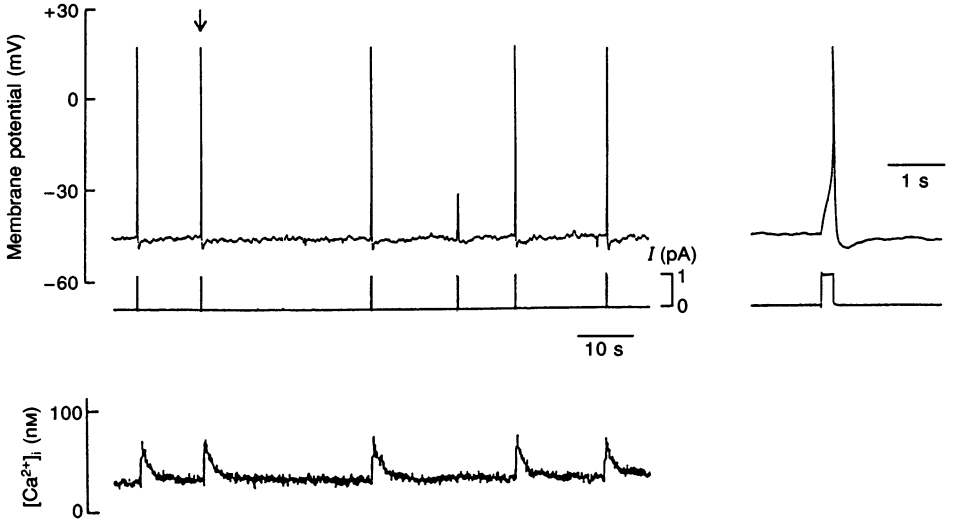

Figure 2. $[Ca^{2+}]_i$ increases associated with action potentials

Simultaneous records of changes in membrane potential (top trace) and $[Ca^{2+}]_i$ (bottom trace) from a cell held under current-clamp mode. Only when a depolarizing current pulse of 200 ms duration and 1 pA amplitude (middle trace) evoked an action potential, did a rapid brief $[Ca^{2+}]_i$ increase occur. The inset to the right shows a time-expanded trace of the section indicated (arrow). Note the similarity of the rapid brief $[Ca^{2+}]_i$ increase following an action potential to the $[Ca^{2+}]_i$ increases generated spontaneously or by exposure to high $[K^+]_o$ (see Fig. 1).

elevated level of $[Ca^{2+}]_i$, as illustrated in Fig. 1*C*. The amplitude and rate of rise of individual brief $[Ca^{2+}]_i$ increases remained more or less constant during the exposure to 30 mM K^+ (duration > 1 min), and were consistent with those of the spontaneously elicited $[Ca^{2+}]_i$ increases. The falling phase of brief $[Ca^{2+}]_i$ increases superimposed on an elevated level of $[Ca^{2+}]_i$ was somewhat shorter than that of those arising from the basal level of $[Ca^{2+}]_i$. The discharge rate of brief $[Ca^{2+}]_i$ increases and the sustained elevated level of $[Ca^{2+}]_i$ varied from one cell to another. Sometimes two to four of these $[Ca^{2+}]_i$ increases were summated to produce a larger stepwise increase in $[Ca^{2+}]_i$ (a compound $[Ca^{2+}]_i$ response; see Fig. 4*A*). During a later period of 20–30 s the mean discharge rate was $1{\cdot}00 \pm 0{\cdot}24$ Hz ($n = 9$). After that the interval between two successive $[Ca^{2+}]_i$ increases was prolonged as the elevated level of $[Ca^{2+}]_i$ declined. On reintroduction of normal PSS, the discharge of brief $[Ca^{2+}]_i$ increases ceased and the initial basal level of $[Ca^{2+}]_i$ was restored.

In cells which spontaneously exhibited $[Ca^{2+}]_i$ oscillations with serrated $[Ca^{2+}]_i$ increases in normal PSS, exposure to high $[K^+]_o$ (30 mM) increased the discharge rate of $[Ca^{2+}]_i$ oscillations but had no or little increasing effect on their amplitude.

When the bath medium (see Methods) was replaced with Ca^{2+}-free solution during prolonged exposure to high $[K^+]_o$ (30 mM), the oscillatory change in $[Ca^{2+}]_i$ was abolished immediately and $[Ca^{2+}]_i$ returned to the initial basal level ($n = 6$). Substantially similar results were obtained with the Ca^{2+} channel blockers nifedipine (1 μM; $n = 6$) and La^{3+} (10 μM; $n = 5$) (data not shown).

A rapid brief increase in $[Ca^{2+}]_i$, just like the serrated $[Ca^{2+}]_i$ increase generated spontaneously, was produced by brief exposure to high $[K^+]_o$, as shown in Fig. 1*B*. The brief exposure to high $[K^+]_o$ was achieved by pressure ejection from a micropipette, which was filled with a high-K^+ (100 mM) solution, as close as possible to the cell (see Methods). The brief increases in $[Ca^{2+}]_i$ were completely blocked by 1 μM nifedipine or 10 μM La^{3+} (data not shown).

The results strongly suggest that the brief increases in $[Ca^{2+}]_i$ evoked by high $[K^+]_o$ and the spontaneously elicited $[Ca^{2+}]_i$ increases arise from Ca^{2+} influx from the extracellular solution associated with discharges of action potentials.

Changes in $[Ca^{2+}]_i$ induced by brief membrane depolarization

In an effort to examine the action potential dependence of these increases in $[Ca^{2+}]_i$, changes in membrane potential were recorded from seven cells in the current-clamp mode of whole-cell patch clamp, at the same time as changes in $[Ca^{2+}]_i$. The cells had a resting membrane potential of $-43{\cdot}2 \pm 2{\cdot}3$ mV and a steady level of $[Ca^{2+}]_i$ of $47{\cdot}1 \pm 6{\cdot}6$ nM ($n = 7$). When a depolarizing current pulse (1–2 pA in intensity and 100–200 ms in duration) was

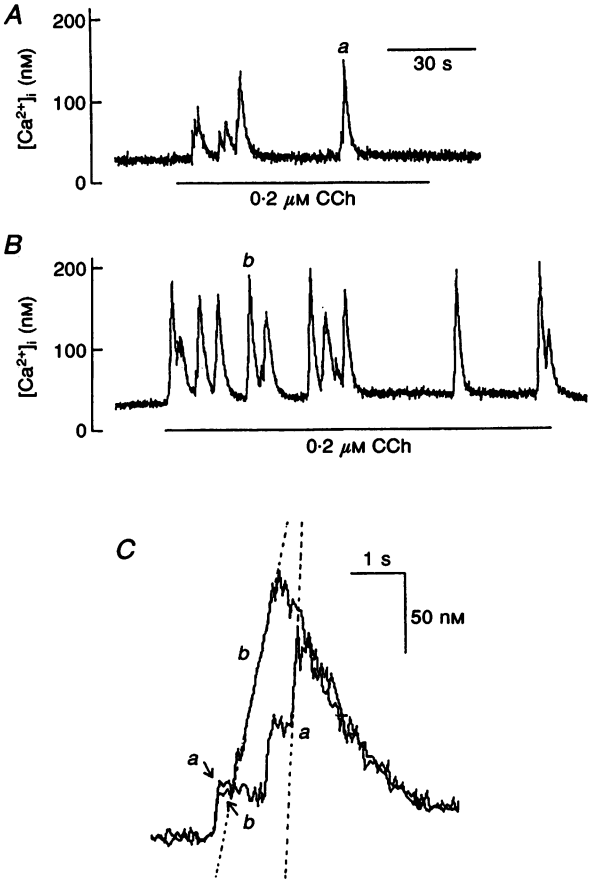

Figure 3. $[Ca^{2+}]_i$ oscillations induced by 0·2 μM CCh
A, $[Ca^{2+}]_i$ oscillations consisting of rapid brief $[Ca^{2+}]_i$ increases which were similar in shape and amplitude to those generated spontaneously and by exposure to high $[K^+]_o$. *B*, $[Ca^{2+}]_i$ oscillations consisting of slower and greater increases in $[Ca^{2+}]_i$ which were usually preceded by a rapid brief $[Ca^{2+}]_i$ increase. *C*, time- and $[Ca^{2+}]_i$-expanded traces of $[Ca^{2+}]_i$ oscillations indicated as *a* and *b* in *A* and *B*. The dashed lines *a* and *b* reflect the rate of rise of the $[Ca^{2+}]_i$ increases. Both traces were graphically superimposed on each other. See text for details. Time calibration in *A* is also applicable to *B*. *A* and *B* are from two different cells.

applied, an action potential was usually evoked with an overshoot of 18·1 ± 1·8 mV, as illustrated in Fig. 2 (top trace), which was followed by a rapid rise in $[Ca^{2+}]_i$ lasting for several seconds (bottom trace). The time to peak, maximum rate of rise and amplitude of the $[Ca^{2+}]_i$ response were measured to be 0·1–0·3 s, 584·2 ± 67·7 nM s^{-1} and 52·0 ± 5·3 nM ($n = 7$), respectively. These values were comparable to those of the spontaneously elicited $[Ca^{2+}]_i$ increases and high $[K^+]_o$-induced brief $[Ca^{2+}]_i$ increases. A membrane depolarization without generation of an action potential did not produce any change in $[Ca^{2+}]_i$ (see the fourth current pulse from the left in Fig. 2).

CCh-induced $[Ca^{2+}]_i$ oscillations

CCh (0·02–20 μM) was applied to cells to see its effect on $[Ca^{2+}]_i$. CCh at 0·02 μM had no effect on $[Ca^{2+}]_i$, but when the CCh concentration was increased to 0·2 μM, ~30% of cells ($n = 12$) tested responded with changes in $[Ca^{2+}]_i$. In half of the responding cells, a small brief rise in $[Ca^{2+}]_i$ was elicited repeatedly with a slightly elevated $[Ca^{2+}]_i$ level. The $[Ca^{2+}]_i$ responses to CCh resembled those to high $[K^+]_o$ (30 mM), as shown in Fig. 3*A*. It can be seen from the time-expanded trace in Fig. 3*C* that a brief increase in $[Ca^{2+}]_i$, which is characterized by a rapid rise, a slow decline and a rather stable amplitude, occurred repeatedly to give rise to a compound $[Ca^{2+}]_i$ response consisting of two or more brief $[Ca^{2+}]_i$ increases. In some cases, such a brief increase in $[Ca^{2+}]_i$ was elicited periodically at intervals long enough to give discrete brief increases in $[Ca^{2+}]_i$ (see Fig. 7*B*). The amplitude and rate of rise of these $[Ca^{2+}]_i$ increases were 44·1 ± 5·8 nM and 678·7 ± 96·5 nM s^{-1} ($n = 6$), respectively. In the remaining cells ($n = 6$), as shown in Fig. 3*B*, CCh elicited oscillatory changes in $[Ca^{2+}]_i$ of which individual oscillatory changes had a relatively constant amplitude (156·8 ± 16·9 nM, $n = 6$) and a slower rate of rise (100·6 ± 18·7 nM s^{-1}, $n = 6$) than a brief increase in $[Ca^{2+}]_i$ as mentioned above. Their discharge interval varied from 4 to 60 s. The slow rise in $[Ca^{2+}]_i$ was always preceded by the fast increase in $[Ca^{2+}]_i$. Figure 3*C* shows time-expanded traces of one of these $[Ca^{2+}]_i$ oscillations recorded from one cell and a compound $[Ca^{2+}]_i$ response recorded from another cell which were artificially superimposed in such a way that their falling phases matched with each other. The rising phase of the former consisted of two components: an initial component, consistent with the rising phase of the latter $[Ca^{2+}]_i$ response, which was followed by a slower component. Occasionally, a brief serrated $[Ca^{2+}]_i$ increase occurred during the latter half of the falling phase of the $[Ca^{2+}]_i$ oscillation with a slower rate of rise. Thus, this type of $[Ca^{2+}]_i$ oscillation (slow $[Ca^{2+}]_i$ oscillation) may be distinct functionally from the brief $[Ca^{2+}]_i$ increases, and may be triggered by the latter.

When CCh was applied at 2 μM, almost all cells responded with $[Ca^{2+}]_i$ oscillations of varied amplitude (30–800 nM) which were of the slow type, accompanied by a sustained elevation of the basal level of $[Ca^{2+}]_i$, as shown in Figs 5 and 6. All oscillations, except for the first, occurred either

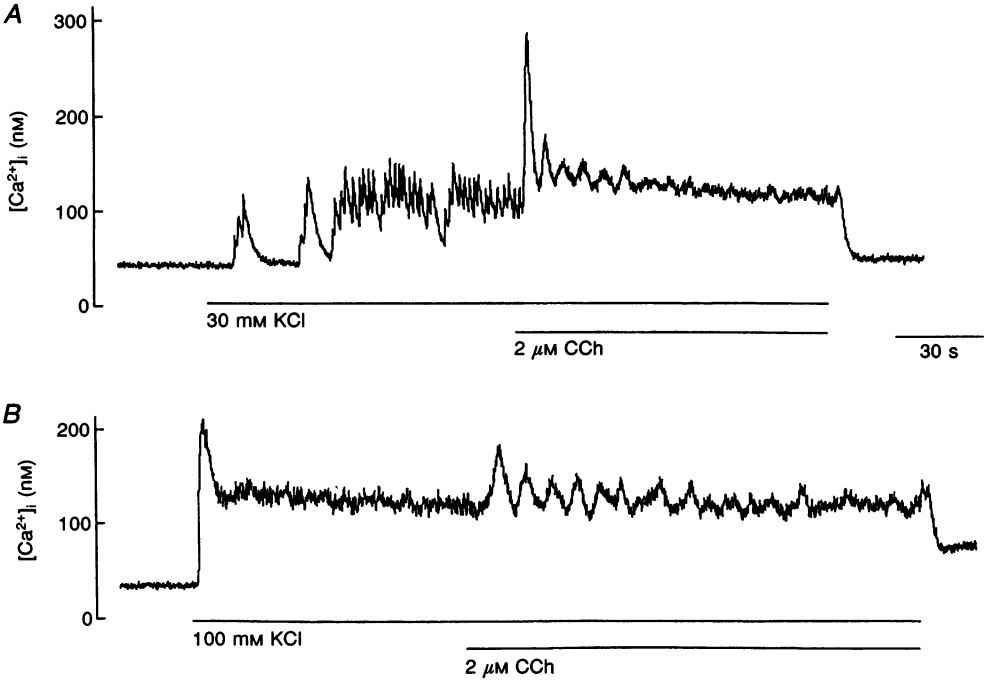

Figure 4. Changes in $[Ca^{2+}]_i$ produced by CCh in the presence of high $[K^+]_o$

A, transition to $[Ca^{2+}]_i$ oscillations at a lower frequency by 2 μM CCh applied subsequent to 30 mM K^+. *B*, oscillatory changes in $[Ca^{2+}]_i$ induced by 2 μM CCh applied during a sustained elevation of $[Ca^{2+}]_i$ in response to 100 mM K^+. Time calibration in *A* is applicable to *B*. *A* and *B* are from two different cells.

in a pattern reminiscent of a pacemaker potential, in that between two successive oscillations there was a slow increase in $[Ca^{2+}]_i$ which was followed by a sudden upstroke of the next oscillation (Fig. 6*B* and *C*), or in a sinusoidal manner, in that without reaching a plateau after a $[Ca^{2+}]_i$ oscillation, the next $[Ca^{2+}]_i$ oscillation occurred (Fig. 5*A* and *B*). The oscillation frequency varied from 0·03 to 0·27 Hz in different cells, giving the mean of 0·13 ± 0·01 Hz ($n = 44$). In some of these cells, CCh-induced $[Ca^{2+}]_i$ oscillations waned with time, to end in a sustained increase in $[Ca^{2+}]_i$ in the continued presence of CCh. The amplitude and rate of rise of the first oscillation were fairly constant in different cells, giving the mean values of 435·6 ± 25·0 nM and 743·9 ± 55·8 nM s^{-1} ($n = 44$), respectively, which were usually greater and faster than those of subsequent oscillations. In response to 20 μM CCh, cells exhibited a biphasic rise in $[Ca^{2+}]_i$: the initial, fast increase reached a peak of 412·7 ± 20·7 nM ($n = 4$) within 2 s and then it declined to a lower but still elevated level which was sustained throughout the period of CCh application (data not shown).

CCh was found to evoke two distinct types of $[Ca^{2+}]_i$ oscillation at different concentrations: one was the slow type elicited predominantly by 2 μM CCh and the other was that elicited preferentially by 0·2 μM CCh, similar to the $[Ca^{2+}]_i$ oscillations evoked by high $[K^+]_o$. To characterize the slow $[Ca^{2+}]_i$ oscillations elicited in response to CCh, the following experiments were performed.

CCh-induced slow $[Ca^{2+}]_i$ oscillations during membrane depolarization produced by high $[K^+]_o$

Figure 4*A* shows a representative $[Ca^{2+}]_i$ response to CCh applied subsequently to high $[K^+]_o$ (30 mM) in four cells. Following application of CCh, rapid brief increases in

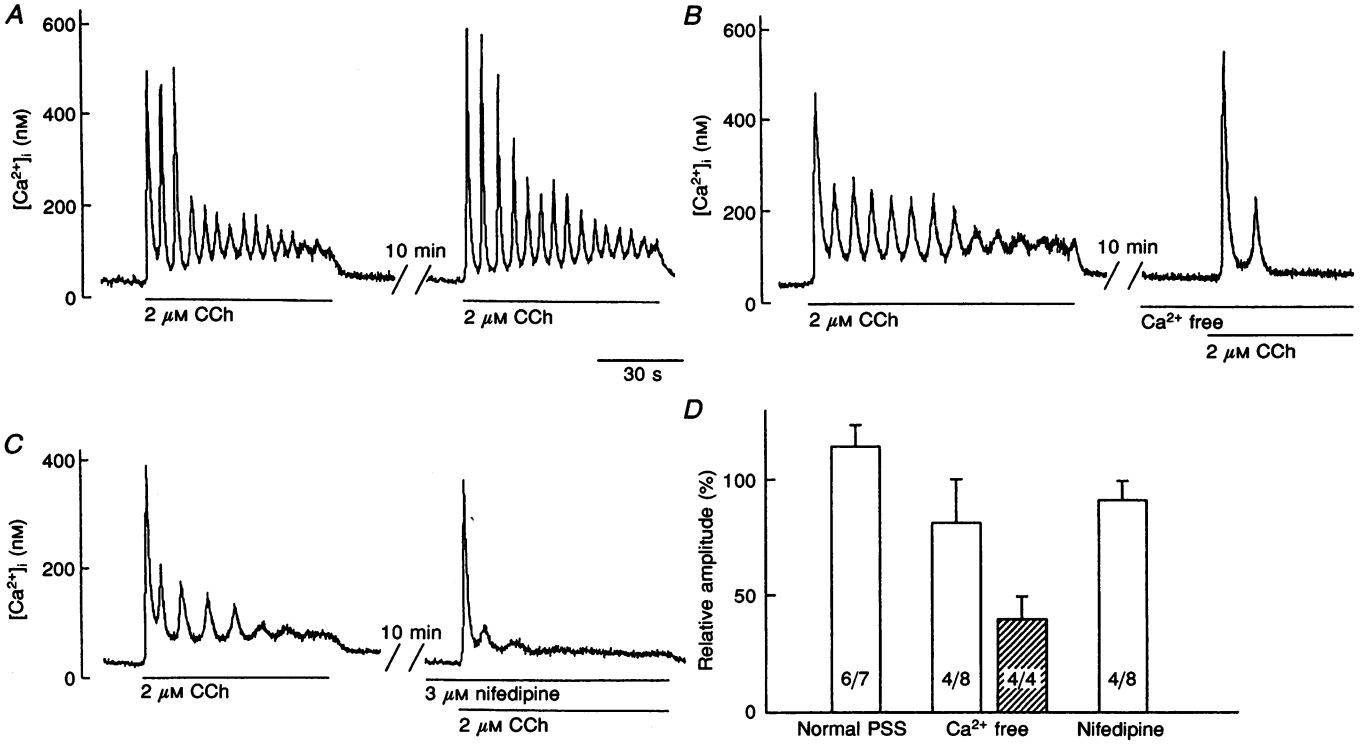

Figure 5. CCh-induced $[Ca^{2+}]_i$ oscillations in the absence of extracellular Ca^{2+} and in the presence of nifedipine

CCh (2 μM) was applied twice at an interval of 10 min (indicated by break in trace), as indicated by lines below recording traces in *A–C*. *A*, $[Ca^{2+}]_i$ responses to the first (left panel) and second application of CCh (right panel) in normal PSS. *B*, $[Ca^{2+}]_i$ responses to the first and second application of CCh before (left panel) and 1 min after removal of extracellular Ca^{2+} (Ca^{2+} free; right panel), respectively. *C*, $[Ca^{2+}]_i$ responses to the first and second application of CCh before (left panel) and 1 min after application of 3 μM nifedipine (right panel), respectively. *D*, the amplitude of the first $[Ca^{2+}]_i$ oscillation for cells which responded to the second application of CCh, expressed as a percentage of the first $[Ca^{2+}]_i$ oscillation of the control response (□): in normal PSS, in the absence of extracellular Ca^{2+} (Ca^{2+} free) and in the presence of nifedipine. The amplitude of a single $[Ca^{2+}]_i$ oscillation for cells which responded to the third application of CCh (100 μM) in the absence of extracellular Ca^{2+} is also shown as a percentage of the first $[Ca^{2+}]_i$ oscillation of the control response (▨). Each column represents the mean and the error bars represent 1 S.E.M. The fraction of responding cells is indicated in each column. Time calibration in *A* is applicable to *B* and *C*. *A–C* are from three different cells.

$[Ca^{2+}]_i$ superimposed on an elevated $[Ca^{2+}]_i$ level were replaced by $[Ca^{2+}]_i$ oscillations of a slow type at a lower frequency of $0{\cdot}12 \pm 0{\cdot}02$ Hz ($n = 4$). The CCh-induced $[Ca^{2+}]_i$ oscillations waned with time to end a sustained $[Ca^{2+}]_i$ plateau.

Exposure to high-K^+ (100 mM) solution has been shown to cause complete depolarization block of action potential discharge (Shimo & Holland, 1966; Bolton, 1979). When applied during a sustained plateau of $[Ca^{2+}]_i$ produced by high-K^+ (100 mM) solution, CCh still triggered $[Ca^{2+}]_i$ oscillations, as shown in Fig. 4*B*. Compared with $[Ca^{2+}]_i$ oscillations during exposure to 30 mM K^+ ($n = 3$), however, the $[Ca^{2+}]_i$ oscillations were smaller in amplitude and ceased more quickly, whereas the oscillation frequency of $0{\cdot}18 \pm 0{\cdot}04$ Hz ($n = 3$) was comparable.

Extracellular Ca^{2+} dependence of the CCh-induced slow $[Ca^{2+}]_i$ oscillations

When application of CCh for 1·5–2·0 min was repeated twice at an interval of 10 min in one cell in normal PSS, the first and second oscillatory $[Ca^{2+}]_i$ responses were fairly well matched in six of seven cells tested (Fig. 5*A*); the amplitude of the first $[Ca^{2+}]_i$ oscillation, mean amplitude of subsequent $[Ca^{2+}]_i$ oscillations and oscillation frequency in the second response were $113 \pm 9{\cdot}2\%$, $109 \pm 18{\cdot}8\%$ and

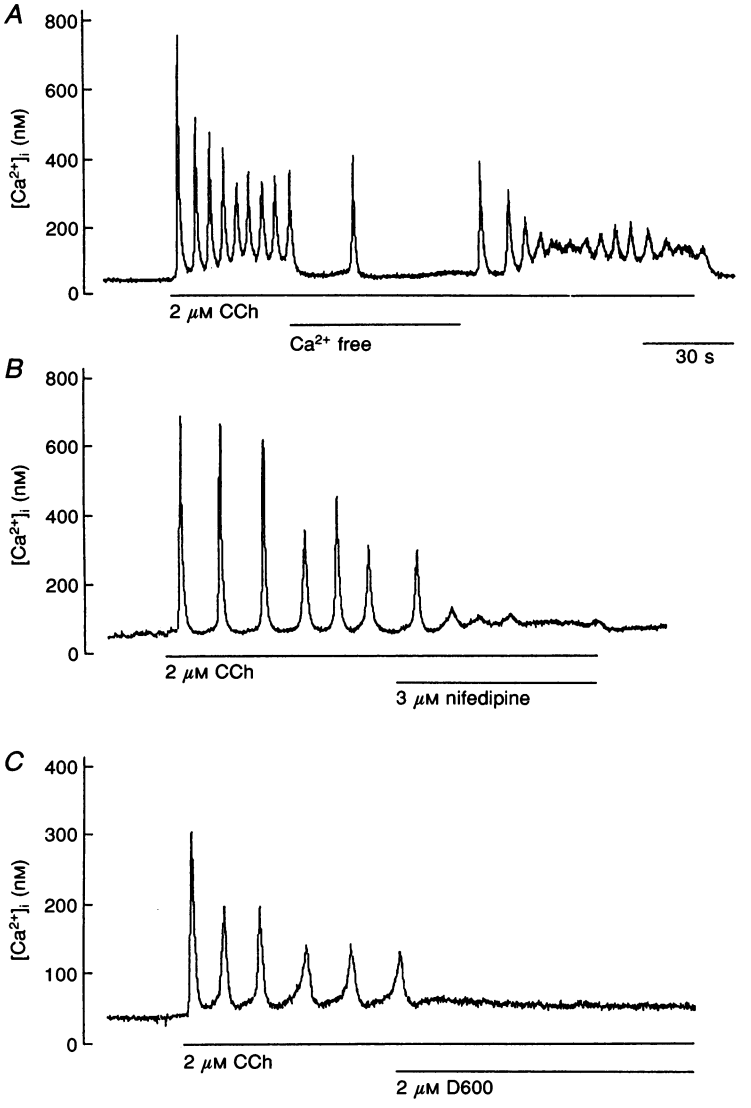

Figure 6. Effect of removal of extracellular Ca^{2+} and application of nifedipine and D600 during the CCh-induced $[Ca^{2+}]_i$ oscillations

A, arrest of 2 μM CCh-induced $[Ca^{2+}]_i$ oscillations upon removal of extracellular Ca^{2+} and their restoration following reapplication of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} was achieved by replacing the bath medium with Ca^{2+}-free solution (2 mM EGTA added). *B* and *C*, arrest of 2 μM CCh-induced $[Ca^{2+}]_i$ oscillations by 3 μM nifedipine and 2 μM D600, respectively. Note occurrence of one $[Ca^{2+}]_i$ oscillation (*A*) and a few $[Ca^{2+}]_i$ oscillations (*B*). Time calibration in *A* is applicable to *B* and *C*. *A–C* are from three different cells.

105·9 ± 14·6% ($n = 6$), respectively, of the corresponding values in the first response. Figure 5*B* shows $[Ca^{2+}]_i$ responses of one cell to CCh applied in the presence of extracellular Ca^{2+} (control) and 1–2 min after its removal (Ca^{2+}-free solution). In the absence of extracellular Ca^{2+}, CCh evoked one or two $[Ca^{2+}]_i$ oscillations in four of eight cells. The mean amplitude of the single and the first of two $[Ca^{2+}]_i$ oscillations was 80·6 ± 18·8% ($n = 4$) of that for the first $[Ca^{2+}]_i$ oscillation of the control response, which was not significantly different from that in normal PSS. The remaining four cells did not respond to CCh with any appreciable change in $[Ca^{2+}]_i$, but they responded to subsequent application of 100 mM CCh with a single $[Ca^{2+}]_i$ oscillation the amplitude of which was 39·1 ± 10·2% ($n = 4$) of the first $[Ca^{2+}]_i$ oscillation of the control response (Fig. 5*D*).

When extracellular Ca^{2+} was removed during the CCh-induced slow $[Ca^{2+}]_i$ oscillations in eight cells, the $[Ca^{2+}]_i$ oscillations disappeared and $[Ca^{2+}]_i$ declined closely to the initial level before the CCh application (Fig. 6*A*). In four of the eight cells, the $[Ca^{2+}]_i$ oscillations increased in interval for a while before they disappeared (see Fig. 6*A*). Reapplication of extracellular Ca^{2+} was followed by the return of $[Ca^{2+}]_i$ oscillations. The results favour a role for extracellular Ca^{2+} in mediating the CCh-induced $[Ca^{2+}]_i$ oscillations.

Effects of Ca^{2+} channel blockers on the CCh-induced slow $[Ca^{2+}]_i$ oscillations

To see how extracellular Ca^{2+} is involved in the CCh-induced slow $[Ca^{2+}]_i$ oscillations, Ca^{2+} channel blockers were used to block Ca^{2+} entry through Ca^{2+} channels.

As shown in Fig. 5*C*, in the four of eight cells to which 1 or 3 μM nifedipine was applied, CCh induced one or a few $[Ca^{2+}]_i$ oscillations. The first oscillation was far larger in amplitude than the subsequent oscillations. The mean amplitude for the single oscillations including the first $[Ca^{2+}]_i$ oscillations was 90·9 ± 8·2% ($n = 4$) of that for the first $[Ca^{2+}]_i$ oscillation of the control response, which was not significantly different from that in normal PSS. The remaining four cells did not respond to CCh with any appreciable change in $[Ca^{2+}]_i$ (Fig. 5*D*).

When applied during CCh-induced $[Ca^{2+}]_i$ oscillations, nifedipine (3 μM) stopped the oscillations immediately or over a period during which a few oscillations still occurred with progressively decreased amplitudes ($n = 11$; Fig. 6*B*). Similar results were obtained with other organic Ca^{2+} channel blockers such as D600 (2 μM, $n = 5$; Fig. 6*C*) and diltiazem (10 μM, $n = 2$). La^{3+} (0·1–1 mM) also caused cessation of the CCh-induced $[Ca^{2+}]_i$ oscillations, but several oscillations still occurred with a gradual decrease in amplitude and frequency before they disappeared, as shown in Fig. 7*A* ($n = 4$). In general, for immediate arrest of the $[Ca^{2+}]_i$ oscillations, 2 mM La^{3+} was required ($n = 3$). The effect of La^{3+} was not readily reversed after wash-out. When tested on repetitive brief $[Ca^{2+}]_i$ increases evoked by 0·2 μM CCh, La^{3+} at a concentration of 10 μM was effective enough to block them immediately ($n = 2$; Fig. 7*B*).

The results suggest that Ca^{2+} channels, through which Ca^{2+} influx is required to sustain the CCh-induced $[Ca^{2+}]_i$ oscillations, are sensitive to these Ca^{2+} channel blockers, but that they are never as sensitive to La^{3+} as Ca^{2+} channels involved in the $[Ca^{2+}]_i$ oscillations induced by high $[K^+]_o$ or 0·2 μM CCh.

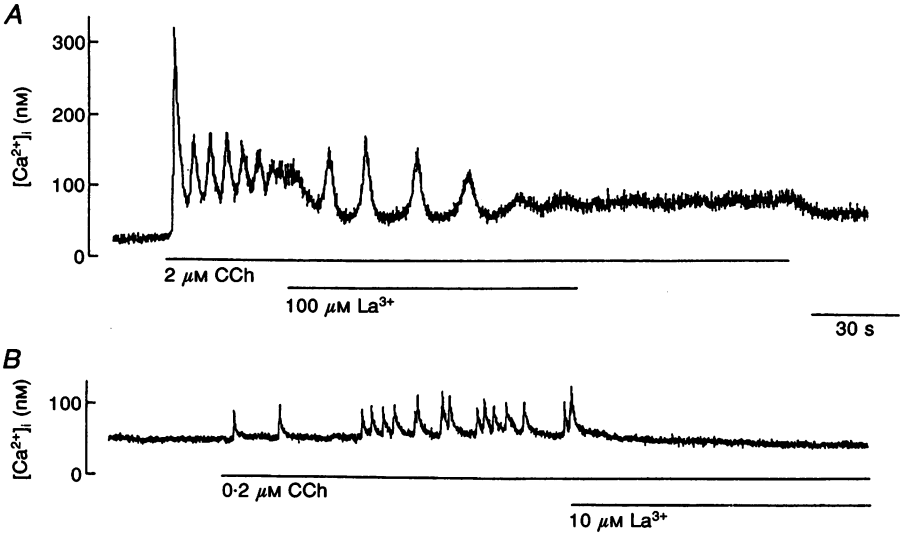

Figure 7. Effect of La^{3+} on the CCh-induced $[Ca^{2+}]_i$ oscillations

A, arrest of 2 μM CCh-induced $[Ca^{2+}]_i$ oscillations by 100 μM La^{3+}. *B*, an immediate arrest of 0·2 μM CCh-induced $[Ca^{2+}]_i$ oscillations by 10 μM La^{3+}. Note a low sensitivity of the 2 μM CCh-induced $[Ca^{2+}]_i$ oscillations to La^{3+}. Time calibration in *A* is applicable to *B*. *A* and *B* are from two different cells.

Role of internal Ca^{2+} stores in CCh-induced $[Ca^{2+}]_i$ oscillations

Cyclopiazonic acid (CPA) and thapsigargin, inhibitors of Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR) (Thastrup *et al.* 1989; Uyama, Imaizumi & Watanabe, 1992), and ryanodine, which locks ryanodine-sensitive channels of SR in an open state (Nagasaki & Fleischer, 1988: Iino, Kobayashi & Endo, 1988), were used to deplete internal Ca^{2+} stores. After treatment with 30 μM CPA for 10 min, the $[Ca^{2+}]_i$ response to CCh was not oscillatory but biphasic: an initial rapid rise in $[Ca^{2+}]_i$ to a peak was followed by a lower but still elevated level which was sustained throughout the period of CCh application, as shown in Fig. 8*A* ($n = 6$). In some cells, there was no appreciable rise in $[Ca^{2+}]_i$ ($n = 3$). The mean amplitude of the initial peak of the biphasic $[Ca^{2+}]_i$ response was 137·9 ± 21·8 nM ($n = 6$), being only 30·4 ± 5·4% of the mean amplitude of the first spike of the control response

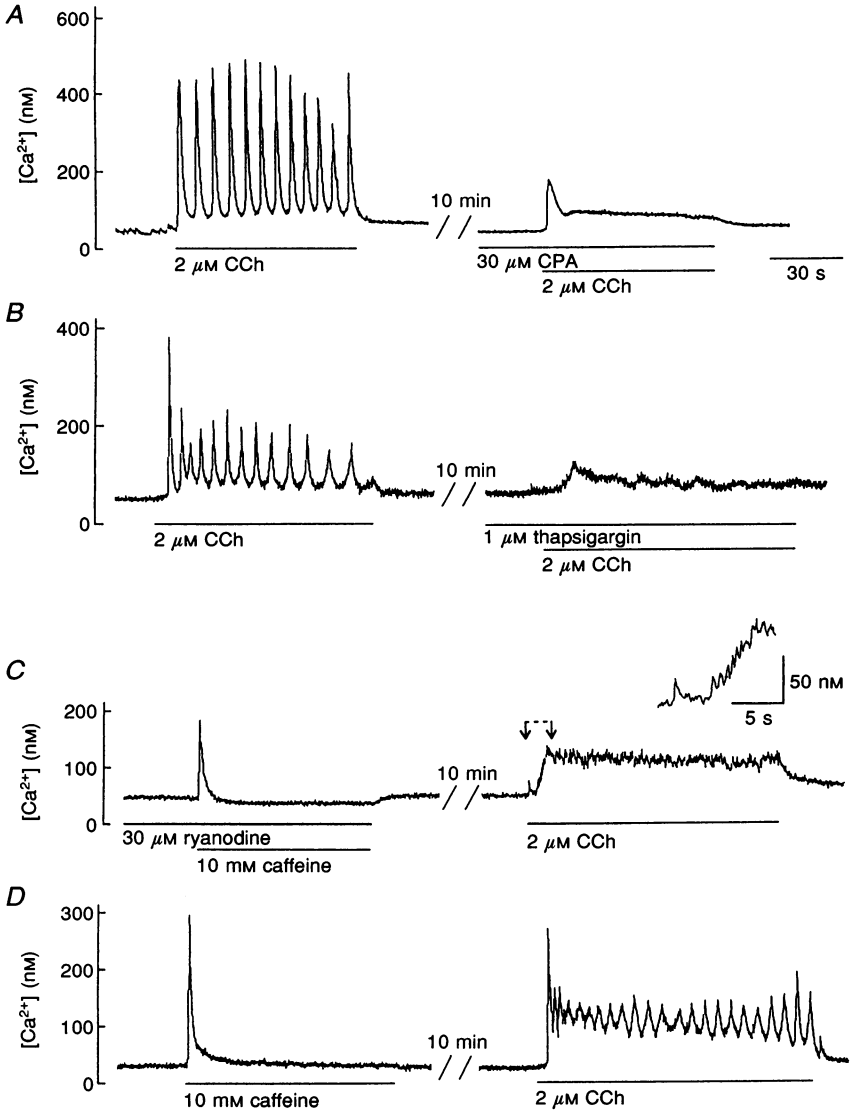

Figure 8. Effects of cyclopiazonic acid (CPA), thapsigargin and ryanodine on the CCh-induced $[Ca^{2+}]_i$ oscillation

A, $[Ca^{2+}]_i$ responses to 2 μM CCh in the absence (left panel) and presence of 30 μM CPA (right panel). CPA was applied 10 min before the second application of CCh. *B*, $[Ca^{2+}]_i$ responses to 2 μM CCh applied in the absence (left panel) and presence of 1 μM thapsigargin (right panel). Thapsigargin was added to the bath medium 1 min before the second application of CCh. *C*, no oscillatory change of $[Ca^{2+}]_i$ in response to 2 μM CCh (right panel) 10 min after a combined treatment with 30 μM ryanodine and 10 mM caffeine to cause functional removal of internal Ca^{2+} stores (left panel). The inset shows a time- and $[Ca^{2+}]_i$-expanded trace of the section indicated by the pair of arrows. *D*, an oscillatory $[Ca^{2+}]_i$ response to 2 μM CCh (right panel) 10 min after treatment with 10 mM caffeine alone to cause only a transient depletion of internal Ca^{2+} stores (left panel). Note that CCh elicited serrated increases especially during the early period of its application. Time calibration in *A* is applicable to *B*, *C* and *D*. *A–D* are from four different cells.

(482·9 ± 65·4 nM, $n = 6$). Thapsigargin (1 μM), applied 1–2 min before, was also effective in changing CCh-induced $[Ca^{2+}]_i$ oscillations to a small ($n = 2$) or no change in $[Ca^{2+}]_i$ ($n = 2$; Fig. 8*B*). When cells were treated with 30 μM ryanodine combined with 10 mM caffeine for 1·0–1·5 min, placed in normal PSS for 10 min, and then exposed to CCh, a sustained rise in $[Ca^{2+}]_i$ was produced (Fig. 8*C*). On the other hand, CCh-induced $[Ca^{2+}]_i$ oscillations were elicited in cells ($n = 8$) treated with 10 mM caffeine alone, which were then placed in normal PSS for 10 min, allowing Ca^{2+} stores to be replenished, with the same oscillatory pattern as seen in normal cells (Fig. 8*D*). After removal of internal Ca^{2+} stores, sometimes a brief serrated increase in $[Ca^{2+}]_i$ was discharged in the singular or in a group especially during the early period of the CCh exposure (Fig. 8*C*).

With high $[K^+]_o$ (30 mM), oscillatory $[Ca^{2+}]_i$ responses similar to those in normal cells were obtained in CPA-treated ($n = 8$), thapsigargin-treated ($n = 7$) and ryanodine-treated ($n = 5$) cells, as shown in Fig. 9*A*, *B* and *C*. Almost complete depletion of Ca^{2+} stores releasable with caffeine after the treatment with CPA, thapsigargin or ryanodine was confirmed either by a very small brief or no rise in $[Ca^{2+}]_i$ in response to application of 10 mM caffeine. In fact, the mean amplitude of $[Ca^{2+}]_i$ responses to 10 mM caffeine of 303·4 ± 39·3 nM ($n = 21$; see Fig. 8*D*) in control cells was reduced to 26·3 ± 8·5 nM ($n = 8$) in CPA-treated cells and to zero in thapsigargin-treated ($n = 3$) and ryanodine-treated ($n = 4$) cells. Consequently, it appears that internal Ca^{2+} stores play an essential role in mediating the slow type of $[Ca^{2+}]_i$ oscillations in response to stimulation of muscarinic receptors.

DISCUSSION

In the present study, the effect of CCh on $[Ca^{2+}]_i$ was investigated in enzymatically isolated smooth muscle cells of the guinea-pig ileum using a Ca^{2+}-sensitive fluorescent dye, fura-2.

The $[Ca^{2+}]_i$ oscillations produced preferentially by 0·2 μM CCh were made up of rapid brief increases in $[Ca^{2+}]_i$. Their temporal and functional characteristics were very similar to those of the high $[K^+]_o$ (30 mM)-induced $[Ca^{2+}]_i$ oscillations; the amplitude (44·1 ± 5·7 nM), rate of rise (678·7 ± 96·5 nM s^{-1}) and duration (3–5 s) of individual $[Ca^{2+}]_i$ increases were much the same as those for the high $[K^+]_o$-induced $[Ca^{2+}]_i$ oscillations (44·1 ± 4·7 nM, 645·9 ± 60·7 nM s^{-1} and 3–5 s, respectively). Two or more of them occurred repeatedly to give rise to a compound $[Ca^{2+}]_i$ response with a rising phase showing stepwise increases. The amplitude and rate of rise of individual $[Ca^{2+}]_i$ oscillations produced by 30 mM K^+ were very similar to

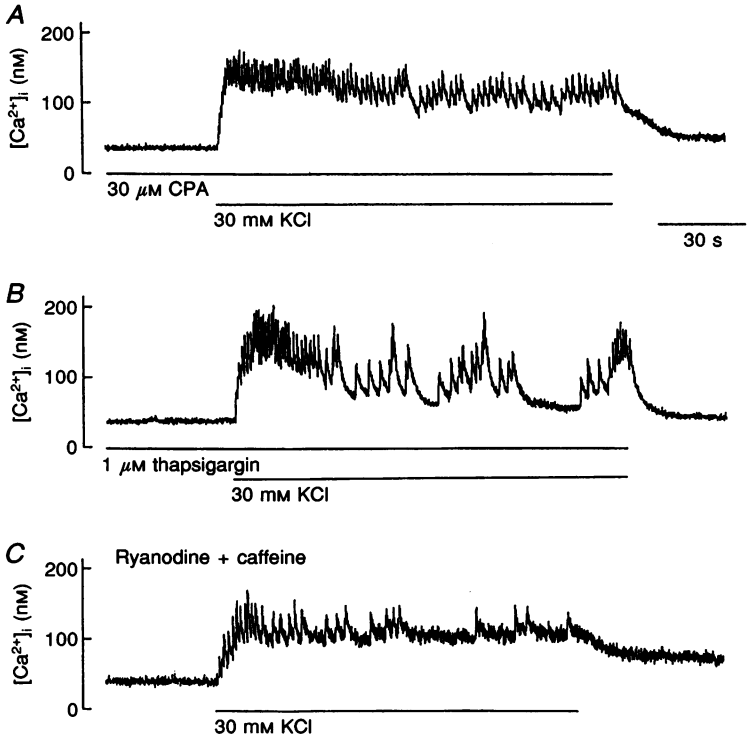

Figure 9. Effects of CPA, thapsigargin and ryanodine on high $[K^+]_o$-induced changes in $[Ca^{2+}]_i$

A and *B*, $[Ca^{2+}]_i$ responses to 30 mM K^+ in the presence of 30 μM CPA and 1 μM thapsigargin, applied 10 min and 1 min before the high $[K^+]_o$ stimulation, respectively. *C*, a $[Ca^{2+}]_i$ response to 30 mM K^+ after combined pretreatment with 30 μM ryanodine and 10 mM caffeine for 1·5 min, as in Fig. 8*C*. Note that oscillatory changes in $[Ca^{2+}]_i$ can still be evoked by high $[K^+]_o$. See text for details. Time calibration in *A* is applicable to *B* and *C*. *A–C* are from three different cells.

those of the $[Ca^{2+}]_i$ increases generated spontaneously, by brief exposure to 100 mM K^+ in normal cells and by action potential evoked by a depolarizing current pulse in current-clamped cells. They were blocked by La^{3+}, an inorganic Ca^{2+} channel blocker, and nifedipine, an organic Ca^{2+} channel blocker, and disappeared upon removal of the extracellular Ca^{2+}. Therefore, the high $[K^+]_o$-induced $[Ca^{2+}]_i$ oscillations appear to result from Ca^{2+} influxes from extracellular solution brought about by increased electrical activities by membrane depolarization. The similar properties of the CCh-induced $[Ca^{2+}]_i$ oscillations to the high $[K^+]_o$-induced $[Ca^{2+}]_i$ oscillations suggest that a common mechanism is operated for these two responses to CCh and high $[K^+]_o$, Ca^{2+} influx through voltage-gated Ca^{2+} channels activated by membrane depolarization.

The $[Ca^{2+}]_i$ oscillations elicited in some cells exposed to 0·2 μM CCh had a distinct difference in the rate of rise (100·6 $\pm$ 18·7 nM s^{-1}) from serrated $[Ca^{2+}]_i$ increases. The appearance of a serrated $[Ca^{2+}]_i$ increase in the falling phase of the $[Ca^{2+}]_i$ oscillation cannot be readily understood by assuming that Ca^{2+} is utilized from the same source for both types of $[Ca^{2+}]_i$ change. Every $[Ca^{2+}]_i$ oscillation was preceded by single or multiple serrated $[Ca^{2+}]_i$ increases. The profile taken together with previous evidence that Ca^{2+} can potentiate IP_3-induced Ca^{2+} release from internal stores (Iino, 1990) suggests that serrated $[Ca^{2+}]_i$ increases may trigger Ca^{2+} release from internal stores resulting in the $[Ca^{2+}]_i$ oscillation. This is not incompatible with the finding that in cells in which internal Ca^{2+} stores had been depleted, CCh (2 μM) elicited serrated $[Ca^{2+}]_i$ increases especially during the early period of its application (see Fig. 8*C*).

The $[Ca^{2+}]_i$ oscillations elicited when CCh was used at 2 μM were more or less regular in periodicity and pattern, and thus did not summate. The oscillations comprised pacemaker potential-like $[Ca^{2+}]_i$ increases or sinusoidal $[Ca^{2+}]_i$ increases which were generally followed by a larger $[Ca^{2+}]_i$ increase. These features resembled those of the $[Ca^{2+}]_i$ oscillations in response to various receptor agonists, including CCh, in a variety of non-excitable cells (Berridge, 1990; Fewtrell, 1993). In addition, the CCh-induced $[Ca^{2+}]_i$ oscillations were elicited even in cells during exposure to 100 mM K^+. Under such conditions, the membrane potential had been clamped at nearly 0 mV, and no discharge of action potentials could be expected. The $[Ca^{2+}]_i$ oscillations can be readily understood when one considers the change in Ca^{2+} release from internal stores. This idea is supported by the fact that they were not elicited after depletion of internal Ca^{2+} stores with CPA, thapsigargin and ryanodine. The occurrence of one or two cycles of this type of $[Ca^{2+}]_i$ oscillation when CCh was applied in Ca^{2+}-free conditions and a delayed abolition of the CCh-induced $[Ca^{2+}]_i$ oscillations after extracellular Ca^{2+} was removed are not incompatible with this hypothesis. Extracellular Ca^{2+} would be required for the $[Ca^{2+}]_i$ oscillation to persist (see below).

The $[Ca^{2+}]_i$ oscillations elicited in response to 2 μM CCh bore certain resemblance to $[Ca^{2+}]_i$ oscillations in voltage-clamped cells (Komori *et al.* 1993) which are attributable to periodic Ca^{2+} release from internal stores via an IP_3-dependent mechanism. In addition, the oscillation frequencies overlap each other (0·03–0·27 Hz in voltage-unclamped cells and 0·08–0·32 Hz in voltage-clamped cells), and the concentrations of CCh required for generation of the $[Ca^{2+}]_i$ oscillations are in the same range. Therefore, similar underlying mechanisms associated with the $[Ca^{2+}]_i$ oscillations seem likely to be operated by stimulation of muscarinic receptors in both voltage-clamped and voltage-unclamped cells, although there is no doubt that voltage-dependent mechanisms would be in working in voltage-unclamped cells.

Ca^{2+} stores involved in the generation of $[Ca^{2+}]_i$ oscillations could be in a close association with the plasma membrane and refilled readily by Ca^{2+} from the extracellular solution to prevent rapid depletion of Ca^{2+}. The cell variation in sensitivity of the CCh-induced $[Ca^{2+}]_i$ oscillations to nifedipine may be attributable to the heterogeneous sensitivity of Ca^{2+} pathways utilized for refilling of Ca^{2+} stores responsible for the $[Ca^{2+}]_i$ oscillations. The Ca^{2+} pathway relatively sensitive to the Ca^{2+} channel blocker may comprise voltage-gated Ca^{2+} channels. However, a question arises as to whether or not the voltage-gated Ca^{2+} channels through which Ca^{2+} entry occurs are fully inactivated during membrane depolarization, since CCh can cause activation of non-selective cation channels leading to membrane depolarization (Benham *et al.* 1985; Inoue & Isenberg, 1990*a*). Voltage-clamp studies on voltage-gated Ca^{2+} channels in smooth muscle cells from various tissues have revealed that during prolonged membrane depolarization, a population of voltage-gated Ca^{2+} channels are not inactivated and they allow sustained influx of extracellular Ca^{2+} (Imaizumi, Muraki, Takeda & Watanabe, 1989; Ganitkevich & Isenberg, 1991).

It is of interest that nifedipine, D600 and diltiazem exerted the inhibitory effect within the same order of concentration as that required to block high $[K^+]_o$-induced $[Ca^{2+}]_i$ responses, whereas La^{3+}, even when used at concentrations 10–50 times higher than that sufficient to block the high $[K^+]_o$-induced responses, allowed the $[Ca^{2+}]_i$ oscillations to be elicited for several cycles before cessation. This suggests the possible existence of another Ca^{2+} entry pathway which can be differentiated by La^{3+} from voltage-gated Ca^{2+} channels. The muscarinic receptor-operated non-selective cation channels (Benham *et al.* 1985; Inoue & Isenberg, 1990*a*, *b*) and/or still unidentified pathway that is activated by depletion of Ca^{2+} stores (Pacaud & Bolton, 1991) might be offered as a candidate.

It has been reported that the activation curve for the Ca^{2+} release process through the IP_3 receptor in internal Ca^{2+} stores activated by $[Ca^{2+}]_i$ is bell-shaped with a peak at 300 nM Ca_i^{2+} (Iino, 1990) and that this activation by $[Ca^{2+}]_i$

is also controlled by the luminal Ca^{2+} of the stores ($[Ca^{2+}]_s$) in such a way that it increases as $[Ca^{2+}]_s$ increases (Missiaen, Taylor & Berridge, 1992; Nunn & Taylor, 1992). In this case, the simplest explanation for the difference in the response profile of cells when Ca^{2+} was removed from the extracellular solution during CCh-induced $[Ca^{2+}]_i$ oscillations is that $[Ca^{2+}]_s$ was reduced below a critical level required to be released by IP_3 accumulated by 2 μM CCh in some cells, but in the other cells $[Ca^{2+}]_s$ could exceed a new critical level and thereby Ca^{2+} release occurred once. The difference in the response profile of cells when CCh was applied 1–2 min after removal of the extracellular Ca^{2+} can also be explained in the same way as above. This is supported by the finding that the cells, in which 2 μM CCh was without effect, responded to subsequently applied 100 μM CCh with a transient rise in $[Ca^{2+}]_i$.

The observed effects may account for the biphasic change in tension in response to stimulation of muscarinic receptors in intestinal smooth muscles (Bolton, 1979): the initial, transient rise in tension may be determined by the membrane activity and massive release of Ca^{2+} from the internal storage sites, and the subsequent sustained rise in tension may result from periodic release of Ca^{2+} from the storage sites to which Ca^{2+} is supplied by Ca^{2+} influx through still non-inactivated voltage-gated Ca^{2+} channels and as yet unidentified channels (Komori, Unno & Ohashi, unpublished observations), since block of action potential discharge is brought about by an excess membrane depolarization (Bolton, 1972) and by a suppression of Ca^{2+} influx through voltage-gated Ca^{2+} channels (Unno *et al.* 1995).

In summary, the mechanisms underlying CCh-induced changes in $[Ca^{2+}]_i$ switched over from the plasma membrane in origin to the internal Ca^{2+} stores in origin in the concentration range between 0·2 and 2 μM. The former reflect Ca^{2+} influx due to the increased electrical activity by a moderate membrane depolarization occurring via a G-protein-dependent activation of non-selective cation channels (Inoue & Isenberg, 1990*a*; Komori, Kawai, Takewaki & Ohashi, 1992). The latter reflect periodic release of Ca^{2+} from internal stores due to significant accumulation of IP_3 (Prestwich & Bolton, 1995) occurring via a G-protein-dependent activation of phospholipase C and hydrolysis of phosphatidylinositol 4,5-bisphosphate (Cockcroft & Gomperts, 1985).

Benham, C. D., Bolton, T. B. & Lang, R. J. (1985). Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature* **316**, 345–346.

Berridge, M. J. (1990). Calcium oscillations. *Journal of Biological Chemistry* **265**, 9583–9586.

Berridge, M. J. & Galione, A. (1988). Cytosolic calcium oscillators. *FASEB Journal* **2**, 3074–3082.

Bolton, T. B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *Journal of Physiology* **220**, 647–671.

Bolton, T. B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiological Reviews* **59**, 606–718.

Cockcroft, S. & Gomperts, B. D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature* **314**, 534–536.

Fewtrell, C. (1993). Ca^{2+} oscillations in non-excitable cells. *Annual Review of Physiology* **55**, 427–454.

Ganitkevich, V. Ya. & Isenberg, G. (1991). Depolarization-mediated intracellular calcium transients in isolated smooth muscle cells of guinea-pig urinary bladder. *Journal of Physiology* **435**, 187–205.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

Hassid, A. (1986). Atriopeptin II decreases cytosolic free Ca in cultured vascular smooth muscle cells. *American Journal of Physiology* **251**, C681–686.

Iino, M. (1990). Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. *Journal of General Physiology* **95**, 1103–1122.

Iino, M., Kobayashi, T. & Endo, M. (1988). Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. *Biochemical and Biophysical Research Communications* **152**, 417–422.

Imaizumi, Y., Muraki, K., Takeda, M. & Watanabe, M. (1989). Measurement and simulation of noninactivating Ca current in smooth muscle cells. *American Journal of Physiology* **256**, C880–885.

Inoue, R. & Isenberg, G. (1990*a*). Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. *American Journal of Physiology* **258**, C1173–1178.

Inoue, R. & Isenberg, G. (1990*b*). Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. *Journal of Physiology* **424**, 73–92.

Kobayashi, S., Kitazawa, T., Somlyo, A. V. & Somlyo, A. P. (1989). Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. *Journal of Biological Chemistry* **264**, 17997–18004.

Komori, S. & Bolton, T. B. (1990). Role of G-proteins in muscarinic receptor inward and outward currents in rabbit jejunal smooth muscle. *Journal of Physiology* **427**, 395–419.

Komori, S. & Bolton, T. B. (1991). Calcium release induced by inositol 1,4,5-trisphosphate in single rabbit intestinal smooth muscle cells. *Journal of Physiology* **433**, 495–517.

Komori, S., Kawai, M., Pacaud, P., Ohashi, H. & Bolton, T. B. (1993). Oscillations of receptor-operated cationic current and internal calcium in single guinea-pig ileal smooth muscle cells. *Pflügers Archiv* **424**, 431–438.

Komori, S., Kawai, M., Takewaki, T. & Ohashi, H. (1992). GTP-binding protein involvement in membrane currents evoked by carbachol and histamine in guinea-pig ileal muscle. *Journal of Physiology* **450**, 105–126.

Meyer, T. & Stryer, L. (1991). Calcium spiking. *Annual Review of Biophysics and Biophysical Chemistry* **20**, 153–174.

Missiaen, L., Taylor, C. W. & Berridge, M. J. (1992). Luminal Ca^{2+} promoting spontaneous Ca^{2+} release from inositol trisphosphate-sensitive stores in rat hepatocytes. *Journal of Physiology* **455**, 623–640.

NAGASAKI, K. & FLEISCHER, S. (1988). Ryanodine sensitivity of the calcium release channel of sarcoplasmic reticulum. *Cell Calcium* **9**, 1–7.

NUNN, D. L. & TAYLOR, C. W. (1992). Luminal Ca^{2+} increases the sensitivity of Ca^{2+} stores to inositol 1,4,5-trisphosphate. *Molecular Pharmacology* **41**, 115–119.

PACAUD, P. & BOLTON, T. B. (1991). Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *Journal of Physiology* **441**, 477–499.

PRESTWICH, S. A. & BOLTON, T. B. (1995). G-protein involvement in muscarinic receptor-stimulation of inositol phosphates in longitudinal smooth muscle from the small intestine of the guinea-pig. *British Journal of Pharmacology* **114**, 119–126.

RUSSELL, S. N. & AARONSON, P. I. (1990). Carbachol inhibits the voltage-gated calcium current in smooth muscle cells isolated from the longitudinal muscle of the rabbit jejunum. *Journal of Physiology* **426**, 23*P*.

SHIMO, Y. & HOLLAND, W. C. (1966). Effects of potassium on membrane potential, spike discharge, and tension in taenia coli. *American Journal of Physiology* **211**, 1299–1304.

THASTRUP, O., DAWSON, A. P., SCHARFF, O., FODER, B., CULLEN, P. J., DRØBAK, B. K., BJERRUM, P. J., CHRISTENSEN, S. B. & HANLEY, M. R. (1989). Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents and Actions* **27**, 17–23.

TOMITA, T. (1981). Electrical activity (spikes and slow waves) in gastrointestinal smooth muscles. In *Smooth Muscle: An Assessment of Current Knowledge*, ed. BÜLBRING, E., BRADING, A. F., JONES, A. W. & TOMITA, T., pp. 127–156. Edward Arnold, London.

TSIEN, R. W. & TSIEN, R. Y. (1990). Calcium channels, stores and oscillations. *Annual Review of Cell Biology* **6**, 715–760.

UNNO, T., KOMORI, S. & OHASHI, H. (1995). Inhibitory effect of muscarinic receptor activation on Ca^{2+} channel current in smooth muscle cells of guinea-pig ileum. *Journal of Physiology* **484**, 567–581.

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

VOGALIS, F., PUBLICOVER, N. G., HUME, J. R. & SANDERS, K. M. (1991). Relationship between calcium current and cytosolic calcium in canine gastric smooth muscle cells. *American Journal of Physiology* **260**, C1012–1018.

WAKAMORI, M., HIDAKA, H. & AKAIKE, N. (1993). Hyperpolarizing muscarinic responses of freshly dissociated rat hippocampal CA1 neurones. *Journal of Physiology* **463**, 585–604.

ZHOLOS, A. V., KOMORI, S., OHASHI, H. & BOLTON, T. B. (1994). Ca^{2+} inhibition of inositol trisphosphate-induced Ca^{2+} release in single smooth muscle cells of guinea-pig small intestine. *Journal of Physiology* **481**, 97–109.

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