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All-or-nothing responses to carbachol in single intestinal smooth muscle cells of rat

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1 Concentration-response relationships for carbachol (CCh)-induced increases in the cytosolic calcium concentration ($[Ca^{2+}]_i$) and membrane currents were studied by use of fura-2 microfluorimetry and nystatin-perforated whole-cell recording in single smooth muscle cells isolated from rat intestine.

2 CCh produced an initial peak rise in $[Ca^{2+}]_i$ followed by a small sustained rise. In individual cells, the peak rise in $[Ca^{2+}]_i$ did not increase in amplitude even with increasing concentrations of CCh, though the threshold concentration varied in different cells. The initial peak rise in $[Ca^{2+}]_i$, but not the sustained rise, was due to the release of stored Ca^{2+}, because it was unchanged after removal of external Ca^{2+} and the addition of nifedipine (1 μM) or La^{3+} (1 mM).

3 CCh elicited an outward and inward current in a cell dialyzed with a pipette solution containing KCl at a holding potential of − 30 mV and with one containing NaCl at − 60 mV, respectively. In individual cells, the amplitude of each current was similar in cells stimulated at over the threshold concentration of CCh, but the threshold was different among cells.

4 The percentage of cells showing Ca^{2+}-transient responses to CCh at given concentrations was similar to those showing current responses and contractile responses.

5 In thin muscle bundles, a concentration-dependent contraction was evoked by CCh in the absence of external Ca^{2+}. Its threshold was similar to those of Ca^{2+}-transient and current responses in single cells.

6 These results suggest that CCh-induced release of stored Ca^{2+} takes place in an all-or-nothing fashion in individual cells of the rat intestinal smooth muscle.

Keywords: Calcium release; carbachol; intestinal smooth muscle cell; intracellular Ca^{2+} stores; ion channel; whole-cell voltage-clamp

Introduction

In various smooth muscle cells, the activation of muscarinic receptors produces an increase of cytosolic calcium concentration ($[Ca^{2+}]_i$) due to the influx of external Ca^{2+} and the release of Ca^{2+} from intracellular stores (Bolton, 1979). It is now widely accepted that inositol 1,4,5-trisphosphate (IP_3) is the intracellular messenger which stimulates the release of Ca^{2+} from intracellular stores following receptor activation (Berridge & Irvine, 1984).

Recently, it has been shown that IP_3-induced Ca^{2+} release is potentiated in the presence of submicromolar concentrations of Ca^{2+} and receives positive feedback regulation by Ca^{2+} (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Iino & Endo, 1992). The feedback mechanism could account for the oscillations in $[Ca^{2+}]_i$ sometimes seen (DeLisle & Welsh, 1992; Miyazaki *et al.*, 1992). Therefore, it is suggested that IP_3-induced Ca^{2+} release is regenerative, that is it occurs in an all-or-nothing fashion, once Ca^{2+} release has been initiated by IP_3 (Iino *et al.*, 1994). From this suggestion, it is expected that CCh-induced release of stored Ca^{2+} occurs dose-independently, if cells are stimulated by CCh concentrations that exceed the threshold for IP_3 formation, resulting in Ca^{2+} release from intracellular stores.

We have recently demonstrated that the muscarinic agonist carbachol (CCh), activates two different ionic channel in rat intestinal smooth muscle cells, probably due to Ca^{2+} release from intracellular Ca^{2+} stores through production of IP_3 (Ito *et al.*, 1993). One is an outward K^+ current as reported in vascular (Byrne & Large, 1988; Amédée *et al.*, 1990a) and visceral smooth muscle cells (Bolton & Lim, 1989; Komori & Bolton, 1991; Ohta *et al.*, 1992), and the other is an inward current mainly due to an increase in membrane Cl^- conductance, the properties of which are similar to those in vascular (Byrne & Large, 1988; Pacaud *et al.*, 1989; Amédée *et al.*, 1990a,b) and tracheal smooth muscle cells (Janssen & Sims, 1992). If CCh causes the release of stored Ca^{2+} in an all-or-nothing fashion, the Ca^{2+}-activated CCh-induced current responses are also expected to occur in a similar manner.

The purpose of the present study was to determine whether CCh causes Ca^{2+} release from intracellular stores and evokes current responses in an all-or-nothing fashion. We investigated, therefore, the effects of various concentrations of CCh on increases in $[Ca^{2+}]_i$ and outward and inward currents in single dispersed intestinal smooth muscle cells of the rat.

Methods

Cell preparation

Male Wistar rats (200–250 g) were killed by stunning and bleeding. Smooth muscle cells were enzymatically isolated from the longitudinal muscle of rat intestine with collagenase and papain as described previously (Ohta *et al.*, 1993). Small aliquots of the dispersed cells were stored in normal external solution on cover-slips in a moist chamber at 4°C for use the same day.

Fluorescence and membrane current measurements

The cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, was estimated in single cells by fura-2 fluorescence with the ratio method using dual-wavelength excitation and single emission (Grynkiewicz *et al.*, 1985). The cells adhering to cover-slips were incubated with fura-2 in the normal external solution containing 5 μM fura-2 AM for 30 min at room temperature (approximately 25°C). Then the cover-slips were transferred to an experimental chamber (volume about 0.2 ml) on the stage of an inverted microscope attached to a fluorometer (CAM-200, Japan Spectroscopic) as described previously (Ohta *et al.*,

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1993). The fluorescence signal was detected with a Nikon CF u.v. lens (× 40) and the emission light, which was passed through a pinhole diaphragm slightly larger than a cell, was collected by a photomultiplier through a 500 nm filter. The ratio of the fluorescence due to excitation at 340 nm to that at 380 nm was calculated and was considered to be an index of $[Ca^{2+}]_i$.

Membrane currents were recorded with the nystatin-perforated patch-clamp technique to prevent the run-down of receptor-mediated responses (Horn & Marty, 1988; Ito *et al.*, 1993) using a patch-clamp amplifier (CEZ-2300, Nihon-Koden, Japan). Nystatin (50–200 $\mu g\ ml^{-1}$) was introduced into the pipette solution. The patch-pipette resistance ranged from 2.5 to 4 MΩ. The experiments were carried out 5–10 min after formation of a gigaseal at which the access resistance decreased to 10–30 MΩ. Data recording and illustration were carried out as described previously (Ohta *et al.*, 1992).

Cells were constantly superfused with the external solution at a flow rate of 2–3 $ml\ min^{-1}$ and CCh was applied to the cell through a puffer pipette placed close to it (Ito *et al.*, 1993). Using this puffer system, the solution around the cell was completely exchanged within 0.5 s. Therefore, when the time required to attain peak response was estimated, this lag time was neglected. All these experiments were performed at room temperature.

Measurement of mechanical activity

Thin longitudinal muscle bundles (0.1 mm in width, 0.7–1 mm in length) were prepared from tension recording. Mechanical activity was measured isometrically using the experimental setup described previously (Ohta *et al.*, 1992). To examine the contractile responses to CCh utilizing Ca^{2+} released from intracellular stores, the following procedure was carried out. After the disappearance of 0.1 mM CCh-induced contraction in Ca^{2+}-free solution containing 2 mM EGTA, the muscle bundles were exposed to the normal external solution containing 2.5 mM Ca^{2+} for 2 min for the purpose of Ca^{2+} loading of intracellular stores. The muscle bundles were washed with a fresh Ca^{2+}-free solution containing 2 mM EGTA for 1 min and then stimulated by various concentrations of CCh under Ca^{2+}-free conditions. These procedures were repeated and the interval between CCh-stimulations was more than 10 min to avoid possible receptor-desensitization.

Solutions

The ionic composition of the normal external solution was as follows (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, glucose 14 (pH 7.2 with NaOH). For the Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mM EGTA was added. The K^+-containing pipette solution contained (mM): KCl 132, $MgCl_2$ 1.2, $ATPNa_2$ 2, HEPES 10, EGTA 0.1 (pH 7.2 with KOH). The Na^+-containing pipette solution was prepared by substitution of equimolar NaCl for KCl (pH 7.2 with NaOH).

Drugs

Chemicals used were $ATPNa_2$ (Boehringer, Germany), carbamylcholine chloride (carbachol), collagenase, nystatin, papain (Sigma, U.S.A.), EGTA, fura-2AM, HEPES (Dojindo, Japan) and nifedipine (Wako Pure Chem, Japan).

Statistics

The results were expressed as the mean value ± s.e.mean (n = number of observations), and statistical significance was assessed with Student's t test. P values of less than 0.05 were considered to be significant.

Results

Change in cytosolic Ca^{2+} concentration induced by CCh

Single smooth muscle cells loaded with fura-2 were stimulated for 10 s by increasing concentrations of CCh at about 4 min intervals. Figure 1a shows typical $[Ca^{2+}]_i$ changes induced by CCh in two cells. In the cell shown in the upper traces, no changes in $[Ca^{2+}]_i$ occurred in response to 0.2 μM CCh. However, 0.5 μM CCh was effective in increasing $[Ca^{2+}]_i$, which occurred in three phases, a slow upstroke, an initial peak and a small sustained phase. When the concentration of CCh was increased to 1 to 10 μM, the rate of increase in $[Ca^{2+}]_i$ became more rapid, being 6.1 ± 0.6 s at 1 μM and 2.8 ± 0.2 s at 10 μM (n = 10), without change in the peak amplitude of $[Ca^{2+}]_i$. As shown in Figure 1b, the amplitudes of the peak rise in $[Ca^{2+}]_i$ were not significantly different among doses of CCh over 0.2 μM. The sustained phase of the Ca^{2+}-transient tended to elevate with increasing concentrations of CCh. Qualitatively, the same results were obtained in another cell, shown in the lower traces, in which a higher concentration of CCh (1 μM) was needed to initiate the rise in $[Ca^{2+}]_i$ than in the cell shown in the upper traces.

The peak rise in $[Ca^{2+}]_i$ elicited by 1 μM CCh was little affected by 2 min exposure of cells to Ca^{2+}-free solution containing 2 mM EGTA (91.9 ± 7.1% of control response, n = 6), a voltage-dependent Ca^{2+} channel blocker, 1 μM nifedipine (96.0 ± 2.2%, n = 4), or a non-specific Ca^{2+} entry blocker, 1 mM La^{3+} (81.9 ± 6.0%, n = 6). These results indicated that the CCh-induced initial peak rise in $[Ca^{2+}]_i$

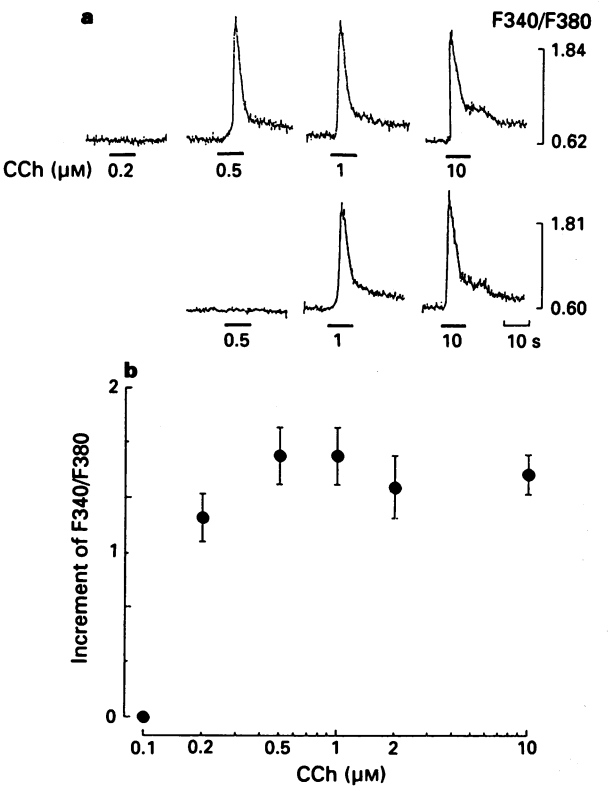

Figure 1 Ca^{2+}-transient responses of single rat intestinal smooth muscle cells to various concentrations of carbachol (CCh). In this and the following two figures, cells were stimulated by CCh for 10 s (—) with increasing concentrations from 0.1 to 10 μM at intervals of more than 4 min. (a) Representative data obtained from two cells; (b) the increment of peak rise in $[Ca^{2+}]_i$ (F340/F380) in the single cells plotted against the concentration of CCh (mean ± s.e.mean, n = 16). Each cell was stimulated with three or four concentrations of CCh, the last concentration being 10 μM. The increment of F340/F380 was estimated by subtracting the resting ratio before stimulation from the peak ratio.

was attributable to Ca^{2+} released from internal stores. In contrast, the sustained rise in $[Ca^{2+}]_i$ evoked by 1 μM CCh was decreased by the external Ca^{2+} removal (17.2 ± 5.2%), nifedipine (42.9 ± 7.8%) and La^{3+} (32.8 ± 3.8%).

Membrane current responses to CCh

To study the membrane currents induced by CCh, the nystatin-perforated whole-cell patch clamp technique was adopted to eliminate run-down of receptor-mediated current responses. First, we examined the effects of various concentrations of CCh on the outward K^+ current, which has been reported to be activated by Ca^{2+} released from intracellular stores, using K^+-containing pipette solution (KCl, 132 mM). Cells were voltage-clamped at − 30 mV to avoid full activation of K^+ channels. The representative data obtained from two different cells are shown in Figure 2. In the cell shown in the upper traces, CCh failed to evoke any current responses at 0.2 μM but elicited a large outward current at 0.5 μM. There was practically no increase in the amplitude of the current with increases in concentration to 1 and 10 μM. The lower traces show that the outward current started to appear at 1 μM CCh and again that no further increase in the amplitude of the current response was observed at 10 μM. As shown in Figure 2b, there was no significant difference between the amplitudes of the outward current induced by CCh with concentrations over 0.2 μM.

The effects of various concentrations of CCh on the inward current were examined in cells dialyzed with KCl-free, NaCl (140 mM)-containing pipette solution to prevent the development of an outward K^+ current at a holding potential of − 60 mV. Similar to the outward current response, an almost full magnitude of the inward current response was elicited by threshold doses of CCh, which differed slightly among cells, and thus there was no further increase in the amplitude with increasing concentrations of CCh (Figure 3).

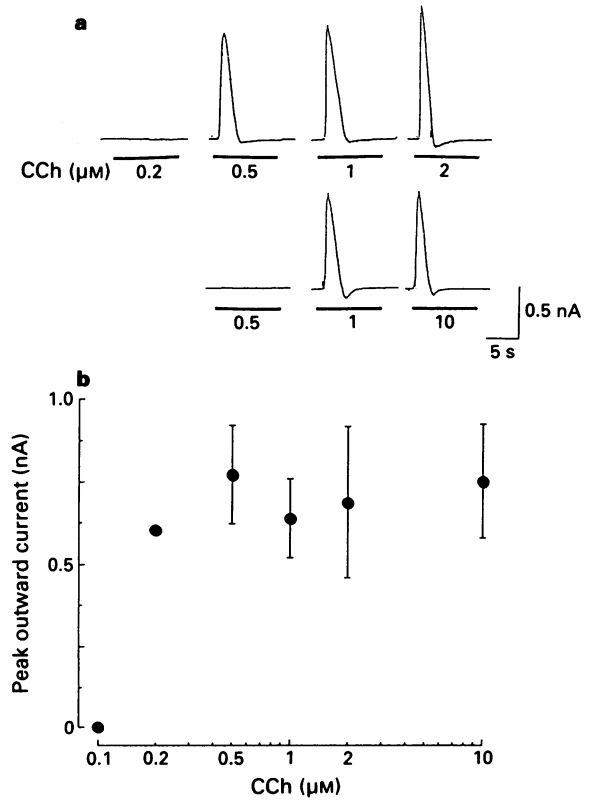

Figure 2 Outward current responses to various concentrations of carbachol (CCh). Membrane currents were recorded with nystatin-perforated whole-cell recording at a holding potential of − 30 mV using a KCl (134 mM)-containing pipette. (a) Representative data obtained from two different cells; (b) the peak amplitude of outward current plotted against CCh concentrations (mean ± s.e.mean, $n = 8$).

Concentration-dependent increase in the number of cells responding to CCh

There was no concentration-dependent increase in the amplitudes of Ca^{2+}-transient and membrane currents evoked by

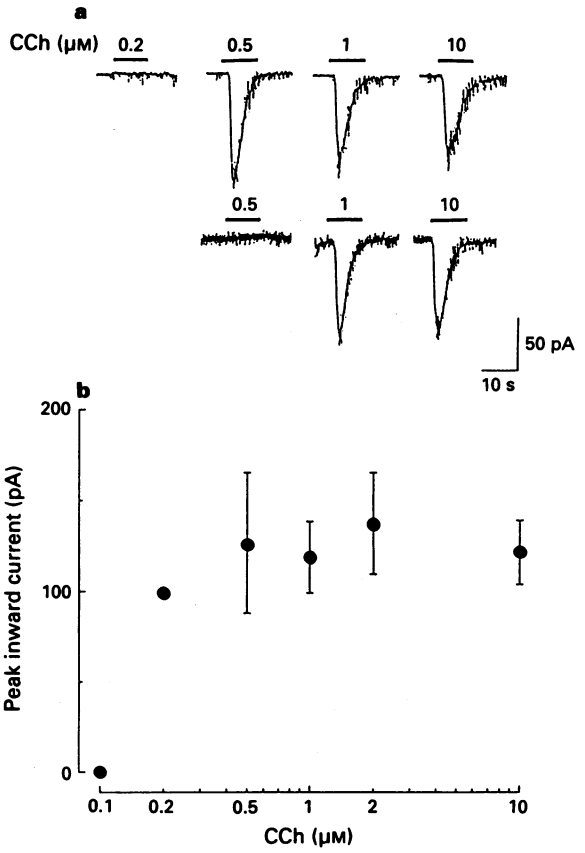

Figure 3 Inward current responses to various concentrations of carbachol (CCh). Holding potential of − 60 mV using an NaCl (134 mM)-containing pipette. (a) Typical inward current responses in two cells; (b) the peak amplitude of inward current plotted against CCh concentrations (mean ± s.e.mean, $n = 11$).

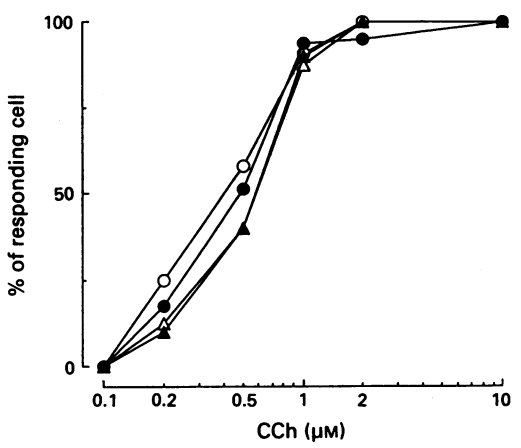

Figure 4 Concentration-dependent increase in the number of single cells responding to given concentrations of carbachol (CCh). The number of cells responding to each concentration of CCh is expressed as a percentage of those responding to 10 μM CCh. The number of contracted cells was counted by direct microscopical observation. (○) CCh-induced Ca^{2+}-transient ($n = 12$); (△) outward current ($n = 10$), (▲) inward current ($n = 8$) and (●) contractile response ($n = 64$).

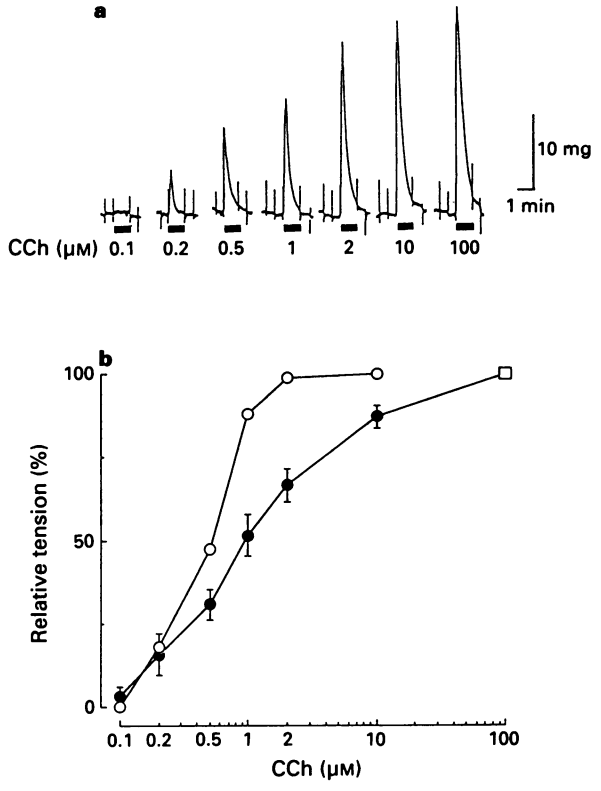

Figure 5 Carbachol (CCh)-induced contractile responses utilizing Ca^{2+} released from intracellular stores in thin muscle bundles. After 2 min exposure of the muscle fibres to the normal external solution containing 2.5 mM Ca^{2+}, they were stimulated by various concentrations of CCh for 1 min (■) in Ca^{2+}-free solution containing 2 mM EGTA. (a) CCh-induced contractile responses in the absence of external Ca^{2+}. (b) (●) the percent amplitude of each contractile response (mean ± s.e.mean, $n = 6$) as compared to that induced by 100 μM CCh (□); (○) the concentration-response curve of the mean value of the number of cells showing Ca^{2+}-transient, outward and inward currents, and contraction in single cells taken from Figure 4.

CCh in the same cells, but the threshold for CCh varied from cell to cell. Therefore, the numbers of cells responding to various concentrations of CCh in evoking Ca^{2+}-transient and current responses were estimated as a percentage of the total number of cells examined. In addition, the numbers of cells contracted by CCh were counted by microscopical observation. The percentage of the responding cells was plotted against the log concentrations of CCh (Figure 4). The concentration-response curves for all CCh-induced responses were sigmoidal and overlapped each other.

CCh-induced contraction utilizing Ca^{2+} released from intracellular stores in muscle bundles

To determine the concentration-response relationships in CCh-induced muscle contractions utilizing Ca^{2+} released from internal stores, thin muscle bundles were loaded with Ca^{2+} and then stimulated by various concentrations of CCh in a Ca^{2+}-free solution containing 2 mM EGTA. Under these conditions, CCh over 0.2 μM evoked a transient contraction, the amplitude of which increased in a concentration-dependent manner (Figure 5a). The threshold concentration of CCh (0.2 μM) inducing transient contraction was similar to those for Ca^{2+}-transient, membrane currents and contractile responses obtained in single cells. As shown in Figure 5b, the concentration-response curve for CCh-induced contraction in muscle bundles extended to higher concentrations of CCh than those obtained from single cells.

Discussion

The present results indicated that CCh evoked a peak rise in $[Ca^{2+}]_i$, and outward and inward currents in an all-or-nothing fashion, that is, there was no dose-dependent increase in the amplitude of each response with concentrations of CCh over threshold in individual cells, though the threshold concentration varied from cell to cell.

CCh has been shown to evoke outward and inward currents by activating Ca^{2+}-dependent K^+ channels and mainly Ca^{2+}-dependent Cl^- channels, respectively, and both current responses were mediated by Ca^{2+} released from intracellular stores in rat intestinal smooth muscle cells (Ito *et al.*, 1993). In the present experiments, both current responses were closely similar to Ca^{2+}-transient responses, with regard to the lack of concentration-dependency and the value of the threshold concentration of CCh. It seems likely therefore that CCh-induced current responses reflect the properties of Ca^{2+}-transient responses. These results may provide further evidence that CCh-induced outward and inward currents are dependent on an increase in $[Ca^{2+}]_i$.

The peak rise in $[Ca^{2+}]_i$ in response to CCh was due to Ca^{2+} released from intracellular stores, because the Ca^{2+}-transient was not influenced by the removal of external Ca^{2+} or by Ca^{2+} channel blockers. In general, IP_3 has been proposed as a second messenger substance provoking Ca^{2+} release from intracellular stores (Berridge & Irvine, 1984) in response to the activation of muscarinic receptor in smooth muscle cells (Gardner *et al.*, 1988; Parekh & Brading, 1991). Recently, it has been reported that IP_3-induced Ca^{2+} release receives positive feedback regulation by $[Ca^{2+}]_i$ and in consequence, Ca^{2+} released by IP_3 further accelerates IP_3-induced Ca^{2+} release (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Iino & Endo, 1992). If this is the case in the present experiments, then our results may be explained by the regenerative all-or-nothing Ca^{2+} release induced by IP_3. Similarly, CCh-induced release of stored Ca^{2+} took place in an all-or-nothing fashion in the freshly dispersed smooth muscle cells of guinea-pig taenia coli (Iino *et al.*, 1994), but not in cultured tracheal smooth muscle cells of the rat (Yang *et al.*, 1993). This difference may be due to the difference in the tissue or cells cultured.

It was noted that the percentage of the cells showing Ca^{2+}-transient responses to various concentrations of CCh agreed well with those responding with contractions in single cells. These results suggest that Ca^{2+} release from intracellular stores plays a role in initiating contractions in single intestinal smooth muscle cells of the rat, as reported in the tracheal (Janssen & Sims, 1992) and gastric smooth muscle cells (Sims, 1992). The threshold concentration of CCh required to evoke a contraction of thin muscle bundles in the absence of external Ca^{2+} was almost equal to that evoking Ca^{2+}-transient, current and contractile responses in single cells. This indicates that the threshold concentration of CCh to induce Ca^{2+} release from intracellular stores was similar regardless of differences in the preparations, muscle bundles or single cells. However, the diffusion rate for CCh should be slower in muscle bundles than in single cells and this may account for why the concentration-response curve for CCh extended to higher concentrations in muscle bundles than in single cells.

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