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Modulation of carbachol-induced $[Ca^{2+}]_i$ oscillations by Ca^{2+} influx in single intestinal smooth muscle cells

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1 Oscillations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) evoked by carbachol (CCh; 2 μM), a muscarinic agonist, were detected as oscillatory changes of muscarinic receptor-coupled cationic current (I_{cat}) in guinea-pig ileal smooth muscle cells by the whole cell patch-clamp technique.

2 Reduction of extracellular Ca^{2+} from 2 mM to 0.2 or 0.05 mM, during CCh-induced I_{cat} oscillations, caused them to disappear or to decrease markedly in frequency. A return to 2 mM Ca^{2+} concentration restored the initial I_{cat} oscillations.

3 Application of nifedipine (1-3 μM) or D600 (2-5 μM) to block the voltage-gated Ca^{2+} channel (VGCC) decreased the frequency of the ongoing I_{cat} oscillations in the cells held at -20 mV, but it was without effect in cells held at -60 mV.

4 Displacement of the holding potential of -20 mV to -60 mV to deactivate VGCC produced a decrease, an increase or no noticeable change in the frequency of the I_{cat} oscillations in different cells. Displacement to 20 mV to inactivate VGCC invariably produced a decrease in the frequency. In nifedipine-treated cells, the I_{cat} oscillations varied in frequency voltage-dependently in a reverse and linear way within the range -80 to 40 mV.

5 Application of thapsigargin (1 or 2 μM), an inhibitor of Ca^{2+}-ATPase in the membrane of internal Ca^{2+} stores, caused CCh-induced I_{cat} oscillations to disappear with a progressing phase during which their amplitude, but not frequency, declined.

6 The results suggest that membrane Ca^{2+} entry has a crucial role to play in regulation of the frequency of CCh-induced $[Ca^{2+}]_i$ oscillations in addition to persistence of their generation, and that the effect is brought about by a potential mechanism independent of Ca^{2+} store replenishment. They also provide evidence that two types of Ca^{2+} permeant channels, VGCC and an as yet unidentified channel, are involved in the Ca^{2+} entry responsible for modulation of $[Ca^{2+}]_i$ oscillations.

Keywords: Calcium oscillations; carbachol; intestinal smooth muscle; patch clamp; membrane current; calcium stores; muscarinic receptor

Introduction

Many different types of cells respond to agonists with repetitive discharge of an increase in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ oscillations) that arises from periodic release of Ca^{2+} from internal stores. The underlying process is that agonists stimulate phosphatidylinositol metabolism leading to increased production of D-myo-inositol 1,4,5-trisphosphate ($InsP_3$) and this causes Ca^{2+} release from internal stores (see for review; Berridge & Galione, 1988; Berridge, 1993). The opening of $InsP_3$-gated channels depends on $[Ca^{2+}]_i$ in such a manner that it is rapidly facilitated until $[Ca^{2+}]_i$ increases to a certain level and then it is slowly inhibited at higher $[Ca^{2+}]_i$ (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). It has been proposed that this mechanism is fundamental for the generation of $[Ca^{2+}]_i$ oscillations (De Young & Keizer, 1992; Keizer & De Young, 1993; Li *et al.*, 1995).

Ca^{2+} influx across the plasma membrane is required for the persistent generation of $InsP_3$-dependent $[Ca^{2+}]_i$ oscillations over the period of agonist stimulation (Jacob *et al.*, 1988; Kawanishi *et al.*, 1989; Hajjar & Bonventre, 1991; Yao & Parker, 1994). Although it can be readily imagined that membrane Ca^{2+} entry primarily contributes to refilling of intracellular Ca^{2+} stores, how it acts as a modulator of $[Ca^{2+}]_i$ oscillations and what sort of pathways are responsible remain unanswered questions.

In electrically non-excitable cells, various types of channels have been proposed to function as a pathway for the Ca^{2+} entry involved in the persistent generation of $[Ca^{2+}]_i$ oscillations, including those activated by $InsP_3$ (Fadool & Ache, 1992) and its metabolite (Luckhoff & Clapham, 1992; Hashii *et al.*, 1993) and those linked with internal Ca^{2+} stores (Hoth & Penner, 1992; Zweifach & Lewis, 1993). These types of Ca^{2+} channel lack a voltage-gating mechanism, and, when activated, admit extracellular Ca^{2+} in a manner dependent on the electrochemical driving force for Ca^{2+} (Luckhoff & Clapham, 1992; Zweifach & Lewis, 1993). In fact, in this cell type, the $[Ca^{2+}]_i$ oscillations increase in frequency as the membrane potential is displaced in the negative direction (Hashii *et al.*, 1993; Yao & Parker, 1994). In smooth muscle cells of the intestine, the voltage-gated Ca^{2+} channel (VGCC) plays an important role in membrane Ca^{2+} entry (Bolton, 1979; Brading & Sneddon, 1980). However, it is unknown how VGCC is involved in the persistent generation of $InsP_3$-dependent $[Ca^{2+}]_i$ oscillations induced by agonists. Furthermore, a receptor-operated channel (Benham & Tsien, 1987) and a Ca^{2+} store depletion-activated channel (Pacaud & Bolton, 1991; Ohta *et al.*, 1995), might be also involved in membrane Ca^{2+} entry during application of these agonists.

Carbachol (CCh) is capable of inducing $InsP_3$-dependent $[Ca^{2+}]_i$ oscillations in single smooth muscle cells of guinea-pig ileum (Komori *et al.*, 1993; Zholos *et al.*, 1994; Kohda *et al.*, 1996). The present study aimed to investigate potential pathways and roles for membrane Ca^{2+} entry in the persistent generation of CCh-induced $[Ca^{2+}]_i$ oscillations. We used the whole-cell patch clamp technique to detect the $[Ca^{2+}]_i$ oscillations as oscillatory changes in CCh-induced cationic current (I_{cat}). I_{cat} arises from the opening of channels coupled with muscarinic receptors via a pertussis toxin-sensitive G protein (Benham *et al.*, 1985; Inoue & Isenberg, 1990; Komori *et al.*, 1992) and can change its intensity in a manner closely correlated with $[Ca^{2+}]_i$ (Pacaud & Bolton, 1991; Komori *et al.*,

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1993). Membrane Ca^{2+} entry was altered by changing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and cell membrane potential and by use of organic Ca^{2+} channel blockers.

The results suggest that membrane Ca^{2+} entry has a crucial role to play in the regulation of discharge frequency, in addition to the persistent generation, of $[Ca^{2+}]_i$ oscillations, and that the effect arises through a mechanism independent of Ca^{2+} store replenishment. They also provide evidence that two types of Ca^{2+} permeant channel, the VGCC and another as yet unidentified channel, are involved in the Ca^{2+} entry responsible for the modulation of $[Ca^{2+}]_i$ oscillations.

Methods

Preparation of cells

Male guinea-pigs, weighing 350–450 g were stunned and killed by exanguination. A 10 to 15 cm length of the ileum was removed and divided into 3 cm segments. The longitudinal muscle layer of the segments was peeled from the underlying circular muscle and washed in physiological salt solution (PSS; composition given below).

Smooth muscle cells were dispersed by a combination of collagenase (0.2–0.6 mg ml^{-1}) and papain (0.3–0.6 mg ml^{-1}), as previously described (Komori *et al.*, 1992). 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 (15 mm in diameter) and kept in a moist atmosphere at 4°C until use on the same day.

Recording of membrane current

A coverslip with cells was placed in a small bath (0.8 ml), which was then filled with PSS of the following composition (mM): NaCl 126, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1.2, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). For preparation of PSS containing a lower concentration of $CaCl_2$ (0.2 or 0.05 mM), the $CaCl_2$ was reduced without using any substitute. The cells were voltage-clamped with patch pipettes filled with a CsCl-based solution which had the following composition (mM): CsCl 134, $MgCl_2$ 1.2, MgATP 1, EGTA 0.05, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). Patch pipettes had a resistance of 4–6 MΩ. Whole-cell membrane current was recorded at room temperature (20–25°C) by the conventional patch-clamp technique. A voltage-clamp amplifier (Nihon Kohden, SEZ-2300; Tokyo, Japan) was used for current recording, and membrane currents were stored on FM tape and replayed onto a thermal array recorder (Nihon Kohden, RTA-1100M; Tokyo, Japan) for illustrations and analysis.

Values in the text are given as means ± s.e.mean with the number of cells (n) used for measurements. Statistical significance was determined by a paired or unpaired Student's t test and differences were considered significant when $P<0.05$.

Drugs

Drugs used were: carbachol chloride, nifedipine (from Tokyo Kasei; Tokyo, Japan), methoxyverapamil (D600), thapsigargin (from Sigma; St.Louis, Mo., U.S.A.) and caffeine (from Wako; Tokyo, Japan).

Application of drugs was made by replacing the bathing medium with the drug-containing solution several times within 10–15 s. A similar solution-exchange method was also used for changing extracellular Ca^{2+}.

Results

I_{cat}, activation of which is very sensitive to a change in $[Ca^{2+}]_i$ (Pacaud & Bolton, 1991; Komori *et al.*, 1993), was recorded to detect oscillations in $[Ca^{2+}]_i$ induced by CCh in single guinea-pig ileal cells. Cells were voltage-clamped by use of patch pipettes filled with a CsCl-based solution to block Ca^{2+}-activated potassium current elicited by CCh at the same time (Komori *et al.*, 1992; 1993; Zholos *et al.*, 1994). CCh was used at 2 μM, an effective concentration in eliciting oscillatory changes in $[Ca^{2+}]_i$ and I_{cat} (Komori *et al.*, 1993; Kohda *et al.*, 1996).

In most cells held at −60 mV or −20 mV, CCh produced oscillatory I_{cat} responses. The I_{cat} oscillations were evoked usually from a sustained I_{cat} component with a more or less regular frequency, as previously described (Komori *et al.*, 1993), and they persisted for the entire or early period during the application of CCh (2–10 min). The frequency varied between 0.08 and 0.65 Hz among cells held at −60 mV, giving a mean of 0.25 ± 0.01 Hz ($n=112$), and between 0.09 and 0.70 Hz among cells held at −20 mV, giving a mean of 0.31 ± 0.02 Hz ($n=75$). The difference between the two mean values was statistically significant ($P<0.05$). The amplitude of individual oscillations usually increased for several of the early cycles after CCh application and then remained constant or declined gradually to reach a stable level. In a fraction of cells (approximately 30%), the amplitude of the first I_{cat} oscillation was greater than that of subsequent I_{cat} oscillations.

Effect of a reduction of $[Ca^{2+}]_o$ on CCh-induced I_{cat} oscillations

In cells held at −60 mV, when $[Ca^{2+}]_o$ was reduced by replacing the bathing medium with a low Ca^{2+}-solution stepwise from 2 mM to 0.2 or 0.05 mM for 60–90 s during CCh-induced I_{cat} oscillations, the I_{cat} oscillations disappeared in 23% cells at 0.2 mM and 36% cells at 0.05 mM. In the remaining cells, the I_{cat} oscillations were still generated but decreased in frequency to 72 ± 2% ($n=10$) at 0.2 mM and to 54 ± 4% ($n=9$) at 0.05 mM, of the control (Figure 1). The difference between the two mean values was statistically significant ($P<0.05$). The amplitude of individual I_{cat} oscillations was transiently increased to a varied extent, which occurred independently of the change in frequency (Figure 1a–c). The persisting I_{cat} oscillations at 0.2 mM $[Ca^{2+}]_o$ were invariably abolished upon subsequent reduction of $[Ca^{2+}]_o$ to 0.05 mM, as shown in Figure 1c ($n=4$).

Figure 1d shows that returning the $[Ca^{2+}]_o$ to the initial level (2 mM) increased the reduction frequencies of 72 ± 2% at 0.2 mM and 54 ± 4% at 0.05 mM to 113 ± 7% ($n=10$) and 96 ± 6% ($n=9$), respectively. In six out of eight cells in which I_{cat} oscillations disappeared following lowering of $[Ca^{2+}]_o$ to 0.2 or 0.05 mM, this treatment was effective in restoring I_{cat} oscillations.

The finding that a reduction of $[Ca^{2+}]_o$ caused CCh-induced I_{cat} oscillations to cease or decrease in frequency indicates an important role for Ca^{2+} influx across the plasma membrane in the persistent generation of the I_{cat} oscillations and/or modulation of their frequency.

Effects of Ca^{2+} channel blockers on CCh-induced I_{cat} oscillations

Nifedipine, known to block VGCC in smooth muscle cells, was applied during ongoing CCh-induced I_{cat} oscillations. In cells held at −20 mV, nifedipine (1 or 3 μM) immediately decreased the oscillation frequency with some reduction in the sustained component of I_{cat}, as shown in Figure 2a and b. Similar results were obtained with another blocker of VGCC, methoxyverapamil (D600; 2 μM). Figure 3 shows plots of the frequencies measured within 0.5–2 min after nifedipine or D600 application, expressed as percentages of the control (the frequency immediately before the drug application). The percentages in nifedipine-treated cells varied from 28 to 95%, giving a mean of 63 ± 5% ($n=20$), and the corresponding data in D600-treated cells varied from 29 to 80%, giving a mean of 59 ± 9% ($n=6$). The inhibitory effects of nifedipine and D600 on the frequency did not depend on the control frequency which ranged from

0.12 to 0.55 Hz (Figure 3). In fact, the correlation coefficient was calculated to be 0.2324 from the twenty data points in the case of nifedipine. The drugs also caused the I_{cat} oscillations to decrease in amplitude. However, the large variation of the effect from one cell to another did not allow any quantification of these results. A gradual decline of the oscillation amplitude with time after application of either drug was often followed by the disappearance of oscillatory changes.

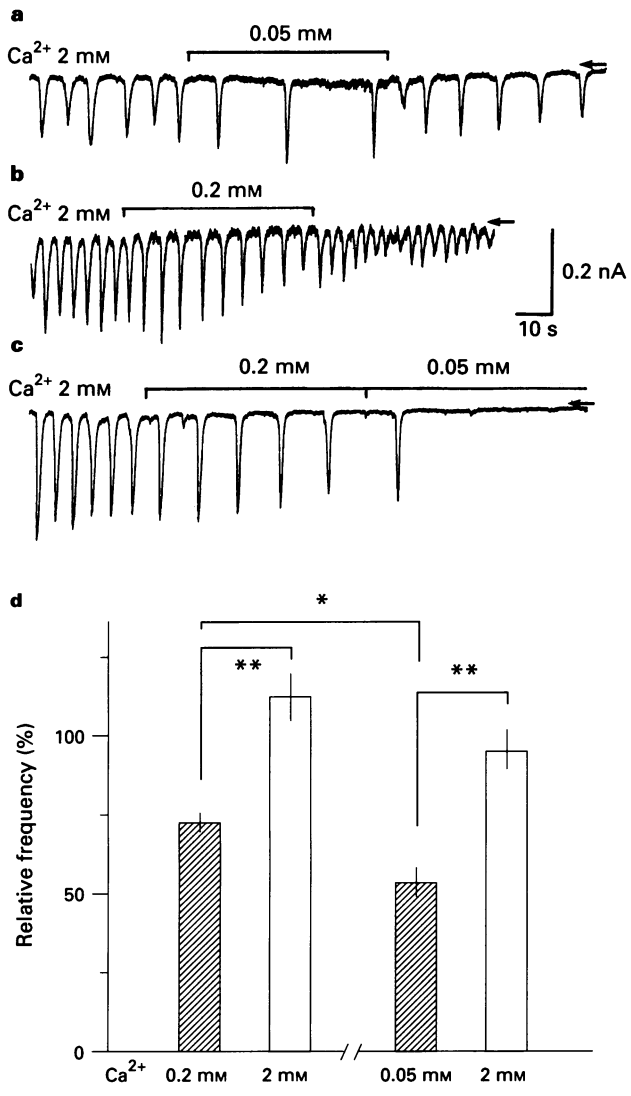

Figure 1 Modulation by extracellular Ca^{2+} of oscillatory inward current (I_{cat}) responses to carbachol (CCh; 2 μM). (a), (b) and (c) Changes in the frequency of I_{cat} oscillations before and during reduction of Ca^{2+} concentration in the bathing solution from 2 mM to 0.05 or 0.2 mM (indicated by horizontal lines above each current trace) and after the initial Ca^{2+} level (2 mM) had been restored, in three different cells held at −60 mV. A decrease in the frequency during the Ca^{2+} reduction occurred with (b) or without (a and c) a progressive decline in oscillation amplitude. In (c), oscillations persisting at 0.2 mM disappeared upon subsequent reduction to 0.05 mM. The calibration bars in (b) are applicable to (a) and (c). Horizontal arrows indicate the level of current before CCh application. (d) A summary of the changes in the frequency of I_{cat} oscillations during reduction of the extracellular Ca^{2+} to 0.2 and 0.05 mM (hatched columns) and after the return to 2 mM (open columns). Oscillation frequencies are expressed as percentages of those before reduction of the extracellular Ca^{2+}. The mean values ±s.e.mean were obtained from 10 and 9 experiments for the left and right pairs of columns, respectively. The differences between the columns connected by solid lines are statistically significant (*$P<0.05$; **$P<0.01$). See text for details.

Figure 2c shows no noticeable effect of nifedipine (3 μM) on the frequency of CCh-induced I_{cat} oscillations in a cell held at −60 mV. It can be seen that the drug somewhat decreased the sustained component of I_{cat}. The inhibitory effect, however, varied in extent with a wide range from one cell to another. The amplitude of individual oscillations was not appreciably changed compared with the control. As shown in Figure 3, the frequencies in the presence of nifedipine (3 μM) and D600 (2-5 μM) were located in a relatively small area around the line of 100% in cells held at −60 mV. The mean percentage was 99±5% ($n=9$) for nifedipine and 95±7% ($n=4$) for D600. The results suggest that VGCC and some other pathway distinct from VGCC are involved in Ca^{2+} influx occurring during the oscillatory I_{cat} response to CCh.

CCh-induced I_{cat} oscillations at different membrane potentials

The membrane potential affects the activity of VGCCs and the electrical driving force for Ca^{2+}, and hence Ca^{2+} influx into the cell.

When the holding potential was displaced from −20 mV, at which VGCCs are operative, to −60 mV, at which VGCCs are almost totally deactivated (Imaizumi *et al.*, 1989), the frequency of CCh-induced I_{cat} oscillations decreased (Figure 4a), increased (Figure 4b) or showed no noticeable change, in different cells. Figure 4e shows percentages of the frequency at −60 mV relative to that at −20 mV in 17 cells (see data points on the left side). The percentages varied from 40 to 160%, giving a mean of 93±8% ($n=17$). In nifedipine (1 or 3 μM)-treated cells, however, the displacement to −60 mV invariably increased the frequency to 135±5% ($n=12$) with a range of 110 to 220% (Figure 4c,e). In addition, the decreased oscillation frequency in the presence of nifedipine was increased by displacing the holding potential from −20 mV to

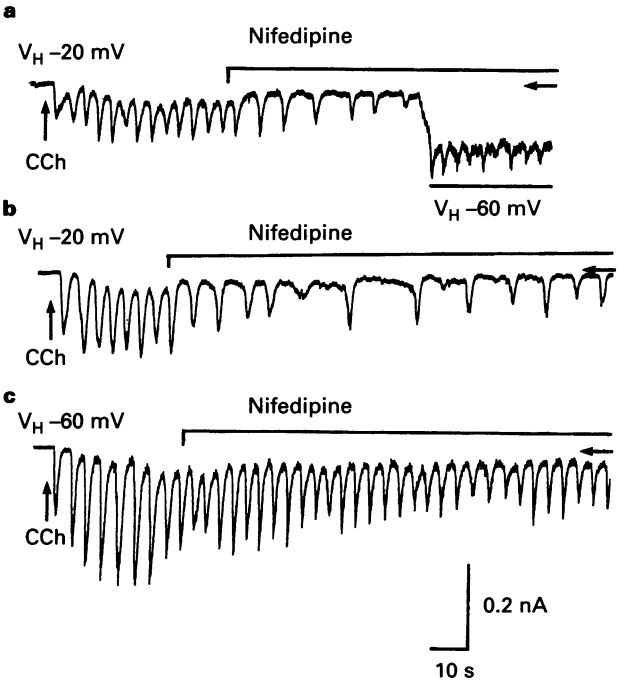

Figure 2 Modulation by nifedipine of carbachol (CCh)-induced I_{cat} oscillations. CCh (2 μM) was applied at the arrow and continued to be present throughout the experiment. (a) and (b) A decrease in the frequency of I_{cat} oscillations after nifedipine (1 μM in (a) and 3 μM in (b)) application (indicated by horizontal lines above each current trace) to cells held at −20 mV. In (a), the holding potential (V_H) was displaced to −60 mV (indicated by the line below the current trace). (c) No noticeable change in the frequency of I_{cat} oscillations was observed after nifedipine (3 μM) application to a cell held at −60 mV.

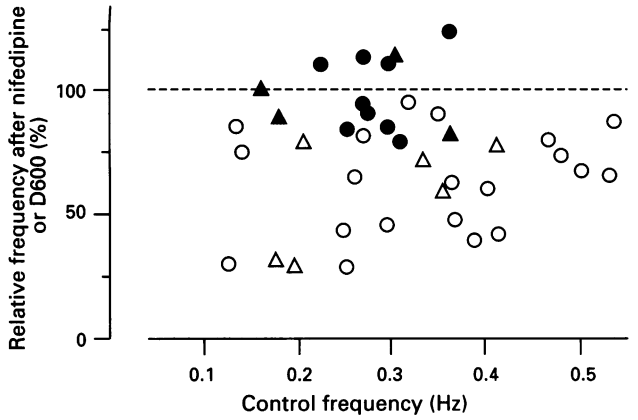

Figure 3 Effects of nifedipine and D600 on carbachol (CCh, 2 μM)-induced I_{cat} oscillations with different frequencies. Abscissa scale: oscillation frequency (Hz) before application of nifedipine (1–3 μM) or D600 (2–5 μM). Ordinate scale: relative frequency (%) after application of either drug compared to the control frequency before application of the drug. Relative frequencies in cells held at −20 mV (○) and in cells held at −60 mV (●), after nifedipine application. Relative frequencies in cells held at −20 mV (△) and in cells held at −60 mV (▲), after D600 application. The correlation coefficient calculated from data points (○) is 0.2314 (n = 20). Note the difference in distribution of the relative frequencies between cells held at −60 mV and −20 mV, irrespective of nifedipine and D600. See text for details.

more negative levels (see Figure 2a). Displacement of the holding potential under the same conditions was also effective in restoring oscillatory changes in I_{cat}, but only when this was done within a short period after they had disappeared following nifedipine application (data not shown). Thus, Ca^{2+} permeant channels distinct from VGCC are operative at −60 mV, resulting in Ca^{2+} entry which modulates the frequency.

When the holding potential of −20 mV was displaced to 20 mV at which VGCCs are almost totally inactivated (Imaizumi *et al.*, 1989), I_{cat} oscillations decreased in frequency in six out of ten cells. The four remaining cells responded with cessation of the oscillations. In the six cells, the frequencies at 20 mV expressed as a percentage of the control ranged from 30 to 75% (Figure 4e), giving a mean of 61 ± 7% (n = 6). As seen from Figure 4d, the oscillations were outwardly directed at 20 mV, in accordance with this voltage (20 mV) being more positive than the reversal potential of around 0 mV for I_{cat} (Pacaud & Bolton, 1991).

To investigate voltage-dependence of the oscillation frequency of I_{cat} after blockade of VGCCs, I_{cat} oscillations were evoked by CCh in the presence of nifedipine (1–3 μM), and the holding potential was displaced from one level (−60 to 40 mV) to different levels ranging between −80 and 40 mV, as illustrated in Figure 5. In all cells tested, the frequency of I_{cat} oscillations became higher as the holding potential was displaced to a more negative potential. Figure 6 shows plots of the frequency against the displaced holding potential obtained

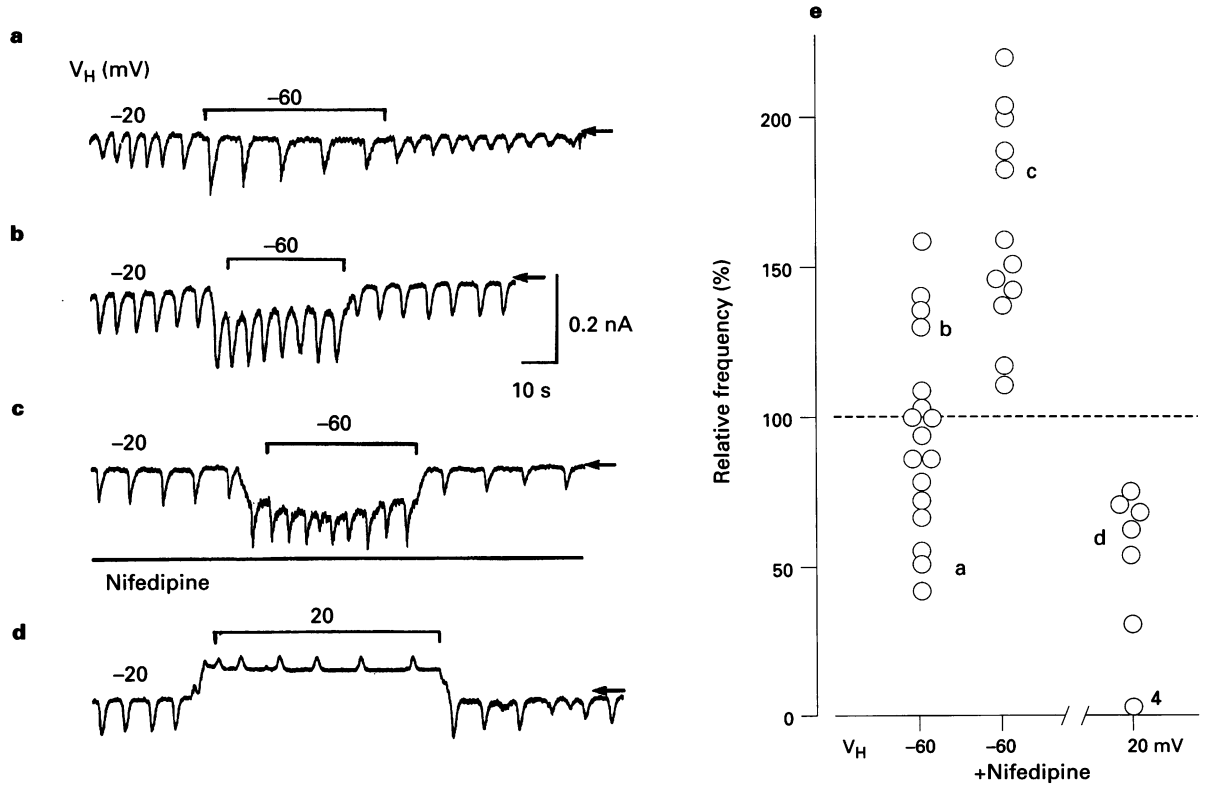

Figure 4 Effect of membrane potential on carbachol (CCh; 2 μM)-induced I_{cat} oscillations. Holding potential (V_H) was displaced from −20 mV to −60 mV or 20 mV, as indicated by lines above each recording trace. (a) and (b) A decrease and an increase in the frequency of I_{cat} oscillations during displacement to −60 mV, respectively. (c) In the presence of nifedipine (3 μM), an increase in the frequency during displacement to −60 mV. (d) A decrease in the frequency during displacement to 20 mV. I_{cat} oscillations were outwardly directed at 20 mV in accordance with this potential being more positive than the reversal potential for I_{cat} (around 0 mV). The calibration bars in (b) are applicable to (a), (c) and (d). Horizontal arrows indicate the level of current before CCh application. (e) Frequencies of I_{cat} oscillations during displacement to −60 mV or 20 mV, expressed as percentages of those before the displacement (at −20 mV). Left to right in normal cells and in nifedipine-treated cells during displacement to −60 mV, and in normal cells during displacement to 20 mV, of which four cells stopped generating I_{cat} oscillations, taken as 0%. Data points with (a–d) are obtained from current traces (a–d), respectively. Note the difference in responses on displacement to −60 mV between, before and after nifedipine application.

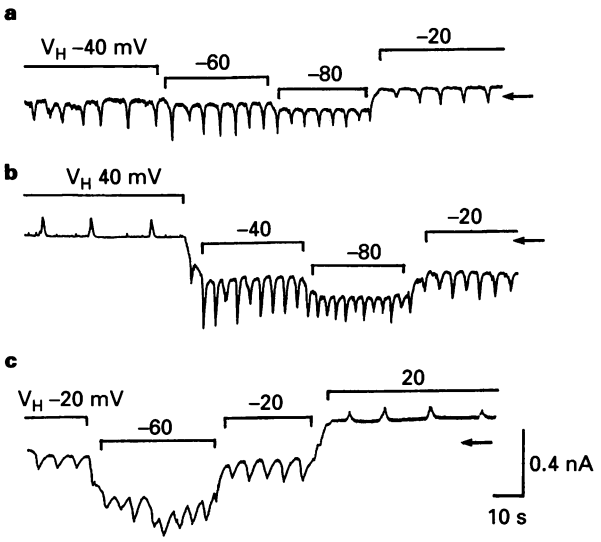

Figure 5 Effect of membrane potential on carbachol (CCh, 2 μM)-induced I_{cat} oscillations in the presence of nifedipine (1–3 μM). Holding potential (V_H) was displaced from −40 mV (a), 40 mV (b) or −20 mV (c) to various levels, as indicated by horizontal lines above each current trace. Traces in (a–c) are from different cells. The calibration bars in (c) are applicable to (a) and (b). Note that the more negative the V_H, the higher the frequency of I_{cat} oscillations.

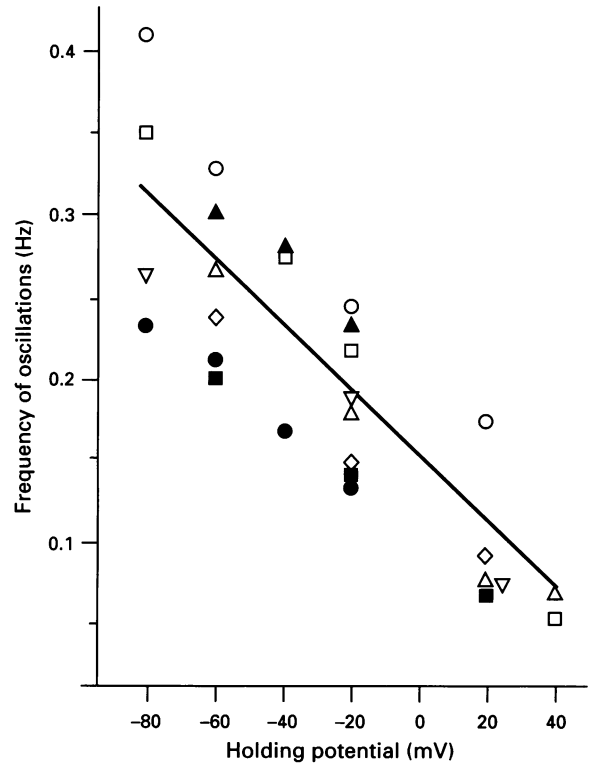

Figure 6 Relationship between holding potential (V_H) and frequency of I_{cat} oscillations in the presence of nifedipine (1–3 μM). As in Figure 5, V_H was displaced within the range −80 to 40 mV in a random order during oscillatory I_{cat} responses to CCh (2 μM). Abscissa scale: the V_H (mV); ordinate scale: frequency of I_{cat} oscillations (Hz). Different symbols are from eight different experiments. The straight line indicates a regression line (Y = 0.157-0.002X, where Y is frequency (Hz) and X V_H (mV)) with a correlation coefficient ($r = -0.8577$, $P < 0.001$) calculated from all 28 data points.

from eight different experiments (see different symbols). Calculation from all the plotted data gave a linear regression line, as indicated in the figure, suggesting a negative and linear dependence of the frequency on membrane potential, at least within the range −80 and 40 mV.

The results suggest that membrane potential functions as a modulator of the frequency of I_{cat} oscillations probably through changes in membrane Ca^{2+} influx via VGCC and the other pathway(s).

Effect of thapsigargin on CCh-induced I_{cat} oscillations

Thapsigargin (1 or 2 μM), a specific inhibitor of Ca^{2+}-transporting ATPase in the Ca^{2+} store membrane (Takemura *et al.*, 1989; Thastrup *et al.*, 1990), when applied during I_{cat} oscillations evoked by CCh at −20 or −60 mV, caused them to disappear. The effect occurred with a latency of up to 1 min

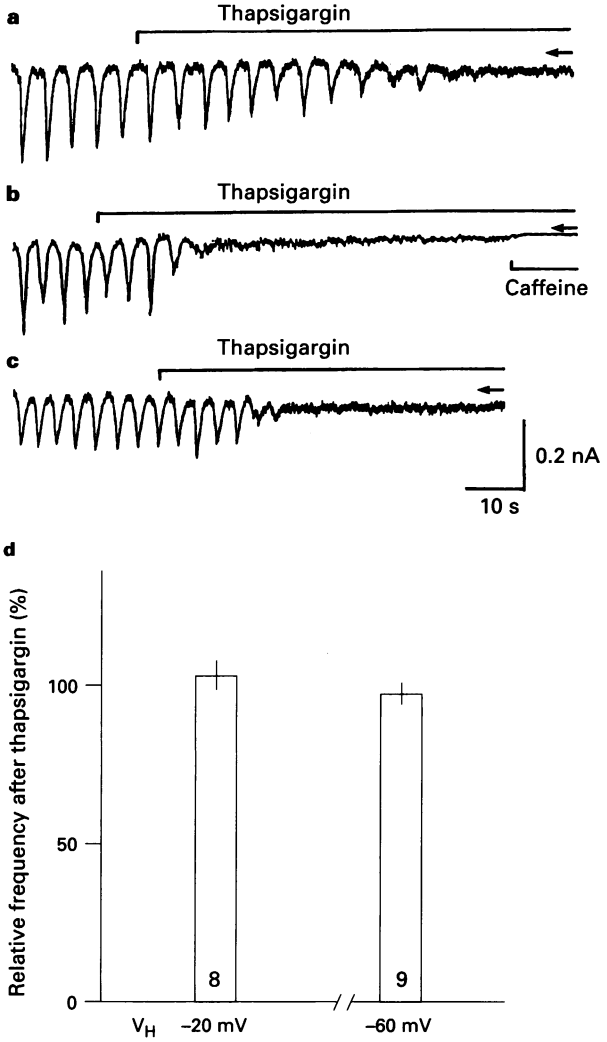

Figure 7 Effects of thapsigargin on oscillatory inward I_{cat} responses to carbachol (CCh; 2 μM). Thapsigargin (1 μM) was applied in the continued presence of CCh (indicated by horizontal lines above each current trace). Traces in (a–c) are from three different cells held at −60 mV (a and b) or −20 mV (c). In (b), 10 mM caffeine was applied (indicated by the line below the current trace). The calibration bars in (c) are applicable to (a) and (b). Horizontal arrows indicate the level of current before CCh application. (d) Frequencies of I_{cat} oscillations after application of thapsigargin (1–3 μM) in cells held at −20 mV (left column) and in cells held at −60 mv (right column). Ordinate scale: relative frequency expressed as a percentage of that before thapsigargin application. Each column represents the mean ± s.e. mean from the number of experiments indicated. Note no noticeable change in the frequency of I_{cat} oscillations during the period in which they persisted following thapsigargin application. See text for details.

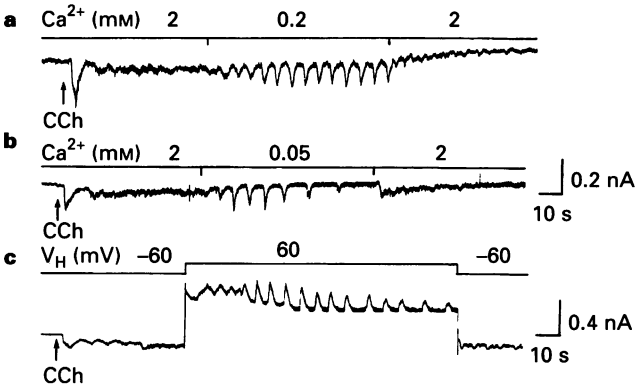

Figure 8 Induction of oscillatory I_{cat} responses to carbachol (CCh) following application of low-Ca^{2+} external solution or displacement of membrane potential. CCh (2 μM) was applied at the arrows and continued to be present throughout the experiment. (a) and (b) Oscillatory responses to a reduction of the external Ca^{2+} from 2 mM to 0.2 mM (a) or 0.05 mM (b) (indicated by lines above each current trace) in cells held at −60 mV. (c) Oscillatory response to displacement of the holding potential (V_H) from −60 mV to 60 mV (indicated by a line above the current trace) in a nifedipine (3 μM)-treated cell. The calibration bars in (b) are applicable to (a).

during which the oscillations decreased in amplitude gradually, but not in frequency, as shown in Figure 7a–c. The percentage frequencies against the control were 104±4% (n=8) and 96±3% (n=9), in respective cells held at −20 and −60 mV (Figure 7d). When 10 mM caffeine was applied subsequent to the disappearance of the oscillations, it produced no increase in the sustained I_{cat} component (Figure 7b; n=8). This indicates that Ca^{2+} stores had been depleted before caffeine was applied, since caffeine can release Ca^{2+} from storage sites if they have not been depleted and produce a transient increase in I_{cat} (Komori *et al.*, 1993; Zholos *et al.*, 1994). We have shown (Kohda *et al.*, 1996) that thapsigargin acts immediately to inhibit Ca^{2+}-transporting ATPase and depletes Ca^{2+} stores within 1–1.5 min when it is applied to single ileal cells.

The finding that inhibition of the Ca^{2+} uptake into the storage sites had no primary effect on the frequency of I_{cat} oscillations suggests that the frequency is not determined solely by the activity of the Ca^{2+} pump in the membrane Ca^{2+} store.

Induction of I_{cat} oscillations by reduction of membrane Ca^{2+} entry

In some cells which did not respond to 2 μM CCh with oscillations in I_{cat}, I_{cat} oscillations occurred only when $[Ca^{2+}]_o$ was reduced from 2 mM to 0.2 or 0.05 mM, as shown in Figure 8a and b. The I_{cat} oscillations readily disappeared after the $[Ca^{2+}]_o$ had returned to initial level. Displacement of the holding potential from −60 mV to 40 or 60 mV exerted a similar effect in the presence of nifedipine (1–3 μM), as shown in Figure 8c.

The results suggest that membrane Ca^{2+} entry can act also to inhibit generation of I_{cat} oscillations under certain circumstances.

Discussion

The results in this study provide evidence that the frequency of CCh-induced $[Ca^{2+}]_i$ oscillations which are driven by $InsP_3$-induced Ca^{2+} release from internal stores (Komori *et al.*, 1993; Zholos *et al.*, 1994; Kohda *et al.*, 1996) can be affected by membrane Ca^{2+} entry in guinea-pig ileal smooth muscle cells. A decrease in the frequency of CCh-induced I_{cat} oscillations was observed following manipulations which reduced membrane Ca^{2+} entry, namely, lowering of $[Ca^{2+}]_o$, application of organic Ca^{2+} channel blockers, or displacement of membrane potential in the positive direction. Two types of Ca^{2+} permeant channels seem likely to be involved in the membrane Ca^{2+} entry responsible for the modulation of the frequency of I_{cat} oscillations ($[Ca^{2+}]_i$ oscillations). The frequency of I_{cat} oscillations was decreased following application of organic Ca^{2+} channel blockers, nifedipine and D600, in cells, the membrane potential of which was held at −20 mV but not at −60 mV. VGCC is activated near to its maximal extent at −20 mV, but it is deactivated at −60 mV in many types of smooth muscle cells (Imaizumi *et al.*, 1989; Ganitkevich & Isenberg, 1991). Therefore, VGCCs appear to be involved. The cell variation in sensitivity to the Ca^{2+} channel blockers (see Figure 3) suggests the varied contribution of Ca^{2+} entry through opening of this channel to the total Ca^{2+} entry in different cells. Recently, we have shown that CCh reduces Ca^{2+} current flowing through VGCCs in the same type of smooth muscle cells, and that the extent of the inhibitory effect varies between cells (Unno *et al.*, 1995). This cell variation might also contribute to the different sensitivity to the Ca^{2+} channel blockers of the frequency of $[Ca^{2+}]_i$ oscillations.

The other Ca^{2+} permeant channel is characterized by its insensitivity to the Ca^{2+} channel blockers, its being accelerated and/or activated by stimulation of muscarinic receptors, and its negative and linear dependence on membrane potential. The relationship between the oscillation frequency and membrane potential suggests that Ca^{2+} entry through opening of this pathway is determined solely by the electrical driving force for Ca^{2+}. Similar membrane potential dependence of the oscillation frequency and membrane Ca^{2+} entry evoked by agonists has been demonstrated in non-excitable cells. It is notable that ionic pathways distinct from VGCC function to allow extracellular Ca^{2+} to enter the cells (Hashii *et al.*, 1993; Yao & Parker, 1994). The muscarinic receptor-coupled cation channels (Benham *et al.*, 1985; Inoue & Isenberg, 1990; Zholos & Bolton, 1994) seem unlikely to be a promising candidate responsible for this component of Ca^{2+} entry, since Ca^{2+} entry does not occur following opening of the cationic channels by CCh (up to 100 μM) in the presence of extracellular Ca^{2+} at the same level as used for the present study (Pacaud & Bolton, 1991).

A Ca^{2+} permeant channel that is activated by depletion of internal Ca^{2+} stores by CCh or caffeine, has been suggested to be present in guinea-pig jejunal cells (Pacaud & Bolton, 1991) and in rat ileal cells (Ohta *et al.*, 1995). Ca^{2+} entry through opening of this type of channel is characterized by resistance to organic Ca^{2+} channel blockers and its dependence on the electrochemical driving force for Ca^{2+} (Pacaud & Bolton, 1991). The similarity with our data leads us to assume that a Ca^{2+} store-dependent pathway is present in the cells used for the present study and Ca^{2+} entry through this channel opening is involved in the modulation of the frequency of $[Ca^{2+}]_i$ oscillations. Recently, the existence of $InsP_3$-activated channels (Fadool & Ache, 1992) and $InsP_4$-modulated channels (Luckhoff & Clapham, 1992; Hashii *et al.*, 1993) has been suggested in non-excitable cells. These types of channels might have a role in the membrane Ca^{2+} entry involved in the observed changes in the oscillation frequency.

The component of Ca^{2+} entry through VGCC opening at the steady state changes in relation to voltage resulting in a bell-shaped curve with a peak at −10 to −20 mV, which is given as a function of both activation and inactivation of VGCCs (Imaizumi *et al.*, 1989). The other component of Ca^{2+} entry changes in proportion to the electrical driving force for Ca^{2+}. Therefore, when membrane potential is displaced to a negative potential from a potential (−20 mV) at which VGCC is activated to its maximal extent, the two components of Ca^{2+} entry are altered in the opposite direction; a shift of membrane potential from −20 mV to −60 mV (membrane hyperpolarization) causes the VGCC-related component to decrease but the other component to increase. Whereas, membrane depolarization from −60 mV to −20 mV causes the former component to increase but the latter component to decrease. Therefore, the cell-to-cell difference in the oscillatory responses

to these changes in membrane potential could be attributable to the variable contribution of the two components of Ca^{2+} entry to the overall Ca^{2+} entry in different cells. On the other hand, when membrane potential is displaced from −20 mV to 20 mV, both components of Ca^{2+} entry are expected to be reduced. As a result, the oscillation frequency would be decreased. In fact, it was invariably decreased in cells in which the holding potential was shifted from −20 mV to 20 mV.

The question arises as to how membrane Ca^{2+} entry modulates the oscillation frequency. It is possible that the cyclical $InsP_3$-induced Ca^{2+} release is accelerated through facilitation of replenishment of the stores brought about by membrane Ca^{2+} entry. However, the result obtained with thapsigargin, an inhibitor of Ca^{2+}-ATPase in Ca^{2+} store membrane, makes this unlikely. The drug did not alter the frequency of I_{cat} oscillations before they ceased. The rate and state of Ca^{2+} store refilling seem to have a minor role in determining the frequency of $[Ca^{2+}]_i$ oscillations, as in some other cells (endothelial cells, Jacob *et al.*, 1988; oocytes, Yao & Parker, 1994). However, the idea is not incompatible with the finding that lowering of $[Ca^{2+}]_o$ or application of Ca^{2+} channel blockers during oscillatory activity resulted in an immediate decrease in the frequency without a progressive process of development. On the other hand, the amplitude of individual oscillations seems likely to be related closely to the refilling state of Ca^{2+} stores at a moment when it discharges. In pituitary gonadotrophs, progressive depletion of Ca^{2+} stores is suggested to underlie a gradual decline in the amplitude of agonist-induced $[Ca^{2+}]_i$ oscillations (Stojilkovic *et al.*, 1993).

The opening of $InsP_3$-gated channels, which are responsible for the release of Ca^{2+} from internal stores, is potentiated by $[Ca^{2+}]_i$ at low concentrations and inhibited at high concentrations (Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). The inhibition of $InsP_3$-induced Ca^{2+} release by $[Ca^{2+}]_i$ occurs with a slower time course than the potentiation (Parker & Ivorra, 1990). In our previous study on guinea-pig ileal cells (Zholos *et al.*, 1994), it was demonstrated that the opening of $InsP_3$-gated channels during muscarinic receptor activation was inhibited when $[Ca^{2+}]_i$ was elevated over a certain level and loss of the inhibition with time allowed $InsP_3$ to act. Simulated models of this situation have revealed that the duality of the action of $[Ca^{2+}]_i$ on $InsP_3$-gated channels can serve for their regenerative opening leading to an oscillatory change in $[Ca^{2+}]_i$ in the presence of steady levels of $InsP_3$ (De Young & Keizer, 1992; Keizer & De Young, 1993; Li *et al.*, 1995). This relation between the $InsP_3$-gated channel and $[Ca^{2+}]_i$ has an analogy to that between membrane excitability of neurones and membrane potential (Li *et al.*, 1995). If the duality of the action of $[Ca^{2+}]_i$ also contributes to the oscillations of $[Ca^{2+}]_i$ in ileal cells, membrane Ca^{2+} entry could affect the oscillation frequency by changing the basal $[Ca^{2+}]_i$; a rise in the basal $[Ca^{2+}]_i$ would promote regenerative opening of $InsP_3$-gated channels and increase the frequency of $[Ca^{2+}]_i$ oscillations. This hypothesis is supported by the finding that a decrease in the oscillation frequency was usually accompanied by some reduction of the sustained component of I_{cat}, reflecting a decline in the basal $[Ca^{2+}]_i$ (Pacaud & Bolton, 1991) (see Figure 1b,c and Figure 2a,b). On the basis of this idea, immediate termination of $[Ca^{2+}]_i$ oscillations after reduction of $[Ca^{2+}]_o$ and displacement of membrane potential to 20 mV or more positive potentials can be explained by assuming that $[Ca^{2+}]_i$ is reduced to a level at which $InsP_3$ cannot act to release Ca^{2+} from the stores. In some cells in which CCh did not elicit I_{cat} oscillations, oscillatory changes in I_{cat} appeared following a reduction of membrane Ca^{2+} entry (see Figure 8). This can be explained by the assumption that $InsP_3$ was unable to release Ca^{2+}, because $[Ca^{2+}]_i$ was not at its normal high level in these cells. A similar hypothesis has been used to explain the result in oocytes that $[Ca^{2+}]_i$ oscillations were triggered by a reduction of membrane Ca^{2+} entry (Yao & Parker, 1994). The present results do not rule out other models proposed for agonist-induced $[Ca^{2+}]_i$ oscillations which involve stimulation of phospholipase C by Ca^{2+} to increase $InsP_3$ formation (Harootunian *et al.*, 1991), or Ca^{2+}-induced Ca^{2+} release from $InsP_3$-insensitive stores (Goldbeter *et al.*, 1990). However, on the basis of these models, it is rather difficult to explain the $[Ca^{2+}]_i$ oscillations induced by a reduction of membrane Ca^{2+} entry in the present study (Figure 8).

In summary, the generation and timing of muscarinic receptor-mediated oscillations of $[Ca^{2+}]_i$ are modulated by membrane Ca^{2+} entry in guinea-pig ileal cells. The Ca^{2+} entry takes place through opening of VGCCs and an as yet unidentified Ca^{2+} permeant channel; the resultant rise in $[Ca^{2+}]_i$ seems to influence the oscillation frequency, through a mechanism independent of the replenishment of internal Ca^{2+} stores available for release in response to $InsP_3$. The results provide some insight into the interplay between Ca^{2+} influx across the plasma membrane and Ca^{2+} release from intracellular stores in muscarinic receptor-mediated $[Ca^{2+}]_i$ signalling in intestinal smooth muscle cells.

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