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Characterization of action potential-triggered $[Ca^{2+}]_i$ transients in single smooth muscle cells of guinea-pig ileum

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1 To characterize increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) associated with discharge of action potentials, membrane potential and $[Ca^{2+}]_i$ were simultaneously recorded from single smooth muscle cells of guinea-pig ileum by use of a combination of nystatin-perforated patch clamp and fura-2 fluorimetry techniques.

2 A single action potential in response to a depolarizing current pulse elicited a transient rise in $[Ca^{2+}]_i$. When the duration of the current pulse was prolonged, action potentials were repeatedly discharged during the early period of the pulse duration with a progressive decrease in overshoot potential, upstroke rate and repolarization rate. However, such action potentials could each trigger $[Ca^{2+}]_i$ transients with an almost constant amplitude.

3 Nicardipine (1 μM) and La^{3+} (10 μM), blockers of voltage-dependent Ca^{2+} channels (VDCCs), abolished both the action potential discharge and the $[Ca^{2+}]_i$ transient.

4 Charybdotoxin (ChTX, 300 nM) and tetraethylammonium (TEA, 2 mM), blockers of large conductance Ca^{2+}-activated K^+ channels, decreased the rate of repolarization of action potentials but increased the amplitude of $[Ca^{2+}]_i$ transients.

5 Thapsigargin (1 μM), an inhibitor of SR Ca^{2+}-ATPase, slowed the falling phase and somewhat increased the amplitude, of action potential-triggered $[Ca^{2+}]_i$ transients without affecting action potentials. In addition, in voltage-clamped cells, the drug had little effect on the voltage step-evoked Ca^{2+} current but exerted a similar effect on its concomitant rise in $[Ca^{2+}]_i$ to that on the action potential-triggered $[Ca^{2+}]_i$ transient.

6 Similar action potential-triggered $[Ca^{2+}]_i$ transients were induced by brief exposures to high-K^+ solution. They were not decreased, but rather increased, after depletion of intracellular Ca^{2+} stores by a combination of ryanodine (30 μM) and caffeine (10 mM) through an open-lock of Ca^{2+}-induced Ca^{2+} release (CICR)-related channels.

7 The results show that action potentials, discharged repeatedly during the early period of a long membrane depolarization, undergo a progressive change in configuration but can each trigger a constant rise in $[Ca^{2+}]_i$. Intracellular Ca^{2+} stores have a role, especially in accelerating the falling phase of the action potential-triggered $[Ca^{2+}]_i$ transients by replenishing cytosolic Ca^{2+}. No evidence was provided for the involvement of CICR in the action potential-triggered $[Ca^{2+}]_i$ transient.

Keywords: Action potential; $[Ca^{2+}]_i$; excitation-contraction coupling; Ca^{2+}-induced Ca^{2+} release; smooth muscle; guinea-pig ileum

Introduction

In smooth muscle cells, an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers activation of the contractile proteins. The $[Ca^{2+}]_i$ increase can also activate cytosolic Ca^{2+}-sensitive ionic channels in the plasma membrane and as a result changes membrane excitability. $[Ca^{2+}]_i$ is increased by entry of Ca^{2+} across the plasma membrane and release of Ca^{2+} from intracellular stores. Many types of smooth muscle can generate action potentials. Each discharge of action potentials is followed by an increase in tension (a contraction) arising from an elevated $[Ca^{2+}]_i$ (Axelsson, 1970). Thus, the action potential discharge is believed to be the primary and most important mechanism for the production of a rise in $[Ca^{2+}]_i$ (for reviews see Bolton, 1979).

Membrane ionic channels involved in the generation of the smooth muscle action potentials have been extensively characterized by use of voltage clamp techniques in single smooth muscle cells (Lang, 1989; 1990; Bolton & Beech, 1993). Voltage-dependent Ca^{2+} channels (VDCCs) of the L-type are largely, if not exclusively, responsible for the upstroke of the action potentials. Of the several types of K^+ channel identified, Ca^{2+}-activated K^+ channels with a large conductance (BK_{Ca}-channels) have been suggested to play an important role in the repolarization phase of action potentials. Simultaneous measurements of Ca^{2+} current through VDCCs (I_{Ca}) upon step depolarizations and $[Ca^{2+}]_i$ revealed a close correlation between I_{Ca} and its concomitant increase in $[Ca^{2+}]_i$; both the I_{Ca} and increased $[Ca^{2+}]_i$ exhibit a bell-shaped voltage-dependence with a peak at -10 to 0 mV (Ganitkevich & Isenberg, 1991; Vogalis *et al*., 1991; Kamishima & McCarron, 1996). However, information on the Ca^{2+} signal in the cytosol following a physiologically relevant membrane event of action potential discharge is limited, even though it is crucial to the understanding of the coupling of membrane excitation to $[Ca^{2+}]_i$-sensitive cellular functions.

The release of Ca^{2+} from intracellular Ca^{2+} stores in smooth muscle is achieved via two separate mechanisms, one is an inositol trisphosphate-induced Ca^{2+} release mechanism and the other a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Chen & van Breemen, 1992). In the CICR mechanism, Ca^{2+}-gated ryanodine-sensitive channels are operative (Xu *et al*., 1994); these can be sensitized to Ca^{2+} by caffeine (Iino, 1989).

Voltage-clamp studies in which ryanodine and caffeine were used to deplete intracellular Ca^{2+} stores provided evidence for a possible involvement of CICR in the elevation of $[Ca^{2+}]_i$ following depolarization-evoked I_{Ca} (Sakai *et al*., 1988; Zholos

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et al., 1991; Ganitkevich & Isenberg, 1992; Grégoire *et al*., 1993). Recently, Imaizumi *et al*. (1996) suggested that operation of the CICR mechanism triggered by an action potential produces an additional rise in $[Ca^{2+}]_i$ which contributes to repolarization of the action potential through activation of BK_{Ca}-channels. However, the involvement of the CICR mechanism in fulfilment of cellular functions which are mediated by an increase in $[Ca^{2+}]_i$ is still uncertain in smooth muscle (Iino, 1989; Somlyo & Somlyo, 1994; Kamishima & McCarron, 1996; Fleischmann *et al*., 1996).

In the present study, we measured changes in membrane potential, at the same time as changes in $[Ca^{2+}]_i$ in single smooth muscle cells isolated from the longitudinal muscle layer of guinea-pig ileum, in an attempt to characterize changes in $[Ca^{2+}]_i$ preceded by the discharge of action potentials and to gain an insight into a role for intracellular Ca^{2+} stores in determining the action potential-dependent change in $[Ca^{2+}]_i$.

The results show that action potentials can be discharged repeatedly every 200 to 300 ms during the early period of a long membrane depolarization and that they undergo a progressive change in configuration, but can each give rise to a constant increase in $[Ca^{2+}]_i$. Intracellular Ca^{2+} stores have a role, especially in the acceleration of the falling phase of the action potential-dependent increase in $[Ca^{2+}]_i$ by replenishing cytosolic Ca^{2+}.

Methods

Preparation of cells

Male guinea-pigs, weighing 350–450 g, were stunned and bled to death. A length of 15 cm of the ileum was removed and divided into five segments (each, about 3 cm long). The longitudinal muscle layer of the intestinal segments was peeled from the underlying circular muscle, washed in physiological salt solution (PSS; composition given below). Single isolated smooth muscle cells were prepared from the muscle layers by use of a combination of collagenase (0.2 to 0.6 mg ml^{-1}) and papain (0.3 to 0.6 mg ml^{-1}), as described previously (Kohda *et al*., 1996).

Fura-2 loading of cells

Cells were suspended in a low Ca^{2+} (0.5 mM)-containing PSS to which fura-2 acetoxymethyl ester (fura-2/AM; 2 μM) was added and placed in a dark room kept at 25°C for 30 min. After the procedure for fura-2 loading, the cell suspension was centrifuged at 700 r.p.m. for 2 min and the cells were resuspended in the low Ca^{2+} PSS without fura-2/AM, placed on coverslips (20 mm in diameter) in a small aliquot and stored in a refrigerator (4°C) under a moist atmosphere. The cells were used for experiments within 8 h of fura-2 loading.

Measurement of fura-2 fluorescence in cells

A shallow chamber (0.5 ml in volume), the bottom of which was formed by a coverslip with fura-2-loaded cells, was mounted on the stage of an inverted fluorescence microscope (Olympus IMT-2, Tokyo), as described previously (Kohda *et al*., 1996). The chamber was perfused with 5 to 10 ml of PSS to wash away contaminants in the cell suspension and then filled with the solution.

Fura-2 fluorescence was measured at room temperature (22 to 25°C) while alternating the two excitation wavelengths (340 and 380 nm) at 100 Hz by an Olympus Ca^{2+} microspectrometric system with an objective ($\times 40$) (OSP-3 model, Tokyo). Fluorescent light was collected from the whole area of a single cell of interest and counted by a photomultiplier tube through a band-pass filter (510 ± 30 nm). Counts of fluorescence at 340 nm (F340) and 380 nm (F380) excitation wavelengths were sampled at 20 Hz. $[Ca^{2+}]_i$ was calculated from the ratio (F340/F380) according to the 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 50 μM 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 F380 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 0.45 ± 0.01, 5.85 ± 0.27 and 4.51 ± 0.23 ($n = 6$), respectively. These mean values and the dissociation constant for Ca^{2+}-fura-2 complex (K_d), 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 potential or membrane current

Fura-2-loaded cells were held under the whole-cell patch clamp by use of the nystatin perforated-technique (Wakamori *et al*., 1993; Fleischmann *et al*., 1996), as described previously (Kohda *et al*., 1996). This technique has the advantage in that it prevents rundown of the activity of ionic channels, such as VDCCs, and diffusion of intracellular functional proteins into the patch pipette (Wakamori *et al*., 1993; Fleischmann *et al*., 1996). Patch pipettes with a resistance of 4 to 6 MΩ were filled with a KCl-based solution for recording of membrane potential and with a CsCl-based solution for that of membrane currents (composition of the solutions given below). Membrane potential and membrane current were measured via a patch-clamp amplifier (CEZ-2400, Nihon Kohden, Tokyo, Japan), stored on a PCM data recorder (RD-111T, TEAC, Musashino City, Tokyo, Japan) and replayed onto a thermal array recorder (RTA-1100M, Nihon Kohden, Tokyo, Japan) for analysis and illustration. The duration of action potentials (APD_{0mV}) was measured as the period between the times when the depolarization phase and repolarization phase of the action potential crossed the 0mV level.

Values in the text are given as means $\pm$ s.e.mean. Statistical significance was tested by use of Student's paired t test and differences were considered significant when $P < 0.05$.

Application of drugs

Application of drugs was made by replacing the solution bathing cells with drug-containing solution 5 to 7 times within 10 s. For brief exposure of cells to high-K^+, a micropipette with a tip diameter of 2.5–5 μM was filled with 100 mM high-K^+ solution (see below) and placed within 10 μm from the cell of interest. The pipette was connected to a nitrogen tank by a polyethylene tube via a three-way solenoid valve (UMGI-T1, CKD corp., Nagoya, Japan), from which the high-K^+ solution was ejected by pressure pulses (1.5 to 2.0 kg cm^{-2}) of 50 to 100 ms duration. The pulse duration and inter-pulse interval were controlled by a stimulator (SEN-3013, Nihon Kohden, Tokyo, Japan).

Solution and drugs

The 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 (titrated to pH 7.2 with NaOH). The high-K^+ solution (100 mM) was obtained by replacing 100 mM Na^+ in PSS with the equivalent amount of K^+. The KCl-based pipette solution had the following composition (mM): KCl 134, HEPES 10.5 (titrated to pH 7.2 with KOH), to which nystatin dissolved in DMSO (4 mg 0.1 ml^{-1}) was added to give a final concentration of 0.2 mg ml^{-1}. The CsCl-based pipette solution was prepared by replacing KCl in the KCl-based pipette solution with the equivalent amount of CsCl.

Drugs used were: thapsigargin, nystatin and nicardipine, all of which were purchased from Sigma (St. Louis, MO, U.S.A.); caffeine, O,O′-bis(2-aminoethyl)ethyleneglychol-*N*,*N*,*N*′-*N*′-tetraacetic acid (EGTA), ryanodine and tetraetylammonium (TEA) (from Wako, Osaka, Japan); charybdotoxin (from Peptide Institute Inc; Osaka, Japan) and fura-2/AM (from Dojin Kagaku, Kumamoto, Japan). All other reagents were of the highest grade commercially available.

Results

Fura-2-loaded cells were held under current clamp mode with a patch pipette filled with a KCl-based solution by use of the nystatin-perforated technique (Kohda *et al*., 1996). Most cells (80%) exhibited no electrical activity with a steady level of $[Ca^{2+}]_i$ of 44.3 ± 3.0 nM ($n = 24$) and a resting membrane potential of -48.0 ± 1.2 mV. In a small fraction of cells (20%), small and irregular fluctuations of membrane potential and $[Ca^{2+}]_i$ were observed, but they were not appreciably correlated to each other.

Relationships between changes in membrane potential and $[Ca^{2+}]_i$ produced by depolarizing current pulses

Figure 1 shows simultaneous recordings of changes in membrane potential and $[Ca^{2+}]_i$ in response to rectangular depolarizing current pulses applied with a duration of 100 ms at various intensities (10, 20 and 30 pA). Membrane depolarization responses to current pulses at 10 and 20 pA developed gradually during the entire period of the pulse duration and then declined more slowly. The amplitude of the depolarization increased with an increase in the current intensity. When the current intensity was increased to 30 pA, the depolarization reached a threshold potential of some -20 mV to generate an action potential (see right panel). The action potential was faster in the declining phase than the subthreshold depolarizations and it terminated within 100 ms. In some cells, the action potential terminated at a more negative level than the resting potential. The duration measured at 0 mV (APD_{0mV}, see Methods), and overshoot potential of action potentials in 24 different cells were 22.0 ± 2.2 ms and 22.5 ± 1.0 mV, respectively. Membrane depolarization responses to the current pulses did not appreciably increase $[Ca^{2+}]_i$ unless they were accompanied by generation of an action potential. The rise in $[Ca^{2+}]_i$ following action potential discharge reached a peak within 100 to 200 ms after its onset and then declined to the basal level in several seconds. The beginning of the $[Ca^{2+}]_i$ rise occurred immediately after the peak of the corresponding action potential. The amplitude, measured as the difference in $[Ca^{2+}]_i$ between the basal level and the peak varied from 20 to 80 nM between the cells, giving a mean of 34.3 ± 2.2 nM ($n = 24$). Hereafter, this rise in $[Ca^{2+}]_i$ was designated as an action potential-triggered $[Ca^{2+}]_i$ transient.

When the duration of depolarizing current pulses was prolonged from 100 ms up to 3000 ms, action potentials were evoked repeatedly every 200 to 300 ms during the early 1000 to 1500 ms of the pulse duration (Figure 2A). The repeatedly-evoked action potentials became progressively smaller in overshoot potential and dull in shape. The

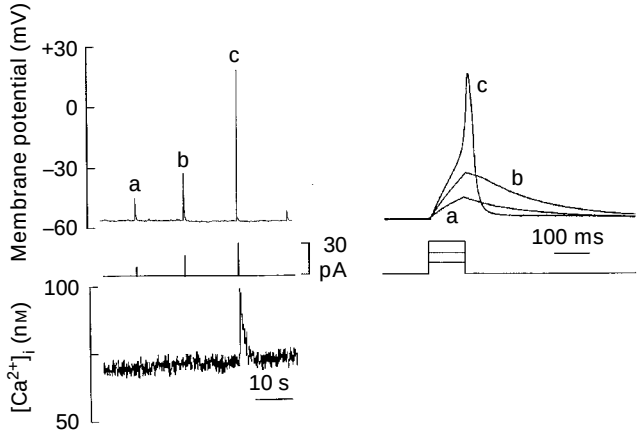

Figure 1 An increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) associated with discharge of an action potential. Cells were preloaded with fura-2 and held under current clamp mode by use of the nystatin-perforated technique with a patch pipette filled with a KCl-based solution. Simultaneous records of changes in membrane potential (top trace) and $[Ca^{2+}]_i$ responses (bottom trace) from a cell. Current pulses of 100 ms duration and 10, 20 and 30 pA intensities (middle trace) were used. A rise in $[Ca^{2+}]_i$ occurred only when an action potential was evoked on a current pulse-induced depolarization. Right panel, time-expanded traces of membrane potential and current pulses indicated by a, b and c in the left panel. The expanded traces were graphically superimposed on one another.

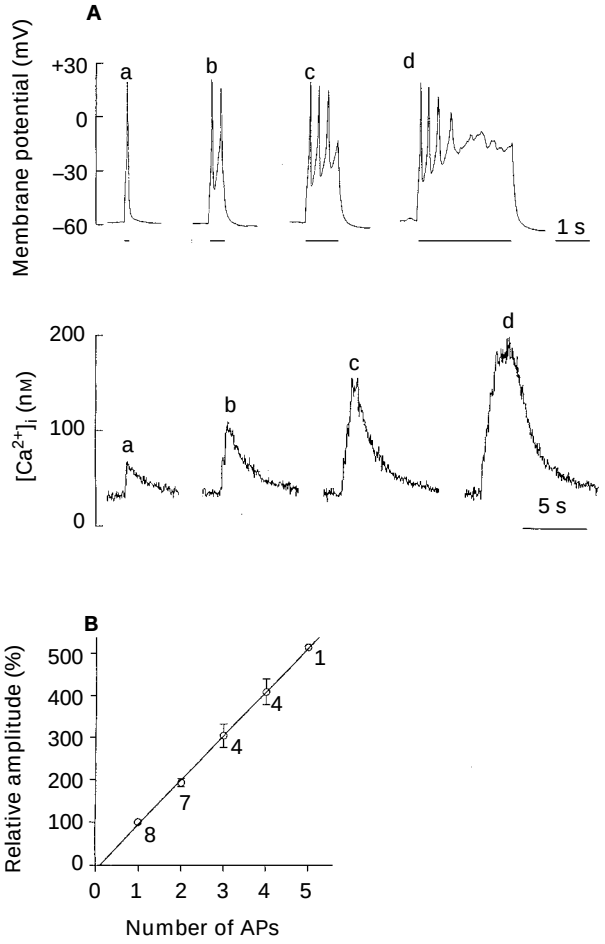

Figure 2 Relationship between the discharge rate of action potentials and $[Ca^{2+}]_i$. Depolarizing current pulses (30 pA in intensity) were applied with different durations of 100 to 3000 ms to evoke action potentials. (A) Simultaneous records of action potentials (upper trace) and $[Ca^{2+}]_i$ increases (lower trace) from a cell held under current-clamp mode. Current pulses of 100 ms (a), 500 ms (b), 1000 ms (c) and 3000 ms (d) duration were applied (as indicated by lines below the upper trace). Note difference in time calibration between the upper and lower traces. (B) A plot of the $[Ca^{2+}]_i$ increase against the number of action potentials. The peak amplitude of $[Ca^{2+}]_i$ increases was expressed as a % of the amplitude of the $[Ca^{2+}]_i$ increase associated with the discharge of a single action potential. Each point represents the mean of the measurements indicated by attached numbers; vertical lines show s.e.mean. The straight line indicates a regression line ($Y = 104.7X - 10.3$, where X is the number of action potentials and Y is the relative amplitude (%) of $[Ca^{2+}]_i$ increases with a correlation coefficient $r = 0.998$).

Table 1 The rate of rise of individual $[Ca^{2+}]_i$ transients triggered by each of action potentials discharged repeatedly in response to depolarizing current pulses in single ileal smooth muscle cells

The number of repeatedly-discharged action potentials	*Rate of rise of successive $[Ca^{2+}]_i$ transients ($nM\ s^{-1}$)*					
	1st	*2nd*	*3rd*	*4th*	*5th*	(n)
Single	448±25					8
2	451±48	452±50				7
3	472±105	495±84	515±80			4
4	407±35	421±66	448±75	456±47		4
5	487	590	572	508	483	1

Values (mean±s.e.mean) were obtained from measurements in the number of cells indicated by *n*. In each case of the different numbers (2 to 4) of action potentials, there was no statistically significant difference between the values for the 1st $[Ca^{2+}]_i$ transient and any subsequent $[Ca^{2+}]_i$ transient.

concomitant response of $[Ca^{2+}]_i$ appeared as one large increase in $[Ca^{2+}]_i$ with notches in the rising phase, the number of which correlated with the number of action potentials evoked. The rising rate of $[Ca^{2+}]_i$ following any notch was comparable to that of an action potential-triggered $[Ca^{2+}]_i$ transient (see Table 1). In addition, the change in $[Ca^{2+}]_i$ between two successive notches was nearly constant so that the amplitude of the large $[Ca^{2+}]_i$ response was in proportion to the number of action potentials evoked, as illustrated in Figure 2B. Thus, the action potentials were found to give rise to a constant increase in $[Ca^{2+}]_i$, although they underwent a progressive change in configuration.

In the following experiments, current pulses of 30 pA intensity and 100 ms duration were used to obtain action potential-triggered $[Ca^{2+}]_i$ transients, unless otherwise stated.

Effects of Ca^{2+} channel and K^+ channel blockers on action potential-triggered $[Ca^{2+}]_i$ transients

Nicardipine (1 μM), a blocker of voltage-dependent Ca^{2+} channels (VDCCs), blocked action potential discharge in response to current pulses and action potential-triggered $[Ca^{2+}]_i$ transients as well. Similar results were obtained with an inorganic Ca^{2+} channel blocker, La^{3+} (10 μM). After blockade of VDCCs with either Ca^{2+} channel blocker, the membrane potential of cells was displaced by strong current pulses from the resting level of -40 to -50 mV to a positive potential of 10 to 30 mV, corresponding to the peak potential of action potentials. The membrane depolarizations were not accompanied by any appreciable change in $[Ca^{2+}]_i$, indicating that an action potential-triggered $[Ca^{2+}]_i$ transient resulted, at any rate, from Ca^{2+} entry through VDCCs.

Charybdotoxin (300 nM), which blocks large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}-channels; Bolton & Beech, 1993), increased action potential duration (Figure 3a). APD_{0mV} was increased by the drug to 131.4±2.9% ($n=5$) of the control value. As seen in Figure 3a, the increased APD_{0mV} is attributable mainly to a decrease in the rate of repolarization of the action potential. The K^+ channel blocker also increased the amplitude of the action potential-triggered $[Ca^{2+}]_i$ transients to 163.1±14.1% ($n=5$). However, the decay rate, which was measured as the time taken to decline to half the maximum amplitude (half decay time), was little or not changed by the drug. Substantially similar results were obtained with TEA, a nonselective K^+ channel blocker, when it was used at a concentration of 2 mM. This concentration of TEA has been shown to block selectively the BK_{Ca}-channel (Bolton & Beech, 1993). APD_{0mV} and the amplitude of the action potential-triggered $[Ca^{2+}]_i$ transient were increased in TEA to 171.4±18.9% and 156.6±9.0% ($n=5$), respectively. Charybdotoxin (300 nM) and TEA (2 mM) decreased the membrane potential by up to 10 mV; this effect varied from one cell to another, giving a mean of 4.2±1.7 mV ($n=5$) for charybdotoxin and a mean of 5.3±3.0 mV ($n=5$) for TEA. The results suggest that opening of BK_{Ca}-channels is involved in action potential repolarization and that blockade of this type of K^+ channel results in a slow action potential repolarization which leads to an increase in Ca^{2+} entry through VDCCs.

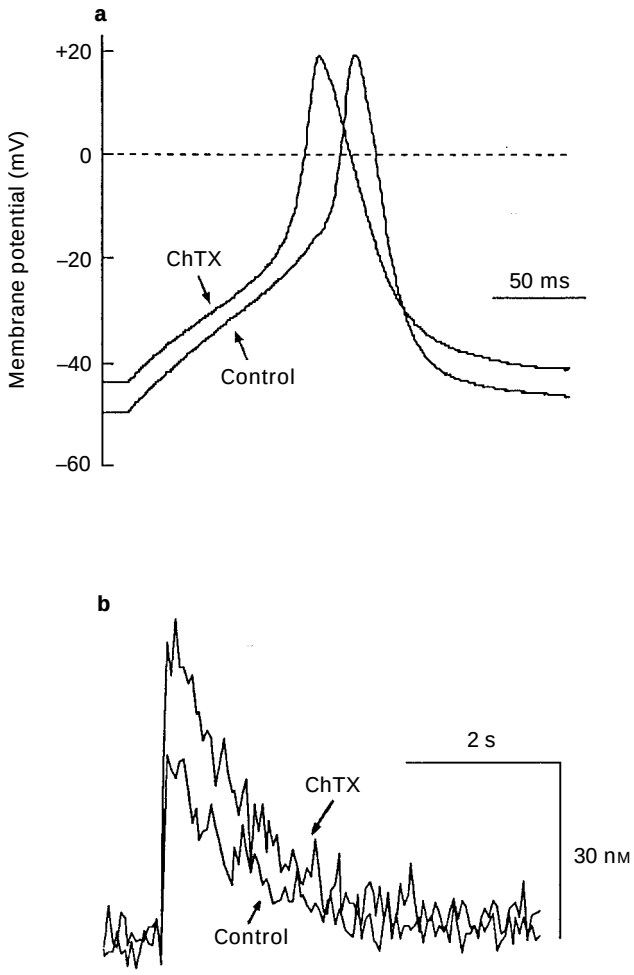

Figure 3 Effect of charybdotoxin (ChTX) on action potential-triggered $[Ca^{2+}]_i$ increases. Simultaneous records of membrane potential and $[Ca^{2+}]_i$ from a cell. (a) Superimposed traces of action potentials evoked by a depolarizing current pulse of 30 pA intensity and 100 ms duration before (control) and 1 min after application of ChTX (300 nM). (b) Superimposed traces of $[Ca^{2+}]_i$ increases associated with the respective action potentials in (a). See text for details.

Effect of thapsigargin on action potential-triggered $[Ca^{2+}]_i$ transients

To see whether intracellular Ca^{2+} stores play a role in determining the elevation of $[Ca^{2+}]_i$ associated with action potential

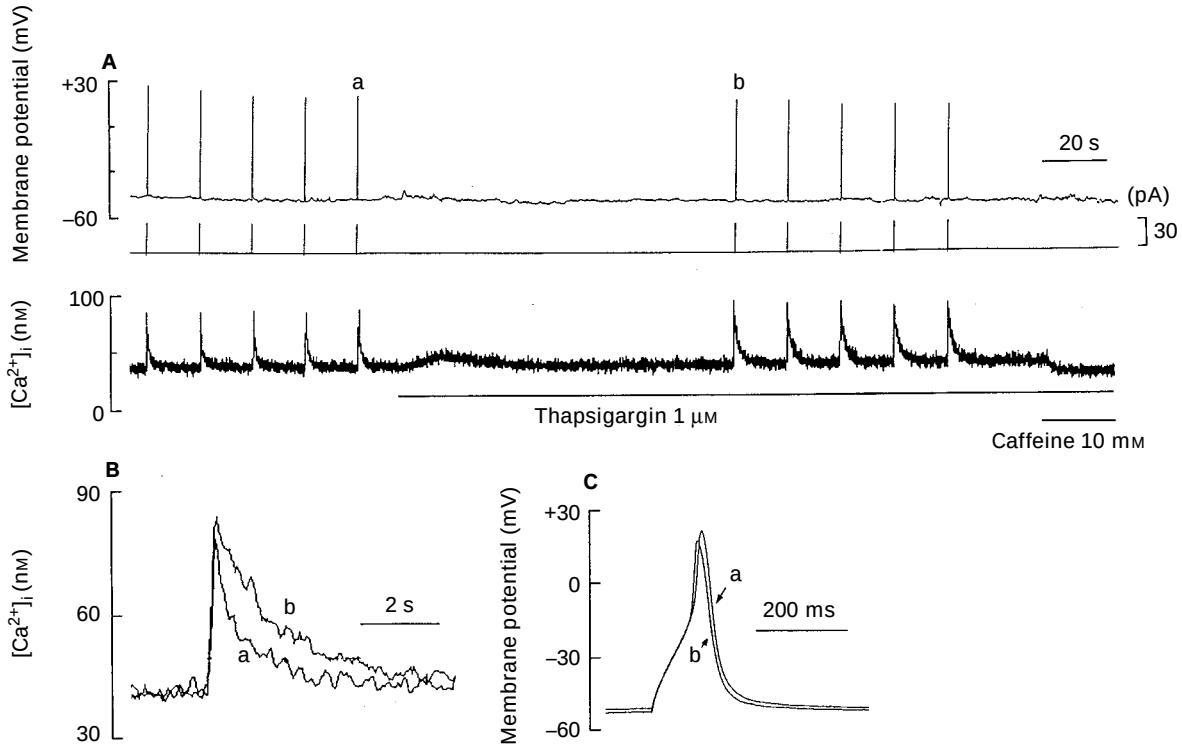

Figure 4 Effect of thapsigargin on action potential-triggered $[Ca^{2+}]_i$ increases. (A) Simultaneous records of changes in membrane potential (top trace) and changes in $[Ca^{2+}]_i$ (bottom trace) from a cell. Action potentials were evoked by depolarizing current pulses of 30 pA intensity and 100 ms duration (middle trace) before and after application of thapsigargin (1 μM) (as indicated by the line below the bottom trace). Caffeine (10 mM) was applied at the end of the trace (indicated by the line below the trace). (B) and (C) Time- and size-expanded traces of $[Ca^{2+}]_i$ increases and action potentials indicated by (a) and (b) in (A). The expanded traces were graphically superimposed on each other. Note a marked increase in the duration of the $[Ca^{2+}]_i$ increase after application of thapsigargin.

discharge, thapsigargin, an inhibitor of SR Ca^{2+}-ATPase, was used to prevent the Ca^{2+} stores from accumulating cytosolic Ca^{2+}.

Thapsigargin, when applied to a cell at 1 μM, had little or no effect on the resting membrane potential, but increased the basal $[Ca^{2+}]_i$ by an amplitude of up to 30 nM ($n=6$). The increase of $[Ca^{2+}]_i$ reached a peak and then declined to close to the initial basal level within 60 s even in the continued presence of the drug (see Figure 4A). Action potentials evoked again after disappearance of the drug-induced $[Ca^{2+}]_i$ elevation were very comparable to those evoked before drug application, as shown in Figure 4A and C. Mean values for the overshoot potential and APD_{0mV} in six different cells were 19.2 ± 1.7 mV and 27.6 ± 4.8 ms ($n=6$) immediately before, and 15.5 ± 1.7 mV and 25.5 ± 4.0 ms ($n=6$) 1.5 to 2 min after the drug application. The difference between the two means for the overshoot potential, but not APD_{0mv}, was statistically significant. The corresponding action potential-triggered $[Ca^{2+}]_i$ transient was increased in amplitude from 43.1 ± 4.6 to 48.7 ± 4.6 nM ($n=6$) and the half-decay time from 0.81 ± 0.19 to 2.00 ± 0.35 s ($n=6$), both changes were statistically significant ($P<0.05$) (Figure 4A and B). The % increase for the half-decay time ($103.1\pm15.4\%$) was much greater than that for the amplitude ($17.8\pm1.8\%$), suggesting that intracellular Ca^{2+} stores may function more effectively to increase the rate of decay of the action potential-triggered $[Ca^{2+}]_i$ transient.

When 10 mM caffeine was applied in the continued presence of thapsigargin, $[Ca^{2+}]_i$ was not increased but rather decreased (Figure 4A), indicating that intracellular Ca^{2+} stores had already been depleted. In other words, such Ca^{2+} stores can be emptied as a result of inhibition of their Ca^{2+}-pump activity by thapsigargin, as previously demonstrated (Kohda *et al.*, 1996).

Effect of thapsigargin on Ca^{2+} current and concomitant rise in $[Ca^{2+}]_i$

In an effort to see whether or not the potentiation by thapsigargin of action potential-triggered $[Ca^{2+}]_i$ transients was due to some increase in Ca^{2+} influx during the total duration of an action potential, the inward Ca^{2+} current (I_{Ca}) flowing through VDCCs was recorded from five cells in the voltage-clamp mode of the nystatin-perforated whole-cell patch clamp, at the same time as changes in $[Ca^{2+}]_i$. The cells were held under voltage clamp at -60 mV and dialyzed with a CsCl-based pipette solution to block K^+ currents. I_{Ca} was evoked by stepping from the holding potential (-60 mV) to 0 mV for such a short period (50 ms) that its concomitant rise in $[Ca^{2+}]_i$ would match an action potential-triggered $[Ca^{2+}]_i$ transient. The I_{Ca} was not affected by application of thapsigargin for 1–2 min, although small decreases or small increases which fell within the range of spontaneous changes, or no detectable change of the peak amplitude of I_{Ca}, were observed in different cells (Figure 5A). Mean values for the peak amplitude and total charge entry calculated from time integral of I_{Ca} were 231.4 ± 13.0 pA and 7.8 ± 0.3 pC ($n=5$) before, and 236.8 ± 13.1 pA and 8.1 ± 0.4 pC ($n=5$) after the drug application. The difference between the two means for either parameter was not statistically significant. On the other hand, the concomitant rise in $[Ca^{2+}]_i$ was increased in amplitude from 30.5 ± 2.9 to 41.1 ± 3.9 nM ($n=5$) and in half-decay time from 1.34 ± 0.14 to 2.19 ± 0.1 s ($n=5$), these changes being significant ($P<0.05$) (Figure 5B).

The results together with those in the previous section strongly suggest that intracellular Ca^{2+} stores play a crucial role in the regulation of cytosolic Ca^{2+} concentration during Ca^{2+} entry following action potential discharge. In addition,

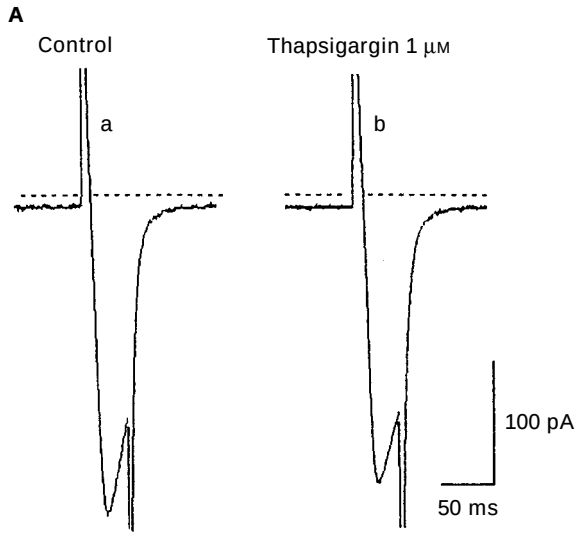

Figure 5 Effects of thapsigargin on Ca^{2+} current (I_{Ca}) and its concomitant $[Ca^{2+}]_i$ increase. Simultaneous records of I_{Ca} and $[Ca^{2+}]_i$ from a cell held under voltage clamp at −60 mV by use of a patch pipette filled with a CsCl-based solution. (A) Traces of I_{Ca} evoked by a 50 ms depolarizing step to 0 mV before (a) and 1 min after (b) application of thapsigargin (1 μM). The dashed lines indicate the current level obtained by the same depolarizing step in the presence of Cd^{2+} (300 μM). (B) Superimposed traces of $[Ca^{2+}]_i$ increases associated with the I_{Ca} indicated by (a) and (b) in (A). Note a marked increase in the duration of the $[Ca^{2+}]_i$ increase after application of thapsigargin. See text for details.

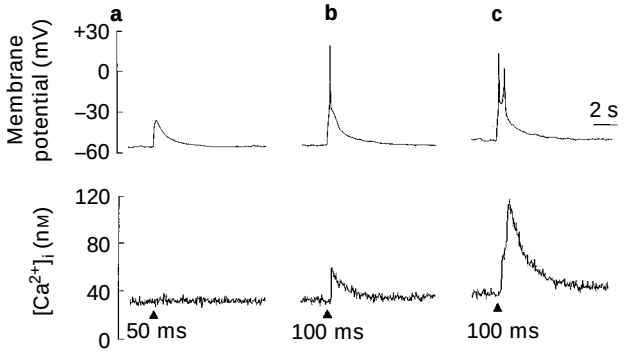

Figure 6 Simultaneous records of changes in membrane potential (upper trace) and changes in $[Ca^{2+}]_i$ (lower trace) in response to brief exposures to high-K^+ solution. High K^+ exposures were achieved by ejection of 100 mM K^+ solution from a micropipette by pressure pulses at points (indicated by ▲ below the lower trace). (a) A depolarization without any change in $[Ca^{2+}]_i$ in response to a 50 ms exposure to high-K^+. (b) and (c) Depolarizations with one and two successive action potentials, respectively, which were followed by $[Ca^{2+}]_i$ increases in response to 100 ms exposures to high-K^+. Traces (a), (b) and (c) were from the same cell.

the results suggest that an action potential-triggered $[Ca^{2+}]_i$ transient does not involve any increase in $[Ca^{2+}]_i$ brought about by activation of CICR mechanism, by which Ca^{2+} is released from the stores upon Ca^{2+} influx via VDCCs (Ganitkevich & Isenberg, 1992; Grégoire *et al*., 1993).

Effect of ryanodine on action potential-triggered $[Ca^{2+}]_i$ transients

To assess further the possible involvement of the CICR mechanism in determining an action potential-triggered $[Ca^{2+}]_i$ transient, ryanodine was used to cause CICR-related channels to open irreversibly (Iino *et al*., 1988). However, preliminary experiments, when ryanodine (30 μM) was applied in combination with 10 mM caffeine to achieve a rapid open-lock of CICR-related channels (Iino *et al*., 1988; Komori *et al*., 1995), the patch pipette was often dislocated from the cell, probably because of its contraction. Therefore, a brief exposure to high-K^+ solution instead of application of depolarizing current pulses was used to elicit an action potential-triggered $[Ca^{2+}]_i$ transient. High-K^+ exposure was achieved by ejecting 100 mM KCl solution from a micropipette by pressure pulses (see Methods). As illustrated in Figure 6, ejection of high-K^+ solution with pressure pulses of 50 ms duration produced a depolarization insufficient to generate an action potential (a subthreshold depolarization). When the duration of pressure pulses was increased to 100 ms, a larger depolarization was produced, during which one or two action potentials were generated. Such action potentials were each followed by a rise in $[Ca^{2+}]_i$ similar to that following the discharge of an action potential in response to depolarizing current pulses. Therefore, the duration of 100 ms of pressure pulses was used in the following experiments.

After application of both ryanodine (30 μM) and caffeine (10 mM) for 1 to 1.5 min, action potential-triggered $[Ca^{2+}]_i$ transients induced by brief exposures to high-K^+ solution were increased in amplitude significantly from 40.5 ± 8.4 nM to 52.3 ± 7.8 nM ($n=4$) ($P<0.05$). The half-decay time of the $[Ca^{2+}]_i$ transients was also increased significantly from 0.93 ± 0.20 s to 1.37 ± 0.26 s ($n=4$) ($P<0.05$) (Figure 7b). When 10 mM caffeine was applied 2 to 3 min after pretreatment with ryanodine and caffeine, $[Ca^{2+}]_i$ was not increased, but rather decreased (Figure 7b), indicating that intracellular Ca^{2+} stores had already been depleted at a time when caffeine was applied.

Thapsigargin was capable of causing depletion of intracellular Ca^{2+} stores through inhibition of the Ca^{2+}-uptake activity (see above). The effect of thapsigargin on the $[Ca^{2+}]_i$ transients was also investigated. Figure 7a shows the $[Ca^{2+}]_i$ transients in the absence and presence of thapsigargin (1 μM). The amplitude of 41.3 ± 4.1 nM was significantly ($P<0.05$) increased to 47.5 ± 5.0 nM ($n=5$) and the half-decay time from 0.98 ± 0.14 s to 1.68 ± 0.33 s ($n=5$).

The results suggest again that the CICR mechanism makes little contribution to the determination of an action potential-triggered $[Ca^{2+}]_i$ transient.

Effect of caffeine and thapsigargin on action potential in cells unloaded with fura-2

In single ileal smooth muscle cells from the guinea-pig, the amplitude and duration of action potentials in response to

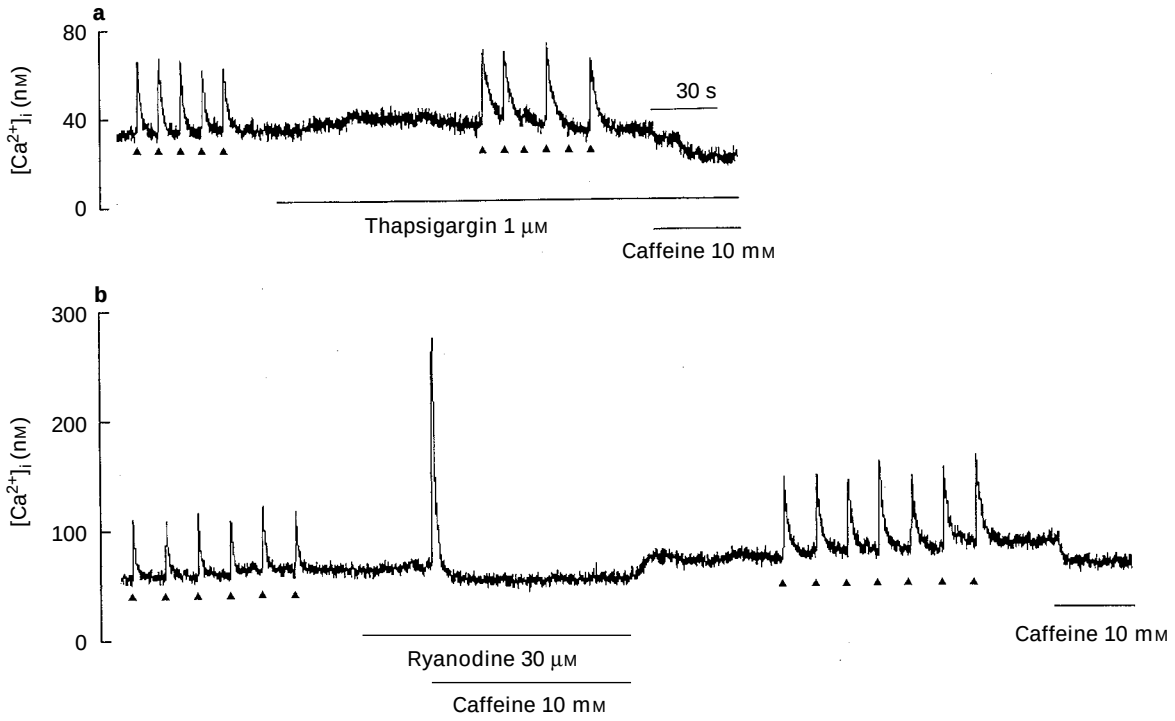

Figure 7 Effects of thapsigargin and ryanodine on action potential-triggered $[Ca^{2+}]_i$ increases in response to high-K^+ exposures. High-K^+ exposures for 100 ms were achieved in the same way as in Figure 6 at points (indicated by ▲ below the traces). (a) $[Ca^{2+}]_i$ increases before and during application of thapsigargin (1 μM). (b) $[Ca^{2+}]_i$ increases before and after application of ryanodine (30 μM) in combination with caffeine (10 mM). In (a) and (b) 10 mM caffeine was applied at the end of the trace (indicated by lines below the traces). Time calibration in (a) is also applicable to (b). Traces (a) and (b) were from different cells.

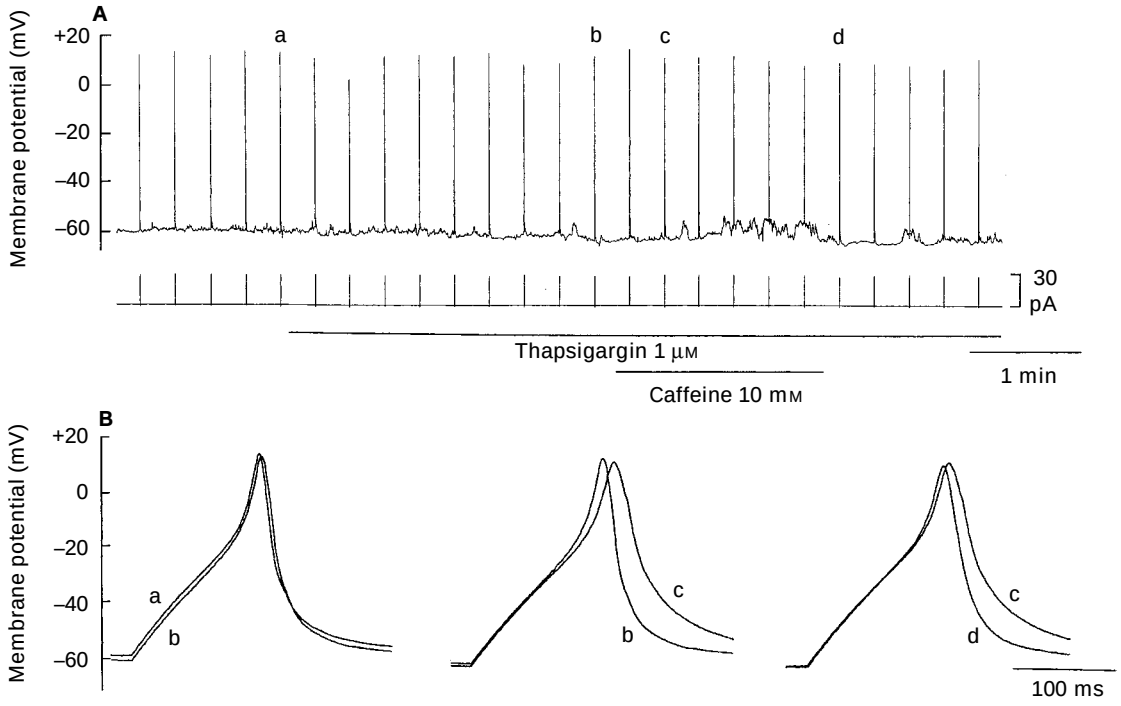

Figure 8 Effects of caffeine and thapsigargin on action potentials. (A) Records of action potentials evoked by depolarizing current pulses (top trace) of 30 pA intensity and 150 ms duration (bottom trace). Thapsigargin (1 μM) and caffeine (10 mM) were applied (indicated by the line below the bottom trace). (B) Time-expanded traces of action potentials indicated by (a), (b), (c) and (d) in (A). The respective pairs of the expanded traces in (B) were graphically superimposed on each other. Note a marked increase in the duration of action potentials after application of caffeine.

current pulses increased after depletion of stored Ca^{2+} with caffeine (Imaizumi *et al*., 1996). This observation is taken as evidence for the idea that Ca^{2+} entry associated with an action potential operates the CICR mechanism and the overall increase in $[Ca^{2+}]_i$ contributes to activation of BK_{Ca}-channels responsible for repolarization of the action potential. It is still possible that the present observation that thapsigargin did not change action potential configuration (see Figure 4) results from use of cells loaded with fura-2.

In cells not loaded with fura-2, caffeine (10 mM) slowed action potentials in both the depolarization and repolarization phases, but thapsigargin (1 μM) was without effect (Figure 8). Caffeine, when applied in the continued presence of thapsigargin, was also effective in increasing the duration of action potentials (APD_{0mV}) to $169.1 \pm 12.0\%$ ($n=3$). The effect disappeared immediately after washout of the caffeine (Figure 8). Therefore, the effect of caffeine on action potentials cannot be attributed to a reduction of stored Ca^{2+}.

Discussion

In the present study, we characterized $[Ca^{2+}]_i$ increases associated with the discharge of action potentials by recording simultaneously changes in $[Ca^{2+}]_i$ and changes in membrane potential from single smooth muscle cells of guinea-pig ileum. An increase in $[Ca^{2+}]_i$ which was preceded by the discharge of a single action potential was stable in amplitude, total duration and shape in one cell, but it varied from 20 to 80 nM in amplitude and within a range of several seconds in total duration from one cell to another, whereas its shape was maintained as a serrated appearance consisting of a rapid rising phase and a slow falling phase, as observed previously (Kohda *et al*., 1996). The relationship between the change in $[Ca^{2+}]_i$ and the discharge of an action potential resembled the situation described in single smooth muscle cells of guinea-pig urinary bladder, in which the configuration of action potentials is just like that in the ileal smooth muscle cells (Schneider *et al*., 1991).

In cells in which VDCCs were blocked with nicardipine or La^{3+}, no appreciable change in $[Ca^{2+}]_i$ was generated by displacing the membrane potential, by use of current pulses to a voltage level comparable to the peak potential of action potentials. This implies that Ca^{2+} entry through VDCCs, but not membrane depolarization itself, during action potential discharge is primarily responsible for an elevation of $[Ca^{2+}]_i$ with a serrated appearance.

During the early period of a long, supra-threshold membrane depolarization, action potentials were discharged repeatedly at intervals of 200 to 300 ms. The concomitant increase in $[Ca^{2+}]_i$ was characterized by a larger amplitude and the occurrence of notches in the rising phase which were correlated to the number of action potentials discharged. It is of interest that such action potentials underwent progressive decreases in overshoot potential, upstroke rate and repolarization rate, but the $[Ca^{2+}]_i$ increase corresponding to every action potential discharge remained almost unchanged, as judged by a similar range of $[Ca^{2+}]_i$ between two successive notches reflecting each action potential-triggered $[Ca^{2+}]_i$ transient (see Figure 2). Thus, it seems likely that the power of such action potentials to produce an increase in $[Ca^{2+}]_i$ stays constant in spite of their progresively-changing configuration. If Ca^{2+} entry through VDCCs during action potential discharge is the primary determinant of an action potential-triggered $[Ca^{2+}]_i$ transient (see above), the period and magnitude of activation of VDCCs would undergo such a progressive change as to make the $[Ca^{2+}]_i$ transients constant. The situation occurring in action potentials is not incompatible with this idea; the decreased overshoot and upstroke rate were invariably accompanied by a decrease in the repolarization rate and an increase in the duration.

Pretreatment of cells with charybdotoxin (300 nM) or TEA (2 mM), which blocks selectively BK_{Ca}-channels (Bolton & Beech, 1993), resulted in a decrease of the repolarization rate of action potentials, suggesting some participation of this type of K^+ channel in the falling phase, as previously determined (Imaizumi *et al*., 1996). In voltage-clamped smooth muscle cells, Ca^{2+} inward current (I_{Ca}) upon step depolarizations is followed by an outward current through BK_{Ca}-channels and the K^+ current is markedly reduced by the inhibition of I_{Ca} with a Ca^{2+} channel blocker or when cytosolic Ca^{2+} is strongly buffered by a chelating substance (Ohya *et al*., 1987; Imaizumi *et al*., 1990; Suzuki *et al*., 1992). Therefore, it seems likely that BK_{Ca}-channels responsible for action potential repolarization are activated by Ca^{2+} entering via VDCCs, which are opened in association with action potential discharge. As activation of BK_{Ca}-channels is somehow involved in the determination of the falling phase of action potentials, its dependence on Ca^{2+} influx rate, which determines temporal and spatial accumulation of Ca^{2+} in the vicinity of the K^+ channels (Benham & Bolton, 1986), may contribute to the prolongation of action potentials. In smooth muscle cells of guinea-pig urinary bladder held under voltage clamp, a ramp-like depolarization leading to a slow influx of Ca^{2+} is much less effective in evoking a current flowing through BK_{Ca}-channels, compared to a step depolarization (Yoshikawa *et al*., 1996). This staying power of action potentials for Ca^{2+} mobilization may play a physiological role in the regulation of tension development during exposure to stimulants, including the neurotransmitter acetylcholine which causes activation of non-selective cation channels leading to membrane depolarization.

A CICR mechanism may not be involved in the determination of action potential-triggered $[Ca^{2+}]_i$ transients in the ileal smooth muscle cells, since the $[Ca^{2+}]_i$ transients were not decreased but rather increased in amplitude after functional removal of intracellular Ca^{2+} stores (by treatment of cells with a combination of ryanodine and caffeine or with thapsigargin). It has been demonstrated that treatment with a combination of ryanodine and caffeine produces open lock of Ca^{2+}-releasing channels responsible for CICR, resulting in depletion of intracellular Ca^{2+} stores (Iino *et al*., 1988; Komori *et al*., 1995), and thapsigargin does not allow intracellular Ca^{2+} stores to replenish Ca^{2+} through its inhibitory action on the SR Ca^{2+}-ATPase, resulting finally in depleted stored Ca^{2+} (Thastrup *et al*., 1989). In fact, in the present study, caffeine-releasable Ca^{2+} stores were abolished after these treatments. In smooth muscle cells from urinary bladder and portal vein, the depolarization-evoked Ca^{2+} entry (I_{Ca}) triggers activation of a CICR mechanism to produce an increased elevation of $[Ca^{2+}]_i$ (Ganitkevich & Isenberg, 1992; Grégoire *et al*., 1993), although the CICR activation is rapidly decreased in repeatedly-evoked I_{Ca} (Grégoire *et al*., 1993). In contrast, the CICR mechanism in arterial smooth muscle can be operated by I_{Ca} only when I_{Ca} is increased by BayK8644 and the extracellular Ca^{2+} concentration is elevated (Ganitkevich & Isenberg, 1995).

One might expect that a greater Ca^{2+} entry following a burst discharge of action potentials would lead to operation of the CICR mechanism even in cells used in the present study. However, the elevation of $[Ca^{2+}]_i$ associated with a burst discharge of action potentials comprised action potential-triggered $[Ca^{2+}]_i$ transients of the same amplitude, and they decayed in a single exponential manner immediately after cessation of the action potential discharge (see Figure 2). However, localized Ca^{2+} signals cannot be resolved due to the limitations of the method used in the present study. Therefore, the possibility that there are some localized Ca^{2+} increments by a CICR triggered by the action potential cannot be excluded. Uyama *et al*. (1993) observed in muscle strips from guinea-pig ileum that depletion of Ca^{2+} stores with CPA, an inhibitor of SR Ca^{2+}-ATPase, decreases the amplitude of the after-hyperpolarization of spontaneously-discharged action potentials. Imaizumi *et al*. (1996) also observed in single smooth muscle cells from the same tissue that caffeine decreases the decay rate and after-hyperpolarization of electrically-evoked action po-

tentials. On the basis of these observations, the authors suggested that CICR is triggered by an action potential and the resultant increase in $[Ca^{2+}]_i$ mediates acceleration of the falling phase of the action potential through activation of BK_{Ca}-channels. However, as mentioned above, Ca^{2+} store depletion by thapsigargin, which has the same effect as CPA on SR Ca^{2+}-ATPase, left I_{Ca} as well as the action potential unaffected. This discrepancy could be due to the different preparations used for the respective studies. Caffeine exerted its potentiating effect on action potentials even after depletion of Ca^{2+} stores (see Figure 8) and, hence, it seems unlikely that the effect is brought about by its ability to deplete such Ca^{2+} stores. Caffeine is known to block various ionic channels including K^+ channels (Noack *et al*., 1990) and Ca^{2+} channels (Imaizumi *et al*., 1989; Hughes *et al*., 1990). Therefore, the channel blocking action of caffeine might account for the observed effect on action potentials in the present study.

The increased amplitude and duration of an action potential-triggered $[Ca^{2+}]_i$ transient by treatment with a combination of ryanodine with caffeine, or with thapsigargin can be explained by assuming that the Ca^{2+} stores have a role in lowering an elevated $[Ca^{2+}]_i$ (Yoshikawa *et al*., 1996).

In summary, in smooth muscle cells of guinea-pig ileum, action potentials are discharged repeatedly every 200 to 300 ms with a progressively-changed configuration during a sustained membrane depolarization, but their power to cause an increase in $[Ca^{2+}]_i$ stays constant. A CICR mechanism may not be involved in the determination of the increase in $[Ca^{2+}]_i$ associated with an action potential. Intracellular Ca^{2+} stores have a role in the lowering an elevated $[Ca^{2+}]_i$ by replenishing cytosolic Ca^{2+} leading to acceleration of the falling rate of the $[Ca^{2+}]_i$ transient.

References

AXELSSON, J. (1970). Mechanical properties of smooth muscle, and the relationship between mechanical and electrical activity. In *Smooth Muscle*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 289–315. London: Edward Arnold.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol*., **381,** 385–406.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev*., **59,** 606–718.

BOLTON, T.B. & BEECH, D.J. (1993). Smooth muscle potassium channels: their electrophysiology and function. In *Potassium Channel Modulators: Pharmacological, Molecular and Clinical Aspects*. ed. Weston, A.H. & Hamilton, T.C. pp. 144–180. Oxford: Blackwell Scientific Publications.

CHEN, Q. & VAN BREEMEN, C. (1992). Function of smooth muscle sarcoplasmic reticulum. In *Advances in Second Messenger and Phosphoprotein Research*. ed. Putney, J.W., Jr. pp. 335–350. New York: Raven Press.

FLEISCHMANN, B.K., WANG, Y.X., PRING, M. & KOTLIKOFF, M.I. (1996). Voltage-dependent calcium currents and cytosolic calcium in equine airway myocytes. *J. Physiol*., **492,** 347–358.

GANITKEVICH, V.Y. & ISENBERG, G. (1991). Depolarization-mediated intracellular calcium transients in isolated smooth muscle cells of guinea-pig urinary bladder. *J. Physiol*., **435,** 187–205.

GANITKEVICH, V.Y. & ISENBERG, G. (1992). Contribution of Ca^{2+}-induced Ca^{2+} release to the $[Ca^{2+}]_i$ transients in myocytes from guinea-pig urinary bladder. *J. Physiol*., **458,** 119–137.

GANITKEVICH, V.Y. & ISENBERG, G. (1995). Efficacy of peak Ca^{2+} currents (Ica) as trigger of sarcoplasmic reticulum Ca^{2+} release in myocytes from the guinea-pig coronary artery. *J. Physiol*., **484,** 287–306.

GRÉGOIRE, G., LOIRAND, G. & PACAUD, P. (1993). Ca^{2+} and Sr^{2+} entry induced Ca^{2+} release from the intracellular Ca^{2+} store in smooth muscle cells of rat portal vein. *J. Physiol*., **474,** 483–500.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*., **260,** 3440–3450.

HUGHES, A.D., HERING, S. & BOLTON, T.B. (1990). The action of caffeine on inward barium current through voltage-dependent calcium channels in single rabbit ear artery cells. *Pflügers Arch*., **416,** 462–466.

IINO, M. (1989). Calcium-induced calcium release mechanism in guinea pig taenia caeci. *J. Gen. Physiol*., **94,** 363–383.

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. *Biochem. Biophys. Res. Commun*., **152,** 417–422.

IMAIZUMI, Y., HENMI, S., NAGANO, N., MURAKI, K. & WATANABE, M. (1996). Regulation of Ca-dependent K current and action potential shape by intracellular Ca storage sites in some types of smooth muscle cells. In *Smooth Muscle Excitation*. ed. Bolton, T.B. & Tomita, T. pp. 337–354. London: Academic Press.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. (1989). Ionic currents in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol*., **411,** 131–159.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. (1990). Characteristics of transient outward currents in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol*., **427,** 301–324.

KAMISHIMA, T. & MCCARRON, J.G. (1996). Depolarization-evoked increases in cytosolic calcium concentration in isolated smooth muscle cells of rat portal vein. *J. Physiol*., **492,** 61–74.

KOHDA, M., KOMORI, S., UNNO, T. & OHASHI, H. (1996). Carbachol-induced $[Ca^{2+}]_i$ oscillations in single smooth muscle cells of guinea-pig ileum. *J. Physiol*., **492,** 315–328.

KOMORI, S., ITAGAKI, M., UNNO, T. & OHASHI, H. (1995). Caffeine and carbachol act on common Ca^{2+} stores to release Ca^{2+} in guinea-pig ileal smooth muscle. *Eur. J. Pharmacol*., **277,** 173–180.

LANG, R.J. (1989). Identification of the major membrane currents in freshly dispersed single smooth muscle cells of guinea-pig ureter. *J. Physiol*., **412,** 375–395.

LANG, R.J. (1990). The whole-cell Ca^{2+} channel current in single smooth muscle cells of the guinea-pig ureter. *J. Physiol*., **423,** 453–473.

NOACK, T., DEITMER, P. & GOLENHOFEN, K. (1990). Features of a calcium independent, caffeine sensitive outward current in single smooth muscle cells from guinea-pig portal vein. *Pflügers Arch*., **416,** 467–469.

OHYA, Y., KITAMURA, K. & KURIYAMA, H. (1987). Cellular calcium regulates outward currents in rabbit intestinal smooth muscle cell. *Am. J. Physiol*., **252,** C401–410.

SAKAI, T., TERADA, K., KITAMURA, K. & KURIYAMA, H. (1988). Ryanodine inhibits the Ca-dependent K current after depletion of Ca stored in smooth muscle cells of the rabbit ileal longitudinal muscle. *Br. J. Pharmacol*., **95,** 1089–1100.

SCHNEIDER, P., HOPP, H.H. & ISENBERG, G. (1991). Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]_i$ and inactivates Ica in myocytes from guinea-pig urinary bladder. *J. Physiol*., **440,** 479–496.

SOMLYO, A.P. & SOMLYO, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature*, **372,** 231–236.

SUZUKI, M., MURAKI, K., IMAIZUMI, Y. & WATANABE, M. (1992). Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells. *Br. J. Pharmacol*., **107,** 134–140.

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 Actions*, **27,** 17–23.

UYAMA, Y., IMAIZUMI, Y. & WATANABE, M. (1993). Cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum, increases excitability in the ileal smooth muscle. *Br. J. Pharmacol*., **110,** 565–572.

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. *Am. J. Physiol*., **260,** C1012–1018.

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

XU, L., LAI, F.A., COHN, A., ETTER, E., GUERRERO, A., FAY, F.S. & MEISSNER, G. (1994). Evidence for a Ca^{2+}-gated ryanodine-sensitive Ca^{2+} release channel in visceral smooth muscle. *Proc. Natl. Acac. Sci. U.S.A.*, **91,** 3294 – 3298.

YOSHIKAWA, A., VAN BREEMEN, C. & ISENBERG, G. (1996). Buffering of plasmalemmal Ca^{2+} current by sarcoplasmic reticulum of guinea pig urinary bladder myocytes. *Am. J. Physiol.*, **271,** C833 – 841.

ZHOLOS, A.V., BAIDAN, L.V. & SHUBA, M.F. (1991). Properties of the late transient outward current in isolated intestinal smooth muscle cells of the guinea-pig. *J. Physiol.*, **443,** 555 – 574.

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