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Ryanodine inhibits the Ca-dependent K current after depletion of Ca stored in smooth muscle cells of the rabbit ileal longitudinal muscle

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1 Effects of ryanodine on the membrane currents were investigated on dispersed smooth muscle cells of rabbit ileal longitudinal layer using voltage and patch clamp procedures.

2 With voltage clamp, membrane depolarization to 0 mV from the holding potential of −60 mV produced an inward Ca current (I_{Ca}) which was followed by transient and sustained outward currents (I_{TO} and I_{SO}, respectively). Prolonged depolarization of the membrane produced spontaneous oscillations of the outward current (oscillatory outward current; I_{OO}) on I_{SO}.

3 Ryanodine (30 μM) modified neither the basal membrane current recorded at the holding potential (−60 mV) nor I_{SO}. Ryanodine inhibited both I_{TO} and I_{OO} in a concentration-dependent manner (IC_{50} = 5.5 and 4.5 μM, respectively, measured 12 min after application of ryanodine). These values were much higher than that observed in skeletal muscle for Ca release.

4 The time course of the ryanodine-induced inhibition of I_{OO} was slow and the inhibition was irreversible. Caffeine (3 mM) enhanced the amplitudes of I_{TO} and I_{OO} in the presence of Ca, and only transiently enhanced I_{OO} in the absence of Ca. However, following application of 10 μM ryanodine, 3 mM caffeine did not increase I_{OO}.

5 Ryanodine (3–30 μM) slightly enhanced the amplitude of I_{Ca} evoked by depolarization pulses at potentials more negative than 0 mV but not that induced by larger depolarizations (positive potentials).

6 With patch clamp procedure, single Ca-dependent K channel currents were recorded in cell free and cell attached configurations. Application of 30 μM ryanodine transiently enhanced the Ca-dependent K current without any detectable changes in the amplitude of the single channel current recorded in the cell attached condition. In the inside-out membrane patch, when the intracellular membrane side was superfused with 1 μM Ca buffered with 10 mM EGTA, bath application of 10 μM ryanodine had no effect on the Ca-dependent K current.

7 It was concluded that both I_{TO} and I_{OO} are generated by Ca released from intracellular stores, mainly sarcoplasmic reticulum. Ryanodine appears to open irreversibly the Ca channel in the store and to inhibit the Ca-dependent K channel due to depletion of the stored Ca.

Introduction

In dispersed smooth muscle cells, several ionic currents were elicited by membrane depolarization and one of them, known as the Ca-dependent K current, was sensitive to increases in the intracellular or extracellular Ca concentration (Inoue *et al.*, 1985; 1986; Benham *et al.*, 1986). Recently, Benham & Bolton (1986) and Ohya *et al.* (1987) have demonstrated that the oscillatory outward current (I_{OO}) which was evoked by membrane depolarization, was activated by caffeine and inhibited by low concentrations of A23187 or procaine. Therefore, they concluded that the I_{OO} was activated by Ca released from intracellular stores, mainly the sarcoplasmic reticulum.

Ryanodine, an insecticidal alkaloid from *Ryania speciosa Vahl* (Wiesner, 1972), has been shown to inhibit or enhance Ca release from intracellular stores (Sutko & Kenyon, 1983; Sutko *et al.*, 1985; Meissner, 1986; Rousseau *et al.*, 1987). In vascular smooth muscle, Ito *et al.* (1986) found that ryanodine diminished Ca release from the store site. Hwang &

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Van Breemen (1987) obtained evidence that ryanodine inhibits Ca release from intracellular stores due to depletion of the stored Ca rather than inhibition of the Ca release mechanism. In Ca loaded 'heavy' vesicles prepared from rabbit skeletal muscle, ryanodine at a low concentration (0.01 μM) increased, but at a high concentration (10 μM) inhibited the Ca efflux (Meissner, 1986). Furthermore, in cardiac cells, submicromolar concentrations of ryanodine inhibited release of Ca and increased Ca leakage from the intracellular stores (Hilgemann *et al.*, 1983; Hunter *et al.*, 1983; MacLeod & Bers, 1987).

In electrophysiological investigations in cardiac muscle, the interpretation of the actions of ryanodine on the action potential or inward current are controversial (Mitchell *et al.*, 1984; Marban & Wier, 1985; Nishio *et al.*, 1986; Shattock *et al.*, 1987; Rasmussen *et al.*, 1987), and there are no data on the actions of ryanodine on electrical events in smooth muscle membranes. The objective of the present study was to clarify the action of ryanodine on the ionic currents of smooth muscle cells, especially on the Ca-dependent inward and outward currents. For this purpose, dispersed single smooth muscle cells of the ileal longitudinal layer were used. The results indicate that ryanodine depletes Ca from intracellular stores. As a consequence, currents due to activation of the Ca-dependent K channel were inhibited. Furthermore, this agent slightly enhanced the inward calcium current (I_{Ca}) during depolarization of the membrane to negative potentials.

Methods

Preparation of single smooth muscle cells

Albino rabbits (Nippon White; 1.8–2.2 kg) of either sex were anaesthetized with sodium pentobarbitone (Pitman & Moor Inc., Washington Cross, NJ, U.S.A.; 40 mg kg^{-1} i.v.) and exsanguinated. The ileum was dissected and the longitudinal muscle layers were peeled from the ileum. Dispersion procedures of the muscle tissue were similar to those described previously (Momose & Gomi, 1980; Inoue *et al.*, 1985). In brief, after 5–10 min incubation in Ca free PSS (physiological salt solution), small segments of the tissue were treated with 0.1% collagenase (Wako Pure Chem., Tokyo, Japan), 0.1% trypsin inhibitor (Type IIs) and 0.2% bovine serum albumin (essentially fatty acid free; Sigma Chemical Co., St. Louis, MO, U.S.A.). After digestion of the tissue, the smooth muscle cells were dispersed by gentle agitation with a glasa pipette. Single cells were collected by centrifugation (600 r.p.m.; 1 min) after removal of debris with a fine nylon mesh and resuspended in PSS.

Recording of membrane currents

Single cells were transfered to a chamber placed on the stage of a differential interference inverted microscope (TMD-Diaphoto, Nihon Kogaku, Tokyo, Japan). A patch electrode made of a Pyrex glass capillary was prepared with an electrode puller and heat polisher (PP-83 and MF-83; Narishige Sci. Inst. Lab., Tokyo, Japan). Contact between the electrode and a cell was made using a three dimensional oil-driven micromanipulator (MO-102; Narishige Sci. Inst. Lab., Tokyo, Japan). After achievement of a 'giga-seal' (> 5 GΩ), the patch membrane was ruptured by negative pressure (10–30 mmH_2O) (Hamill *et al.*, 1981; Ohya *et al.*, 1986).

In the present experiments, a fragmented smooth muscle cell (smooth muscle ball: SMB, shorter than 100 μm in length) was used rather than a spindle shaped smooth muscle cell (200–300 μm in length) (Ohya *et al.*, 1986). The solution in the chamber was rapidly exchanged by flushing with 5–10 ml of the test solution (within 1 min) or continuous superfusion and the overflow siphoned off.

Membrane currents were recorded through a single electrode voltage clamp (SEVC) amplifier (Ishizuka *et al.*, 1984; Finkel & Redman, 1985) or a patch clamp amplifier (EPC-5; List Medical Electronic, Darmstadt, F.R.G.), and monitored with a high-gain storage oscilloscope (VC-10; Nihon Kohden, Tokyo, Japan). The conditions of the SEVC amplifier were the same as those described by Ohya *et al.* (1986). The frequency of the sample and hold circuit was 10 kHz and the duty cycle was set at 0.05. A resistor (100 MΩ) was used as a current-voltage converter, and all electrical responses were stored by means of a video-cassette recorder (NV-750, National, Tokyo, Japan) via a PCM data-recording system (d.c.-3 kHz; sampling rate of 22 KHz; PCM-501ES, SONY, Tokyo, Japan).

Amplitude histograms were drawn using a mini-computer (ATAC-450; Nihon Kohden, Tokyo, Japan). For measurements of the amplitude of membrane currents, capacitative and leak currents were subtracted by the 'P/n' method introduced by Almers & Palade (1981) on a digital oscilloscope (4094; Nicoret Inst. Co., Madison, Wisc., U.S.A.), and hard copies were obtained using an X-Y plotter (HP-7440; Hewlett-Packard Co., San Diego, CA, U.S.A.). The amount of total charge carried by I_{oo} was calculated with a mini-computer by integrating the current obtained during a 1 min recording period.

Solutions and drugs

For whole-cell voltage clamp experiments, PSS of the following ionic composition was used in the bath

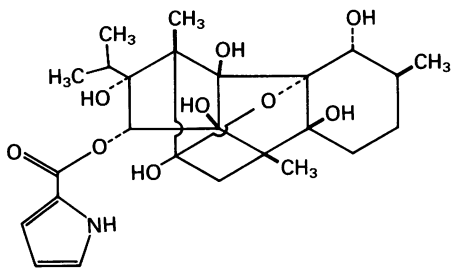

Figure 1 Chemical structure of ryanodine ($C_{25}H_{35}NO_9$), molecular weight 493.54.

(mM): NaCl 134, KCl 6, $CaCl_2$ 2.5 and glucose 12. Ca-free solution was prepared by removal of Ca from PSS and adding an equimolar amount of $MgCl_2$ or $MnCl_2$. High K solution containing the following ionic composition was used as the pipette solution (mM): KCl 145, $MgCl_2$ 5, Na_2ATP (adenosine triphosphate disodium) 5, EGTA (ethylene *bis*glycol N,N,N′,N′-tetraacetic acid) 0.3. Some experiments were performed with high-Cs solution which was made by replacement of KCl with 120 mM CsCl and 25 mM TEACl (tetraethylammonium chloride). The pH of the bath and pipette solutions was kept at 7.3 ± 0.05 by 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid) titrated with Tris (trishydroxyaminomethane).

For single channel recording, the pipette was filled with PSS and the bath was superfused with the high K solution containing an appropriate Ca concentration. The pH of these solutions was adjusted to 7.3 ± 0.05 by addition of 10 mM HEPES with Tris. The final Ca concentration ($[Ca]_{free}$) in the bath for the inside out patch experiments was calculated from the following equation:

$$[EGTA] = ([Ca]_{total} - [Ca]_{free}) \times (1 + K'[Ca]_{free})/K'[Ca]_{free},$$

where [EGTA] and $[Ca]_{total}$ represent the concentration of applied EGTA and Ca, respectively, and K′ is the apparent stability constant of EGTA to Ca. In the present experiments, two different concentrations of EGTA (0.3 and 10 mM) were used as buffer solutions to obtain the desired free Ca concentrations.

All experiments were performed at room temperature (22–25°C).

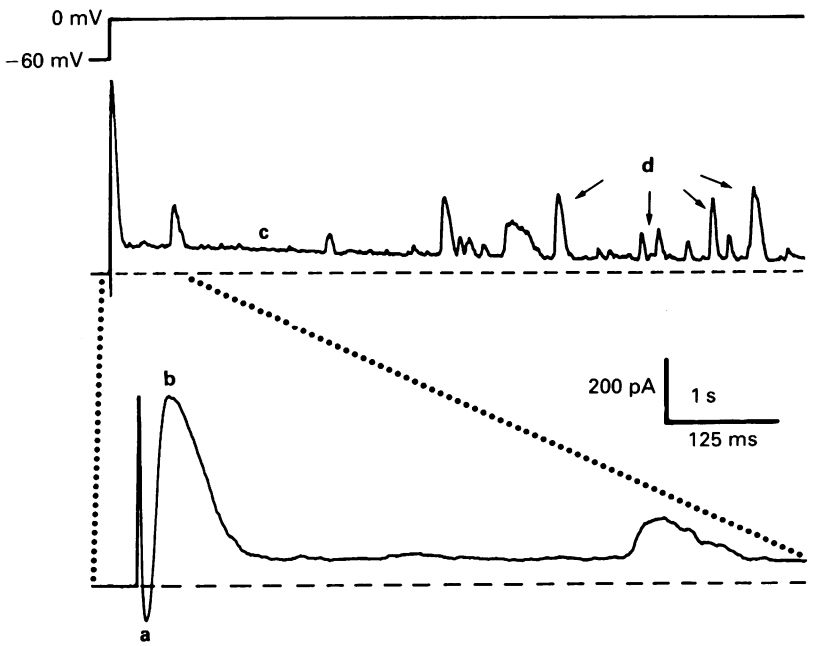

Figure 2 Membrane currents recorded in response to a depolarization pulse to 0 mV from the holding potential of −60 mV. The bottom trace shows the early part of the middle trace on a faster time scale. (a), (b), (c) and (d) indicate the inward (a), transient outward (b), sustained outward (c) and oscillatory outward (d) currents respectively. Broken lines on both traces indicate the basal membrane current level observed at −60 mV. The pipette was filled with a high K solution and the bath solution was physiological salt solution (PSS). Capacitative and leak currents were not subtracted. Note that the initial upward deflection in the lower trace was a capacitative current.

Drugs used in the present experiments were ryanodine (Figure 1; gift from Prof. K. Ito, Miyazaki University, Miyazaki, Japan) and caffeine (Wako Pure Chem., Tokyo, Japan). Drugs were added in the bath solutions.

Results

Effects of ryanodine on the ionic current recorded with the whole cell voltage clamp method

When the bath was superfused with PSS and the high K solution was in the pipette in the whole cell configuration, membrane depolarization to 0 mV (from the holding potential of −60 mV) evoked a transient inward current and subsequently a transient and a sustained outward current (I_{TO} and I_{SO}, respectively). As shown in Figure 2, I_{TO} occurred transiently within 100 ms (indicated as b) and oscillatory outward currents (I_{OO}) were superimposed (indicated as d) on I_{SO} (indicated as c) when the membrane was kept at 0 mV for over 10 s. The transient inward current (indicated as a) was blocked in Ca-free solution containing EGTA (3 mM), in $MnCl_2$ (1 mM) or in the presence of Ca antagonists. I_{TO} and I_{OO} were inhibited by application of high concentrations of caffeine (above 3 mM), procaine (above 1 mM), low concentrations of A23187 (0.1–1 nM) or Ca-free solution containing 1 mM EGTA in the bath, or intracellular perfusion of Ca-free solution containing 4 mM EGTA. Therefore, I_{TO} and I_{OO} were thought to be generated by activation of Ca-dependent K channels as indicated by Ohya *et al.* (1987).

Actions of ryanodine on the individual ionic currents were investigated. To investigate the action of ryanodine on the amplitude of the isolated inward current (I_{Ca}), a Cs solution containing 4 mM EGTA was used instead of the high K solution, as the pipette solution and amplitudes of I_{Ca} were measured at various test potentials (−40 to +30 mV). The maximum amplitude of I_{Ca} was observed with a membrane depolarization to 0 mV from the holding potential of −60 mV. Ryanodine (10 μM) enhanced the amplitude of I_{Ca} at negative test potentials without changes in the decay, but did not affect that evoked by depolarization to positive potentials (Figure 3a and b). To compare the action of ryanodine (1–30 μM) on I_{Ca} evoked by three different depolarization pulses, the amplitudes of I_{Ca} recorded at −20, 0 and +20 mV were plotted (those amplitudes recorded in the absence of ryanodine were normalised as 1.0). As shown in Figure 3c, the amplitude of I_{Ca} evoked by depolarization to −20 mV was enhanced by application of ryanodine in concentrations above 10 μM, and that evoked by depolarization to +20 mV inhibited by concentrations of ryanodine above 30 μM.

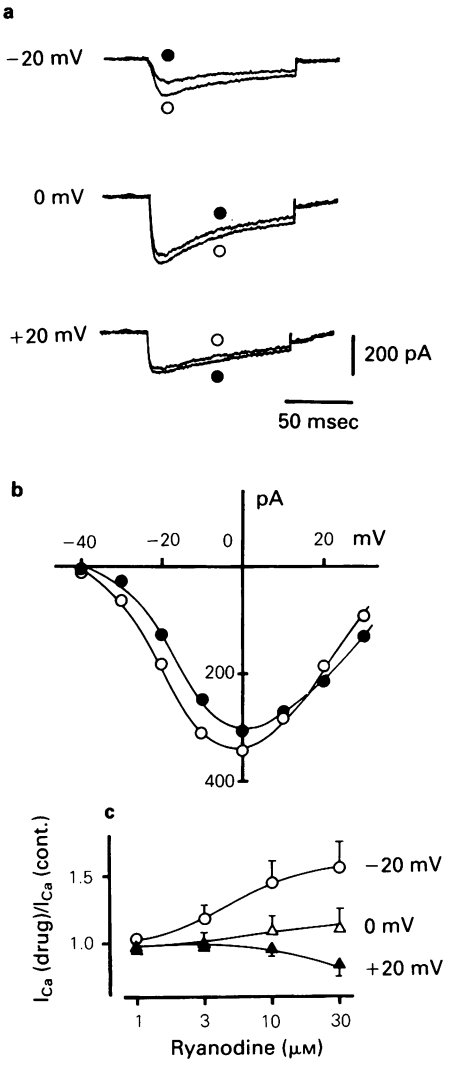

Figure 3 Effects of ryanodine on the inward calcium current (I_{Ca}). (a) I_{Ca} evoked by three different depolarizing pulses (to −20, 0 and +20 mV) in the absence (●) and presence (○) of ryanodine 10 μM. Traces in the presence of ryanodine were obtained 15 min after administration. Depolarizing pulses (100 ms) were applied. The holding potential was −60 mV. (b) The current-voltage relationships of I_{Ca} recorded from the same cell in the absence (●) and presence (○) of ryanodine 10 μM. The peak amplitude of I_{Ca} was plotted. (c) Relationships between the concentration of ryanodine and the relative amplitude of I_{Ca} evoked by three different depolarizations (to −20, 0 and +20 mV). The amplitude of I_{Ca} in the absence of ryanodine was normalized as 1.0. Each symbol represents the mean value of the peak amplitude of I_{Ca} of 3–5 observations; vertical lines show s.d. The pipette was filled with the Cs solution and the bath solution was PSS.

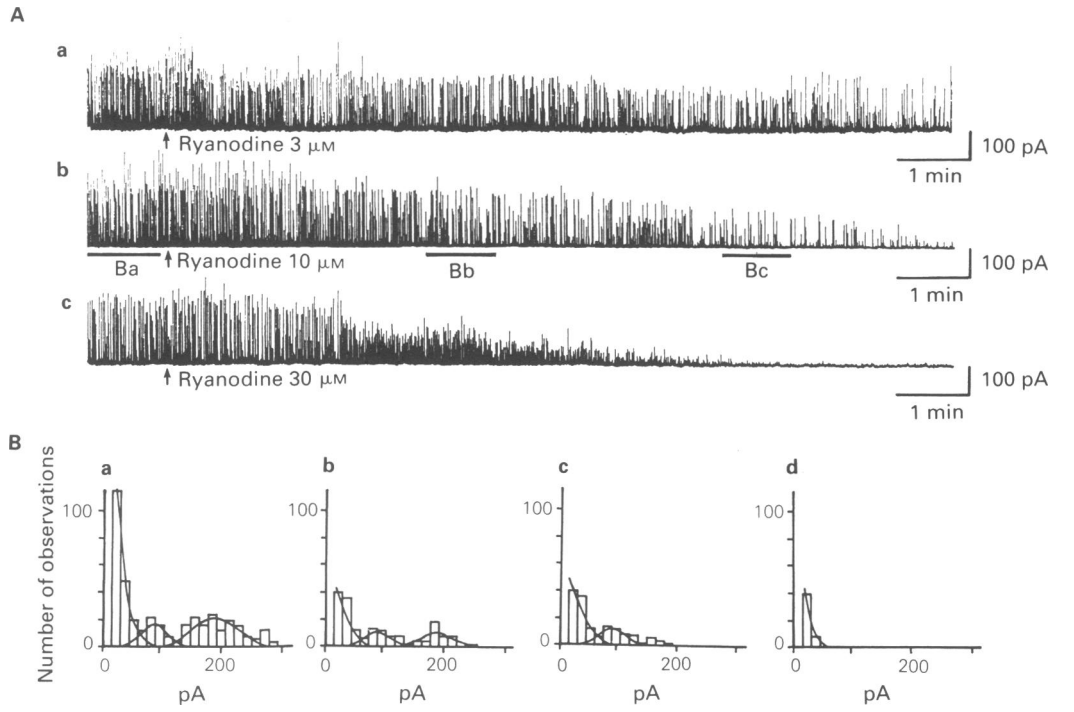

Figure 4 Effects of ryanodine on the oscillatory outward current (I_{OO}). (A) Ryanodine (3, 10 and 30 μM) was added at the time indicated by the arrows. The membrane potential was kept at 0 mV throughout the experiments. (Aa), (Ab) and (Ac) were obtained from different cells. (B) Amplitude histograms of I_{OO} before (a), 4 min (b), 8 min (c) and 12 min (d) after application of 10 μM ryanodine. Amplitude histograms were obtained by counting the amplitude of I_{OO} indicated by bars under Figure Ab for 1 min. Curves were drawn by the following equation:

$$Y = A_1 \exp(-X_1/M_1) + A_2 \exp(-(X_2 - M_2)^2/2S_2) + A_3 \exp(-(X_3 - M_3)^2/2S_3),$$

where A_n, M_n and S_n are the maximum number of observations, the mean amplitude and variance of the nth component of the amplitude, respectively. Each regression curve was fitted with following parameters; A_1 = 342 (control), 117 (4 min), 100 (8 min) and 100 (12 min); A_2 = 9 (control), 5.7 (4 min) and 5.7 (8 min); A_3 = 11.1 (control) and 5.5 (4 min); M_1 = 14.2 Pa (control), 18.3 pA (4 min), 21.9 pA (8 min) and 18.8 pA (12 min); M_2 = 88.5 pA (control), 96.8 pA (4 min) and 90.5 pA (8 min), M_3 = 191 pA (control) and 188.9 pA (4 min); S_2 = 30.2 pA^2 (control), 38.3 pA^2 (4 min) and 49.4 pA^2 (8 min); S_3 = 127 pA^2 (control) and 39.8 pA^2 (4 min).

Figure 4A shows the effect of various concentrations of ryanodine (3, 10 and 30 μM) on I_{OO} evoked by continuous depolarization. Reduction in the amplitude of I_{OO} by 3 μM ryanodine was weak, but 10 μM ryanodine inhibited both the amplitude and frequency of I_{OO} (Figure 4Ab). With application of 30 μM ryanodine, generation of I_{OO} ceased but more than 8 min superfusion was required (Figure 4Ac). Figure 4B shows the amplitude of I_{OO} recorded before and during (4, 8 and 12 min) application of 10 μM ryanodine. Before application of ryanodine, the amplitude of I_{OO} could be classified into three groups (small, medium, and large amplitudes). The distribution of the small amplitude showed a skew curve and the amplitude histograms of the medium and large I_{OO} were roughly Gaussian with mean values of 90 pA and 190 pA, respectively. Ryanodine (10 μM) reduced the frequency of I_{OO}.

To observe the effects of ryanodine on the isolated I_{TC} with minimum interference by I_{Ca}, the membrane current was evoked by 0 mV depolarization to avoid the effects of 10 μM ryanodine on I_{Ca} (Figure 5A). The response evoked in the presence of caffeine was subtracted from that evoked in the absence of 3 mM caffeine, because changes in the amplitudes of I_{Ca} and I_{SO} by caffeine (3 mM) were negligible (Figure 5Aa vs e). After subtraction, only the transient outward current remained (Figure 5Aa'–d'). No change in the amplitude of I_{SO} evoked by any depolarization was observed on application of ryanodine up to 30 μM. Ryanodine (10 μM) slowly inhibited isolated I_{TO} time-dependently and 12 min after the application the

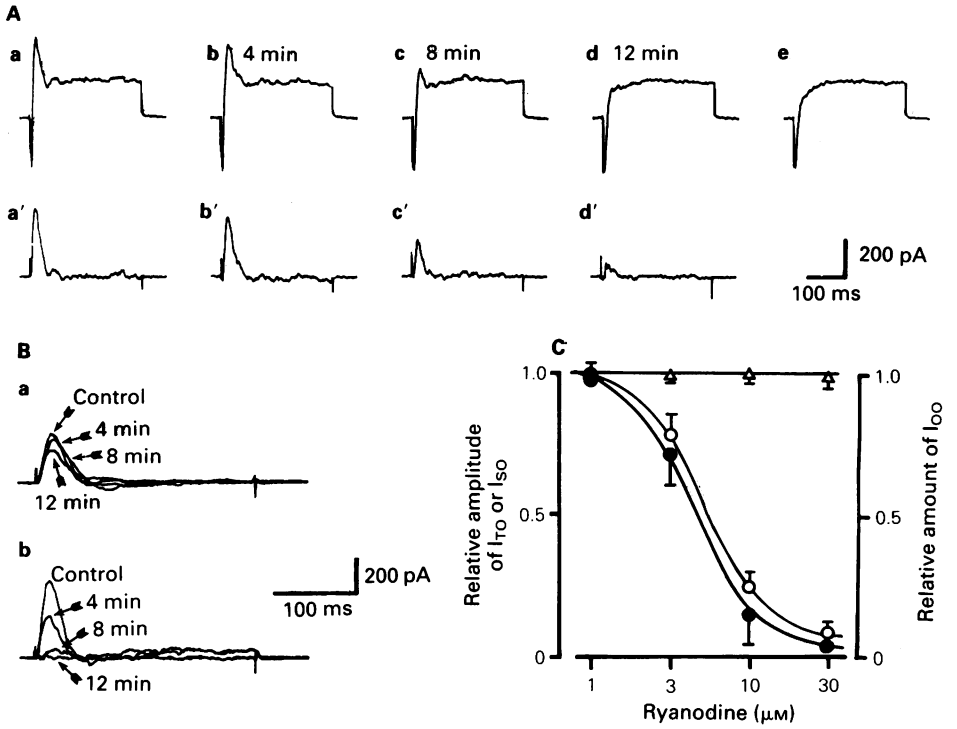

Figure 5 Effects of ryanodine on isolated transient outward current (I_{TO}), and the concentration-inhibition curves on isolated I_{TO}, oscillatory outward current (I_{OO}) and sustained outward current (I_{SO}). (A) The membrane currents were evoked by a depolarization to 0 mV before (a) and during (b–d) application of 10 μM ryanodine. Time indicates the period after drug application. Immediately after obtaining trace (d), caffeine (3 mM) was added, and after 3 min, trace (e) was obtained. Traces (a'), (b'), (c') and (d') were obtained by subtracting the membrane current in the presence of caffeine (trace e) from membrane current before application of caffeine (traces a, b, c and d, respectively). A depolarizing pulse (300 ms in duration) was applied every 30 s. (B) Effects of ryanodine at 3 (a) and 30 μM (b) on isolated I_{TO}. Isolated I_{TO} was obtained before (control) and 4, 8, and 12 min after application of ryanodine. I_{TO} was isolated by the same procedures as in (A). (C) Relationships between concentration of ryanodine and isolated I_{TO} (○), I_{OO} (●) and I_{SO} (△). Each symbol indicates mean of 5–6 observations; vertical lines show s.d. The amount of I_{OO} and amplitudes of isolated I_{TO} and I_{SO} before application of ryanodine were normalized as 1.0, respectively. The amount of I_{OO} represents the total amount of charge carried by I_{OO}, calculated by integration of the current for a 1 min recording period. The pipette was filled with the high K solution. PSS was used for measurement of isolated I_{TO} and I_{OO}, and the Ca-free solution (2.5 mM Mn) for I_{SO}. I_{OO} was evoked by a depolarizing pulse to 0 mV. Isolated I_{TO} and I_{SO} were evoked by depolarizing pulses (300 ms in duration) to 0 mV and +20 mV, respectively.

amplitude of the isolated I_{TO} was reduced to a quarter of the control value. Longer superfusion of ryanodine (10 μM) (15 min), blocked the isolated I_{TO}. A higher concentration of ryanodine (30 μM) blocked the isolated I_{TO} (Figure 5B) 12 min after its application. On removal of ryanodine the amplitude of the isolated I_{TO} was not restored to the control level (more than 30 min) nor was the progressive inhibition of the isolated I_{TO} prevented. Figure 5C shows the relationship between the concentration of ryanodine and the relative amplitude of the isolated I_{TO} measured 12 min after application. The IC_{50} value was calculated to be 5.5 μM ($n = 6$).

I_{SO} was isolated from other currents (I_{Ca}, I_{TO} and I_{OO}) by either removal of Ca or addition of 1.5 mM Mn instead of Ca in the PSS. The isolated I_{SO} was evoked by membrane depolarizations above −20 mV and the amplitude of I_{SO} was increased in a voltage-dependent manner. Ryanodine (30 μM) did not change the current-voltage relationship of I_{SO} (data not shown).

The effects of ryanodine on isolated I_{TO}, I_{OO} and I_{SO} are summarized in Figure 5C. The amount of I_{OO} observed before application of ryanodine was calculated from the integral of I_{OO} ($\int$ Idt) observed in 1 min (nanocoulomb) and expressed as a relative value of 1.0, and the amplitudes of isolated I_{TO} and I_{SO} recorded before application of ryanodine were also normalized to a relative value of 1.0, respectively. The IC_{50} value for ryanodine on I_{OO} was

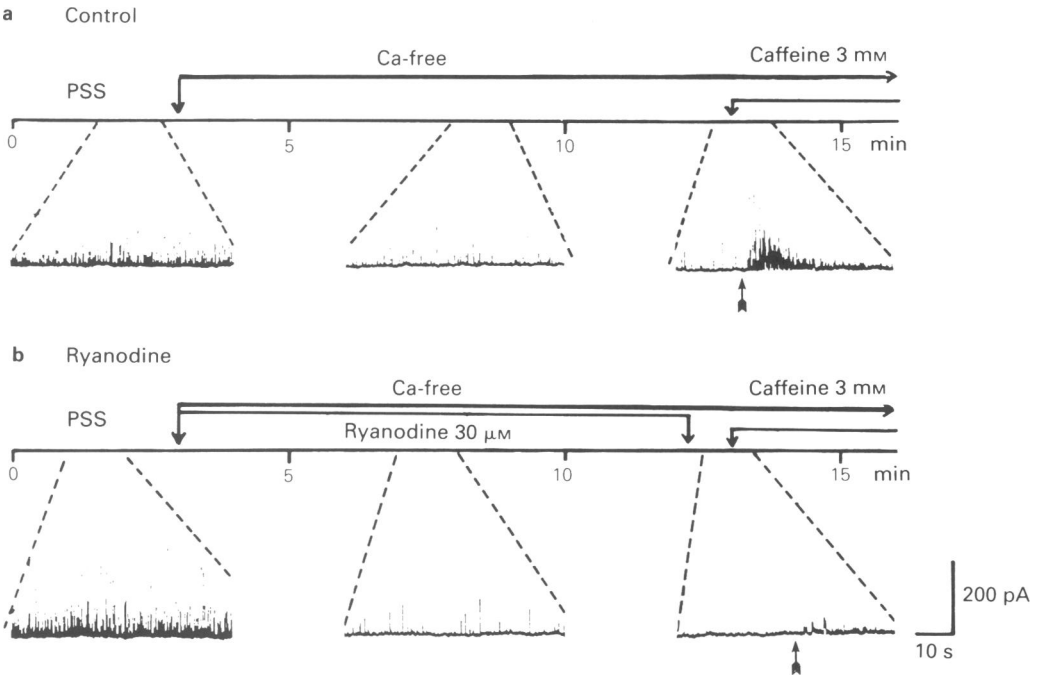

Figure 6 Effects of 3 mM caffeine on the oscillatory outward current (I_{OO}) before (a) and after (b) pretreatment with ryanodine (30 μM) in Ca-free solution containing 2.5 mM $MgCl_2$. The membrane was depolarized to 0 mV at 0 min. Ca-free solution was superfused at 3 min and 3 mM caffeine was added at 13 min (both indicated as arrow with bar). In (b), 30 μM ryanodine was added in Ca-free solution and 3 mM caffeine was added at 13 min (indicated as arrow with bar). Traces were obtained at the times indicated by dotted lines on the time scales.

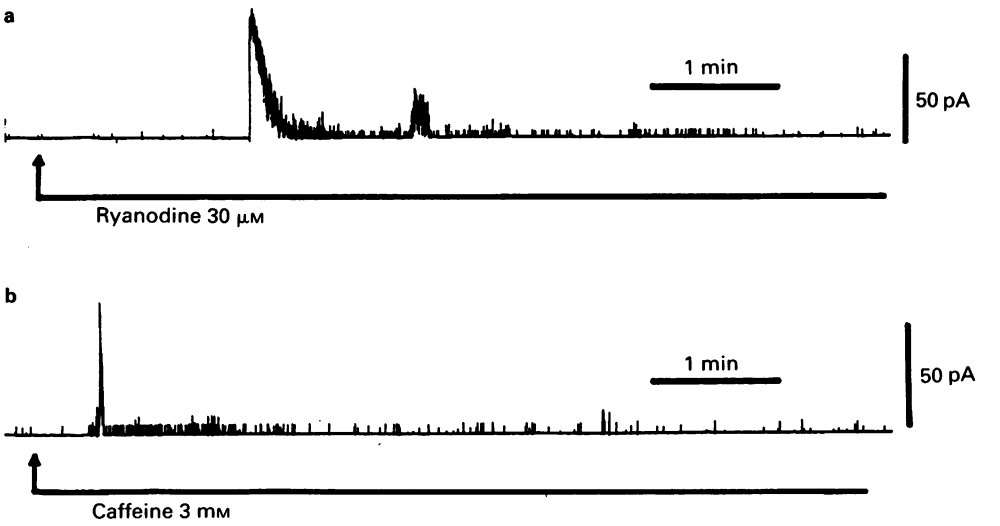

Figure 7 Effects of 30 μM ryanodine (a) and 3 mM caffeine (b) on the unitary currents obtained in the cell-attached patch clamp configuration. Ryanodine and caffeine were added at the time indicated by arrows to the bath solutions. The pipette was filled with PSS and the bath was superfused with the high K solution (0.1 mM EGTA). The membrane potential in the patch pipette was kept at 0 mV. (a) and (b) were obtained from different cells.

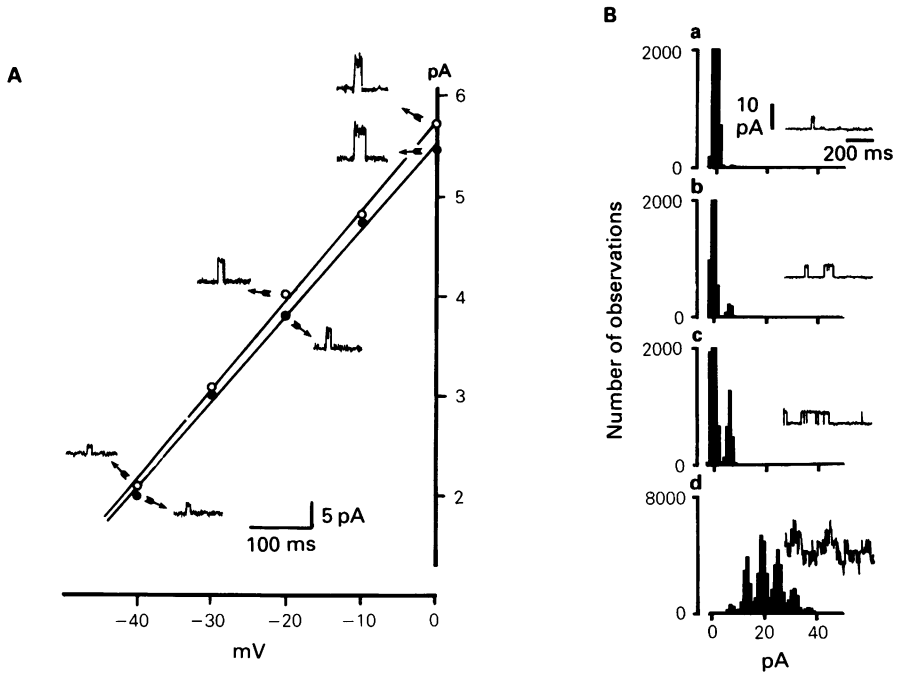

Figure 8 The current-voltage relationship of the unitary current in the presence and absence of 10 μM ryanodine (A) and effects of Ca concentration on the single channel currents recorded with the inside-out membrane patch procedure (B). (A) The amplitudes of the unitary current were measured before (●) and 15 min after application of ryanodine (○) at various levels of the patch potential. The traces of unitary currents measured at different potential levels are also shown in (A). The pipette was filled with PSS and the bath solution was the high K solution containing 1 μM Ca (buffered with 10 mM EGTA). Lines were drawn by the least squares method. Concentration of EGTA was 10 mM. (B) Amplitude histograms were drawn at four different concentrations of Ca in the bath. (a) 0.3 μM, (b) 1 μM, (c) 3 μM and (d) 10 μM Ca; (a–d) were obtained from the same patch membrane. The patch potential was 0 mV. Note that the scale of the vertical axis in (d) is different from those in (a–c). The pipette was filled with PSS and the bath was superfused with high K solution with various concentrations of Ca (buffered with 10 mM EGTA). Currents in (A) and (B) were obtained from different membrane patches.

4.5 μM ($n = 6$). This value was roughly the same as that observed on the isolated I_{TO}. Ryanodine had no effect on I_{SO} up to a concentration of 30 μM.

In nominal Ca-free solution (2.5 mM $MgCl_2$ and no addition of EGTA) the frequency of I_{OO} was reduced but I_{OO} was still generated more than 10 min after superfusion (Figure 6a). Application of caffeine (3 mM transiently enhanced and then inhibited I_{OO}. When the smooth muscle cell was pretreated with Ca-free solution containing 30 μM ryanodine for 10 min, 3 mM caffeine no longer enhanced I_{OO} (Figure 6b). These results indicate that pre-application of ryanodine suppresses the caffeine-induced I_{OO}.

Effects of ryanodine on the Ca-dependent K current measured with a cell attached or inside out patch clamp method

Actions of ryanodine on the unitary current were investigated by means of the patch clamp technique (cell attached configuration). Figure 7 shows a typical example of the actions of 30 μM ryanodine and 3 mM caffeine on the outward current. When the bath was superfused with the high K solution (0.3 mM EGTA) and the pipette was filled with PSS, unitary currents appeared with a low frequency at the patch potential of 0 mV. The amplitude of the unitary current was 5 pA at 0 mV and the conductance was 78 pS. A few min after application of ryanodine (30 μM) the net current was markedly but transiently increased and the peak amplitude reached 75 pA. The net current was then decreased, but after several min, the rate of appearance of the unitary current was much higher than that observed before application of ryanodine (Figure 7a). Ten minutes after the application of ryanodine, addition of 3 mM caffeine did not enhance the frequency of the unitary current (data not shown). On the other hand, in the absence of ryanodine, 3 mM caffeine increased the frequency of the unitary current (Figure 7b). The

onset of the action of caffeine occurred earlier than that of ryanodine (within 30 s). These enhancing actions of ryanodine were seen in 5 out of 9 cells.

To clarify the ionic nature of this unitary current, the inside-out membrane patch was used. Figure 8A shows the current-voltage relationship in the presence and absence of 10 μM ryanodine. A unitary current was not generated when the membrane potential was kept more negative than −50 mV, but when the membrane was depolarized (up to 0 mV), the amplitude of the unitary current was increased (control in A). The conductance calculated from the linear component was 85 pS. Application of ryanodine to the bath (cytoplasmic site) did not change the amplitude of the unitary current, but the current was abolished by superfusion with Cs solution. Figure 8B shows the effects of various Ca concentrations on the unitary current. When Ca concentration was 0.3 μM (buffered by 10 mM EGTA), this current was rarely seen (a). An increase in the Ca concentration up to 3 μM enhanced the activity of the unitary current (b and c). When 10 μM Ca was applied, at least 6 channels opened simultaneously (d). Thus, this unitary current is Ca-dependent.

Effects of ryanodine on the unitary current obtained from the inside out membrane patch were investigated (Figure 9). In this particular fragment, at least 2 channels were open simultaneously in the presence of 1 μM Ca (buffered by 10 mM EGTA) at a membrane potential of −10 mV. Application of 30 μM ryanodine did not produce any obvious changes in the appearance of the unitary current. Amplitude histograms observed before and 6 min after addition of 30 μM ryanodine are shown in Figure 9B. The probability of simultaneous opening of 2 channels was low in the present conditions, and the values for the open probability (p) times the number of channels in the patch (N), Np, were 0.085 in the absence of ryanodine and 0.10 in the presence of ryanodine. Furthermore, when the change in the ionic conductance of this unitary current was estimated in the presence of 30 μM ryanodine (ryanodine in Figure 9A), the value obtained (88 pS) was identical to that observed before application of ryanodine.

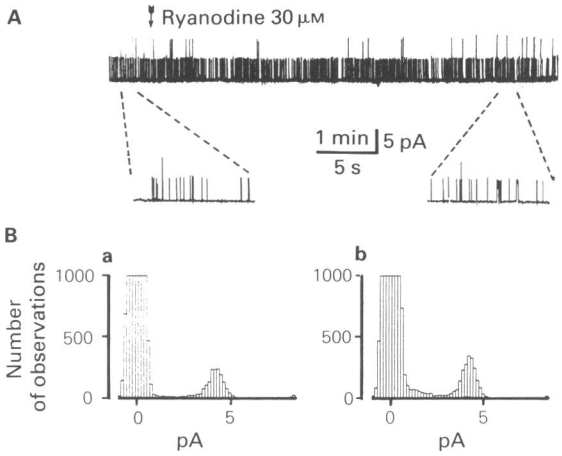

Figure 9 (A) Effects of 30 μM ryanodine on the unitary current. The calculated Ca concentration in the bath solution was 1 μM (buffered with 10 mM EGTA). The pipette was filled with PSS and the bath was superfused with the high K solution. Ryanodine (30 μM) was added at the time indicated by the arrow. Traces with faster time sweep are also shown. (B) Amplitude histograms of the unitary current before (a) and 6 min after (b) application of ryanodine. (a) and (b) were obtained for the same period (1 min) with a sampling interval of 1 ms.

Discussion

Benham & Bolton (1986) and Ohya *et al.* (1987) concluded that the generation of I_{OO} is closely related to the increase in Ca concentration in the cytosol released from the sarcoplasmic reticulum (SR) rather than Ca influx from the extracellular space. This conclusion was based on several observations: (a) caffeine (above 3 mM) initially accelerated and then inhibited the generation of I_{OO}, (b) I_{OO} persisted over 10 min in Ca free external solution, (c) I_{OO} ceased on intracellular perfusion of high concentrations of EGTA, (d) low concentrations (below 1 nM) of A23187, which is known to deplete Ca from the SR selectively (Itoh *et al.*, 1985), inhibited I_{OO}. They also postulated that the I_{OO} was composed of a Ca-dependent K current, since I_{OO} was not generated when Cs solution was in the pipette, I_{OO} was abolished with high concentrations of EGTA in the pipette and was very sensitive to tetraethylammonium (TEA), a selective Ca-dependent K current blocker in smooth muscle cells (Ohya *et al.*, 1987).

I_{TO} is also found to be closely related to Ca released from the SR, though generation of I_{TO} required the generation of I_{Ca}. Both I_{Ca} and I_{TO} ceased on perfusion with PSS containing $MnCl_2$ (Ohya *et al.*, 1986). Following application of low concentrations of A23187, procaine or caffeine, I_{TO} was completely inhibited but there was no effect on I_{Ca} (Ohya *et al.*, 1986; 1987). Presumably, I_{TO} may be generated by Ca released from the SR following activation by influx of Ca (Ca-induced Ca release mechanism).

Ryanodine is thought to inhibit Ca release or to deplete Ca stored in the SR (Sutko & Keynon, 1983;

Sutko et al., 1985; Meissner, 1986; Ito et al., 1986; Rousseau et al., 1987; Hwang & van Breeman, 1987), and as a consequence I_{OO} may be inhibited. A direct action of ryanodine on the Ca-dependent K channel could be ruled out from the present experiments, because the Ca-dependent K channel current recorded with the inside out patch was not modified by 30 μM ryanodine. The present results indicate that ryanodine may not inhibit the release of Ca from the SR but continuous release of Ca may deplete the amount of Ca stored in the SR. In fact, following application of ryanodine I_{OO} was transiently enhanced and then ceased, as recorded with the cell attached configuration. Recently Rousseau et al. (1987) studied the action of ryanodine on the Ca channel of the SR vesicle prepared from rabbit skeletal muscle cells. They concluded that ryanodine shifts Ca channels from a full conductive state to a subconductive state with an increase in the open probability to unity and, consequently, depletes Ca in the SR. Using the whole cell voltage clamp procedure, a transient enhancement of the I_{OO} was not observed. The discrepancy observed between the actions of ryanodine in the two different experimental procedures may be caused by the use of EGTA in the pipette in the case of the whole cell voltage clamp procedure. EGTA may rapidly chelate the Ca released from the SR.

The amplitude of the isolated I_{Ca}, evoked by small depolarization of the membrane (below 0 mV), was enhanced by ryanodine in concentrations above 10 μM. This enhancement was not due to attenuation of the I_{TO} or reduction in the free Ca in the cytosol following depletion of stored Ca, because after abolition of I_{TO} and I_{OO} using a pipette solution containing 4 mM EGTA, ryanodine still enhanced I_{Ca}. Thus, ryanodine may act not only on the SR but also on the voltage-dependent Ca channel. In the rat and rabbit ventricular muscles (Mitchell et al., 1984; Shattock et al., 1987) ryanodine prolonged the action potential, whereas in the guinea-pig ventricular muscle, Nishio et al. (1986) demonstrated that ryanodine had no effect on the I_{Ca}. In the present experiments, at test potentials more positive than +20 mV, no enhancement (but a slight inhibition) of the I_{Ca} was observed in the presence of 30 μM ryanodine. Further experiments are required to determine the actions of ryanodine on I_{Ca}.

In the present experiments high concentrations of ryanodine (above 3 μM) were required to inhibit I_{TO} and I_{OO}. The requirement of a similar concentration of ryanodine was noted in the guinea-pig and rabbit aorta (Ito et al., 1986; Hwang & Van Breemen, 1987). On the other hand, in cardiac and skeletal muscle cells, nanomolar concentrations of ryanodine were enough to produce a response (Meissner, 1986; Seguchi et al., 1986; Lattanzio et al., 1987; Hasselbach & Migala, 1987). Ito et al. (1986) suggested several possible reasons to explain the low sensitivity of smooth muscle cells to ryanodine, such as use- and depolarization-dependence of ryanodine (Sutko et al., 1985). On the other hand, using SR vesicles prepared from the canine ventricle, two binding sites for ryanodine were found to occur, with K_D's of 6.8 nM and 17 μM (Alderson & Feher, 1987). The membrane potential of smooth muscles is higher than that of skeletal and cardiac muscle, but if ryanodine inhibits I_{TO} and I_{OO} in a depolarization-dependent manner I_{OO} would be more potently inhibited by ryanodine than I_{TO} because to record the I_{OO}, the membrane was depolarized to 0 mV for over 10 s, while in the case of I_{TO} short depolarization (0 mV) was applied with a long interval (30 s) from the holding potential of −60 mV. However, the IC_{50} values for ryanodine on I_{OO} and I_{TO}, as estimated from the concentration-response relationship, were similar. This may indicate that the SR membrane of smooth muscle cells has only a low affinity binding site for ryanodine.

From the amplitude histogram I_{OO} could be classified into three groups (small, medium and large I_{OO}) and the medium and large I_{OO} groups showed Gaussian distributions with mean amplitudes of 90 and 190 pA, respectively at a clamp potential of 0 mV, whereas at the same membrane potential (0 mV), the mean amplitude of the single Ca-dependent K channel current was 5.5 pA. This means that 16 and 35 channels were simultaneously opened at the peak of the medium and large I_{OO} groups, respectively. Assuming that the Ca sensitivity of each Ca-dependent K channel is the same and each channel opens independently, the amplitude of I_{OO} may reflect the amount of Ca releaesd from each SR. The main action of ryanodine on I_{OO} was a reduction in the frequency rather than inhibition of the amplitude. If ryanodine irreversibly opened the Ca channel in the SR, burst releases of Ca from some SR vesicles distributed just beneath the cell membrane may deplete Ca in such vesicles and the available number of SR vesicles for the Ca release would be decreased. As a consequence the frequency of I_{OO} would be lowered.

In conclusion, ryanodine (above 3 μM) enhanced I_{Ca} when the current was evoked by low depolarizations (below 0 mV) but markedly inhibited I_{TO} and I_{OO} due to depletion of Ca stored in the SR. Ryanodine may irreversibly open the Ca releasing site in the SR and cause the depletion of Ca. However, the site of Ca release or the Ca releasing mechanism in smooth muscle may differ from those observed in skeletal and cardiac muscle because the potency of ryanodine, for depletion of the stored Ca, was about 1000 times lower in smooth muscle than the other muscle tissues.

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