Regulation of Calcium Current by Intracellular Calcium in Smooth Muscle Cells of Rabbit Portal Vein

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Effects of concentrations of intracellular calcium, [Ca\textsuperscript{2+}], on the voltage-dependent Ca\textsuperscript{2+} current (I\textsubscript{Ca}) recorded from dispersed single smooth muscle cells of the rabbit portal vein were studied, using a whole cell voltage clamp method combined with an intracellular perfusion technique. Outward currents were minimized by replacement of Cs\textsuperscript{-}-rich solution in the pipette and 20 mM tetraethylammonium in the bath. The I\textsubscript{Ca} was evoked by command pulses of above -30 mV, and the maximum amplitude was obtained at about 0 mV. This I\textsubscript{Ca} was dose dependently inhibited by increases in the [Ca\textsuperscript{2+}], above 30 nM. The K\textsubscript{v} value of the [Ca\textsuperscript{2+}] required to inhibit the I\textsubscript{Ca} was about 100 nM. The Ba\textsuperscript{2+} current was also inhibited by increases in the [Ca\textsuperscript{2+}]. Conversely, perfusion of Ba\textsuperscript{2+} into the cell up to 100 μM did not suppress the I\textsubscript{Ca}. Changes in the [Ca\textsuperscript{2+}] did not modify the steady-state inactivation curve. The inhibition of the I\textsubscript{Ca} evoked by the test pulse is most prominent when the preceding influx of Ca\textsuperscript{2+} during the conditioning pulse was large, as estimated using a double pulse protocol. This inhibition was proportionally reduced by increases in the concentration of the Ca\textsuperscript{2+} chelator, ethylene glycol bis(\beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Therefore, the Ca\textsuperscript{2+}-dependent inactivation of the Ca\textsuperscript{2+} channel may contribute toward regulating [Ca\textsuperscript{2+}], in smooth muscle cells of the rabbit portal vein. (Circulation Research 1988;62:375–383)

Mechanisms of inactivation of the voltage-dependent Ca\textsuperscript{2+} channel have been investigated using various excitable tissues.\textsuperscript{1,2} The Ca\textsuperscript{2+} channel of egg cell membranes of marine polychaetes was inactivated only in a voltage-dependent manner,\textsuperscript{3} as in the Na\textsuperscript{+} channel of squid giant axons.\textsuperscript{4} On the other hand, there is evidence that an increase in the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), either due to released or influxed Ca\textsuperscript{2+}, participates in the initiation of inactivation of the Ca\textsuperscript{2+} channel of neurons and skeletal and cardiac muscle cells. For example, 1) increases in the [Ca\textsuperscript{2+}] have been shown to suppress the amplitude of Ca\textsuperscript{2+} current (I\textsubscript{Ca}),\textsuperscript{5,6} 2) the inactivation curves obtained from double pulse studies are "U" shaped, which indicates that the inactivation depends on the Ca\textsuperscript{2+} inflow during conditioning of conditioning pulse.\textsuperscript{7-10} 3) Intracellular application of ethylene glycol bis(\beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) has been shown to slow the decay of I\textsubscript{Ca} and weaken the inhibition of I\textsubscript{Ca} as observed using the double pulse protocol.\textsuperscript{11,12} 4) When charge carriers of the Ca\textsuperscript{2+} channel are replaced from Ca\textsuperscript{2+} to Ba\textsuperscript{2+}, decay of the Ba\textsuperscript{2+} current (I\textsubscript{Ba}) has been shown to be slower than the decay of the I\textsubscript{Ca}.\textsuperscript{13,14}

In a previous paper, we proposed the possible involvement of a Ca\textsuperscript{2+}-dependent mechanism in the inactivation of I\textsubscript{Ca} obtained in intestinal smooth muscle cells\textsuperscript{12} because the inactivation curve obtained using the double pulse protocol was U shaped and because decay of the inward currents slowed when Ba\textsuperscript{2+} was used instead of Ca\textsuperscript{2+} as the charge carrier of the Ca\textsuperscript{2+} channel. Similar conclusions were reported by Jmari et al\textsuperscript{15} and Ganitkevich et al\textsuperscript{16} who used rat myometrial tissue and guinea pig tenia cecum, respectively. However, this evidence was derived from experiments performed using the double pulse protocol alone, and investigations involving the direct regulation of [Ca\textsuperscript{2+}], were apparently not carried out.

Using an intracellular perfusion technique\textsuperscript{17} to alter the [Ca\textsuperscript{2+}], and using various concentrations of a Ca\textsuperscript{2+} chelator, we obtained evidence that an increase in the [Ca\textsuperscript{2+}], inhibits the Ca\textsuperscript{2+} channel in smooth muscle cells of the rabbit portal vein.

Materials and Methods

Preparations of Single Smooth Muscle Cells

Single smooth muscle cells of the rabbit portal vein were prepared by enzymatic treatment as described by Inoue et al.\textsuperscript{18} Briefly, male albino rabbits (Nippon White, 1.8–2.0 kg) were anesthetized with sodium pentobarbital (40 mg/kg i.v.) and exsanguinated. The portal vein was excised, connective tissues were carefully removed, and the endothelium was rubbed off with a cotton swab. The muscle layer was then cut into small pieces in Ca\textsuperscript{2+}-free solution containing (mM) NaCl 145, KCl 6, 4-(2-hydroxyethyl)-1 piperazine ethansulfonic acid (HEPES) 10, glucose 12, pH 7.3 titrated with tris (hydroxymethyl) aminomethane (Tris\textsuperscript{•}). After 15 minutes incubation in Ca\textsuperscript{2+}-free solution, the tissues were transferred into another vessel filled with the same solution containing 2 mg/ml collagenase (clostridio peptidase A; Boehringer-
Mannheim, Mannheim, Federal Republic of Germany). After 30 minutes incubation for digestion of the tissue at 36°C, single cells were dispersed by gentle agitation using a glass pipette in fresh Ca²⁺-free solution. The cells were stored in a stock solution (0.5 mM CaCl₂ was added to the Ca²⁺-free solution) containing 2 mg/ml bovine serum albumin (essentially fatty acid free) and 1 mg/ml trypsin inhibitor (type II-s; Sigma Chemical, St. Louis, Missouri). Spindle-shaped single smooth muscle cells (5-10 μm diameter and 150-250 μm length) were obtained. Experiments were performed within 3 hours after the cell harvest.

Electrical Recordings

The cell suspension was taken into a small chamber (0.2 ml) and placed on the stage of a microscope (TMD-Microscope, Nihon Kogaku, Tokyo, Japan). Whole cell voltage clamp was performed with a suction pipette through a single electrode voltage clamp amplifier. The recording conditions of the amplifier were the same as those described in our previous reports.12,16,18 Patch pipettes (resistances of 2-4 MΩ) made of Pyrex glass capillary tubes were prepared as described by Hamill et al.21 After formation of the pipette-membrane seal with a high resistance (over 5 GΩ), the patch membrane was disrupted by a negative pressure (10–30 mm H₂O).

Electrical responses were displayed on a storage oscilloscope and simultaneously stored in a video-cassette recorder (NY-880HD, National, Osaka, Japan) through a PCM converting system (PCM-501ES, Sony, Tokyo, Japan; 16 bit, sampling frequency of 44 kHz) with a built-in low-pass filter (four poles Bessel type active filter, 3 kHz).

Experiments were carried out using short cells (150 μm length) to achieve an isopotential throughout the cell and a rapid internal perfusion. Although difficulties for the satisfactory voltage clamping of longer cells have been previously mentioned,12 the five times higher input resistance achieved in the present experiments compared with that reported previously12 (5 GΩ versus 1 GΩ) and the small amplitude of Iₜₚ was improved voltage control.22 Further evidence of the isopotential state of the cells was provided by the current-voltage (I-V) curve in which no abrupt increase in the inward current was seen, which would have indicated loss of voltage control.23

To prevent a run-down phenomenon of the Iₜₚ or Iᵣₑ, a 250-msec command pulse to 0 mV (for Iₜₚ) or to −10 mV (for Iᵣₑ) from the holding potential of −60 mV was applied every 30 seconds, except for measurements of the current-voltage relation (I-V curve) and the double pulse studies. Five millimolar ATP present in the pipette solution also prevented the run-down of Iₜₚ in smooth muscle cells24 as is also the case with neurons and cardiac muscle cells.25,26 Under such conditions, the amplitude of Iₜₚ was preserved for more than 20 minutes after initiation of the current recording.19,20

Solutions

The bath solution of the following composition was used throughout the current recording (mM): NaCl 120, tetraethylammonium chloride (TEA Cl) 20, CaCl₂ (or BaCl₂) 2.8, HEPES 10, glucose 8, pH 7.3 with Tris. To isolate the Iₜₚ,12,22 the pipette was filled with high-Cs⁺ solution of the following composition (mM): CsCl 125, HEPES 20, adenosine 5'-triphosphate (dissodium salt, ATP-2Na) 5.1, various concentrations of MgCl₂ to adjust a free-Mg²⁺ concentration to 1, pH 7.3 with CsOH. Concentrations of free Ca²⁺ (3-1,000 nM), free Ba²⁺ (1-100 μM), and EGTA (0.1, 1, and 10 mM) were varied and are given in Table 1. The free Ca²⁺, Ba²⁺, and Mg²⁺ concentrations of the pipette solutions were obtained by making use of multiequilibrium equations, using the association constants for CaEGTA²⁻, MgEGTA²⁻, BaEGTA²⁻, CaATP²⁻, MgATP²⁻, and BaEGTA²⁻ as cited by Itoh et al27 and Sato et al.27 (The apparent binding constants were 4.3 × 10⁻⁸ M⁻¹, 2.14 × 10⁻⁹ M⁻¹, 2.57 × 10⁻⁹ M⁻¹, 4.0 × 10⁻⁸ M⁻¹, 1.0 × 10⁻⁷ M⁻¹, and 1.95 × 10⁻⁸ M⁻¹, respectively.27-30) In preparing the pipette solutions, the concentration of EGTA was fixed at 10 mM, and appropriate amounts of CaCl₂, BaCl₂, and MgCl₂ were added (Table 1). The calculated Ca²⁺ and Ba²⁺ concentrations in the pipette solution were termed as the [Ca²⁺] and [Ba²⁺], respectively. All experiments were performed at room temperature (20–26°C).

Intracellular Perfusion Procedures

To control the intracellular constituents while recording the current, the pipette solution was exchanged using the modified "intracellular perfusion technique" introduced by Soejima and Noma.19 Apparatus and procedures were the same as described by Ohyama et al.19,20 In brief, a heat-formed fine polyethylene tube, connected to a reservoir filled with a test solution, was inserted into the suction electrode at a distance of 100-300 μm behind the tip. Exchange of the solution was performed by applying a negative pressure to the suction pipette and by a positive pressure to the inserted tube by lifting the reservoir. Exchange of the pipette solution was confirmed by checking the volume of the solution leaving the pipette holder (0.03–0.05 ml/min).

### Table 1. Compositions of Divalent Cations Present in Pipette Solutions

<table>
<thead>
<tr>
<th></th>
<th>[Ca²⁺] (mM)</th>
<th>[Ba²⁺] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂ (mM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BaCl₂ (mM)</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>MgCl₂ (mM)</td>
<td>6.0</td>
<td>5.9</td>
</tr>
<tr>
<td>EGTA (mM)</td>
<td>10</td>
<td>10</td>
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Morphometric Observations of the Cell

Shortening of the dispersed single cells induced by perfusions of Ca\(^{2+}\) into the cytosol was observed in the Ca\(^{2+}\)-free bath solution containing Mg\(^{2+}\) (2.5 mM MgCl\(_2\) was added to the Ca\(^{2+}\)-free solution). Pipette solutions and procedures for the pipette-membrane seal and rupture were the same as those used for the whole cell voltage clamp study. After rupture of the patch membrane, the contraction was initiated. The cell length was measured on photos taken before and after the contraction. The shortening was expressed as a relative length. (The cell length before rupture of the patch membrane was normalized as 1.0.)

Data Analysis

To estimate the relation between the inhibition of \(I_{Ca}\) against the [Ca\(^{2+}\)]\(_i\), the extent of influx of Ca\(^{2+}\) provoked by the conditioning pulse was estimated from the integral of the \(I_{Ca}\) (\(\Delta I_{Ca}\)) evoked by the conditioning pulse (200 msec) and expressed as nanocoulomb (nC).

To normalize amplitudes of the \(I_{Ca}\), obtained from individual cells, the \(I_{Ca}\) was expressed as a current density and calculated as follows: the maximum amplitude of the \(I_{Ca}\) was divided by the cell capacitance (\(\mu\)A/\(\mu\)F), estimated from the capacitive current evoked by the potential step from -60 to -50 mV.

The steady-state inactivation curve of \(I_{Ca}\) was drawn by fitting the data to the Boltzmann distribution:

\[
P = \frac{1}{1 + \exp\left(\frac{V - V_h}{k}\right)}
\]

where \(P\) is the probability of being available to open to a test depolarization; \(V\) is the conditioning command potential; \(V_h\) is the conditioning command potential required for the half inhibition of the \(I_{Ca}\); \(k\) is the Boltzmann coefficient.

If it is assumed that inactivation of the Ca\(^{2+}\) channel would be caused by the Ca\(^{2+}\) binding, the dissociation constant (\(K_C\)) and the Hill's coefficient (\(n\)) for the inhibition of \(I_{Ca}\) can be determined by fitting the data to the following equation:

\[
\frac{I_{Ca}}{I_{Ca0}} = 1 - \frac{1}{1 + \left(K_C[Ca^{2+}]^n\right)}
\]

where \(I_{Ca}\) is the maximum current amplitude recorded in various [Ca\(^{2+}\)], and \(I_{Ca0}\) is that recorded in the absence of Ca\(^{2+}\) (10 mM EGTA) in the pipette.

Fitting the data to each equation was performed using the nonlinear least-squares method. The data are given as mean \(\pm\) SD. Statistical significance was determined by Student's \(t\) test (paired or unpaired), and level of significance was \(p<0.05\).

Results

Effects of [Ca\(^{2+}\)], on Amplitude of \(I_{Ca}\)

To investigate the effects of [Ca\(^{2+}\)], on the \(I_{Ca}\), the [Ca\(^{2+}\)], was altered using an intracellular perfusion technique. Figure 1A shows inhibition of the \(I_{Ca}\) induced by increases in the [Ca\(^{2+}\)], from 3 to 100 nM. The \(I_{Ca}\) was evoked by a command potential of 0 mV from the holding potential of -60 mV. After the amplitude of \(I_{Ca}\) had reached a steady level (registered as the control) using a pipette solution containing 3 nM [Ca\(^{2+}\)], (5 minutes after rupture of the patch membrane), the pipette solution was exchanged with the solution containing 100 nM [Ca\(^{2+}\)]. Two minutes after the intracellular perfusion, the amplitude of \(I_{Ca}\) gradually decreased and reached a new steady level at 6 minutes. (The \(I_{Ca}\) was reduced to 0.45 times the control.) In contrast, the intracellular perfusion with the same [Ca\(^{2+}\)], (3 nM) slightly reduced the amplitude of \(I_{Ca}\) (0.9 times control measured at 15 minutes after starting the perfusion). Figure 1B shows the effects of various [Ca\(^{2+}\)], on the amplitude of \(I_{Ca}\). To obtain the above relations, 3 nM [Ca\(^{2+}\)], was initially applied (the control), and various [Ca\(^{2+}\)], (10-1,000 nM) were perfused into the cell. Increases in the [Ca\(^{2+}\)], significantly inhibited the amplitude of \(I_{Ca}\) in a dose-dependent manner (10 nM, 0.85 \(\pm\) 0.12, \(n=7\); 100 nM, 0.53 \(\pm\) 0.18, \(n=8\); 1,000 nM, 0.11 \(\pm\) 0.14 times the control, \(n=5\)). Half inhibition of the \(I_{Ca}\) was observed at about 120 nM [Ca\(^{2+}\)]. On the other hand, when [Ca\(^{2+}\)], was reduced from 100 to 3 nM (the amplitude of \(I_{Ca}\) in 100 nM [Ca\(^{2+}\)], was normalized as 1.0), the \(I_{Ca}\) was gradually enhanced, and the amplitude was increased to 2.2 times the control.
Effects of \([\text{Ca}^2+]\), on \(I_{\text{Ca}}\) and Effects of \([\text{Ba}^2+]\), on \(I_{\text{Ca}}\)

Inhibitory effects of \([\text{Ca}^2+]\), on the \(I_{\text{Ca}}\) channel were also determined from measurements of the \(I_{\text{Ca}}\). When \(\text{Ca}^2+\) in the bath solution was replaced with \(\text{Ba}^2+\), the \(I_{\text{Ca}}\) could be recorded. \([\text{Ca}^2+]\), was increased from 3 nM (control) to 100 nM using the intracellular perfusion technique, and reductions in amplitudes of the \(I_{\text{Ca}}\) were investigated (Figure 3A). Amplitudes of the \(I_{\text{Ca}}\) were gradually decreased in proportion to increases in the \([\text{Ca}^2+]\), to 0.45 times the control. Increases in the \([\text{Ca}^2+]\), inhibited the \(I_{\text{Ca}}\) to much the same extent as observed in the case of the \(I_{\text{Ca}}\). (The \(I_{\text{Ca}}\) was inhibited in 100 nM \([\text{Ca}^2+]\), to 0.49 ± 0.05 times the control, \(n = 5\)).

As the decay phase of \(I_{\text{Ca}}\) was slow, compared with that of the \(I_{\text{Ca}}\), it seemed unlikely that \(\text{Ba}^2+\) would have any inhibitory effect on the \(I_{\text{Ca}}\) channel. To confirm this, we investigated the effect of \([\text{Ba}^2+]\), (1–100 μM) on the \(I_{\text{Ca}}\). Figure 3B shows a typical result obtained in 100 μM \([\text{Ba}^2+]\). As the control, the \(I_{\text{Ca}}\) was recorded with the \(\text{Ca}^2+\)-free pipette solution containing 10 mM EGTA, and subsequently, the pipette solution containing 100 μM \([\text{Ba}^2+]\), was intracellularly perfused. Introduction of \(\text{Ba}^2+\) (100 μM) into the cytosol did not inhibit the \(I_{\text{Ca}}\) for over 20 minutes (reduced to 0.9 times the control). Similar experiments were performed using other cells, and we concluded that there is no significant effect with applications of 1–100 μM \([\text{Ba}^2+]\), (1 μM, 0.95 ± 0.06, \(n = 4\); 10 μM, 0.91 ± 0.07, \(n = 4\); 100 μM, 0.90 ± 0.12 times the control, \(n = 5\)).

Effects of \([\text{Ca}^2+]\), on Current-Voltage Relation

Figure 4 shows the I-V curves of the \(I_{\text{Ca}}\) at two different concentrations of \([\text{Ca}^2+]\), (3 and 100 nM) using the same cell. In the presence of 3 nM \([\text{Ca}^2+]\), the threshold potential required to evoke the \(I_{\text{Ca}}\) was about −30 mV, and the maximum amplitude was obtained by application of the command pulse of 0 mV. When the \([\text{Ca}^2+]\), was increased to 100 nM, the \(I_{\text{Ca}}\) was consistently suppressed at any given command potential (to 0.5–0.65 times the control).

Effects of \([\text{Ca}^2+]\), on Inactivation Curve of \(I_{\text{Ca}}\)

A steady-state inactivation curve of the \(I_{\text{Ca}}\) was determined using the double pulse protocol. To reach the steady state of the \(I_{\text{Ca}}\), conditioning pulses of 5 seconds with various intensities were applied, and subsequently, to evoke the \(I_{\text{Ca}}\), the test pulse (0 mV, 50 msec) was applied at the holding potential of −80 mV. (The interval of two pulses was 10 msec.) Similar steady-state inactivation curves were obtained with 3 and 100 nM \([\text{Ca}^2+]\), as shown in Figure 5 (3 nM, \(k = 7.2\) and \(V_h = -38\) mV; 100 nM, \(k = 7.3\) and \(V_h = -40\) mV; \(n = 5–7\)).

Inhibitions of the \(I_{\text{Ca}}\) were also studied using a different double pulse protocol with a short conditioning pulse (200 msec). The conditioning (up to +50 mV) and test (0 mV, 50 msec) pulses were separated.
by an interval of 100 msec to minimize the influence of the noninactivated Ca\(^{2+}\) current and of contaminating currents such as the Ca\(^{2+}\)-dependent Cl\(^-\) current, etc. Figure 6A shows a typical result obtained from one cell for which two EGTA concentrations (0.1 and 1 mM) were used for the pipette solution. In this experiment, CaCl\(_2\) was not added to the pipette solution. The amplitudes of I\(_{\text{Ca}}\) were larger in the presence of 1 mM EGTA than those of 0.1 mM EGTA. With application of the conditioning pulse of -30 mV, the I\(_{\text{Ca}}\) evoked by the test pulse was inhibited, and application of the conditioning pulse of 0 mV further inhibited the I\(_{\text{Ca}}\). However, when the conditioning pulse of +50 mV was applied, the amplitude of I\(_{\text{Ca}}\) was partly restored. Figure 6B shows the relative amplitudes of the I\(_{\text{Ca}}\) evoked by the test pulse plotted against the conditioning potential. The amplitudes of I\(_{\text{Ca}}\) recorded without application of the conditioning pulse, in the presence of 0.1 and 1.0 mM EGTA, were both normalized as 1.0. The maximum inhibition of the I\(_{\text{Ca}}\) was obtained by a conditioning pulse of 0 mV or +10 mV; that is, the inhibition curve was U shaped. However, the fraction of the maximal inhibition was smaller in the presence of 1 mM EGTA than that in 0.1 mM EGTA (to 0.4 times in 0.1 mM EGTA, and to 0.5

**Figure 4.** Current-voltage relation observed in the presence of 3 nM or 100 nM \([\text{Ca}^{2+}]\). A, \(I_{\text{Ca}}\) was recorded before (3 nM, open circles) and 10 minutes after perfusion of 100 nM \([\text{Ca}^{2+}]\), (100 nM, closed circles). The holding membrane potential was kept at -60 mV, and command potentials are shown in the left side of traces. B, \(I-V\) curve obtained in the presence of 3 nM or 100 nM \([\text{Ca}^{2+}]\). Peak amplitudes of \(I_{\text{Ca}}\) provoked by various command potentials were plotted. The holding potential was kept at -60 mV. A and B were obtained from the same cell.

**Figure 5.** Steady-state inactivation curves observed in the presence of 3 nM and 100 nM \([\text{Ca}^{2+}]\). Various amplitudes of conditioning pulse (5 seconds) were applied before application of the test pulse (0 mV, 50 msec) at an interval of 10 msec. The holding potential was kept at -80 mV. Continuous curves were obtained by fitting data to the Boltzmann distribution (3 nM, \(k = 7.2, V_0 = -38 mV\); 100 nM, \(k = 7.3, V_0 = -40 mV\); see "Materials and Methods"). Each point was expressed as the mean±SD of 5–7 observations.

**Figure 6.** Inactivations of \(\text{Ca}^{2+}\) channel measured using the double pulse protocol in the presence of two different concentrations of EGTA in the pipette solution (0.1 mM, 1 mM). The holding potential was kept at -60 mV. Inset, a test command pulse (0 mV and 50 msec) was applied after application of the conditioning pulse (200 msec and various steps of depolarization) with an interval between conditioning and test pulses of 100 msec. After completion of a series of the experiment for 0.1 mM EGTA, the pipette solution was replaced with that containing 1 mM EGTA, and then the experiment for 1 mM EGTA was repeated. A, \(I_{\text{Ca}}\) measured in the presence of 0.1 mM (closed circles) or 1 mM (open circles) EGTA. B and C, Inactivation of \(I_{\text{Ca}}\) induced by the conditioning pulse. \(I_{\text{Ca}}\) evoked by the test pulse in the presence of 0.1 mM or 1 mM EGTA without application of the conditioning pulse was normalized as 1.0. Relative amplitude of \(I_{\text{Ca}}\) evoked by the test pulse was plotted against potentials of the conditioning pulse (B) and the \(\text{Ca}^{2+}\) influx (expressed as nanocoulomb, nC; see "Materials and Methods") provoked by the conditioning pulse (C). A, B, and C were obtained from the same cell.
The shortening of the single smooth muscle was expressed as a relative cell length after exposure to the pipette solution. (The cell length before exposure to the pipette solution was normalized as 1.0; see "Materials and Methods"). Individual records were obtained in the test pipette solution containing in the absence (EGTA 10 mM without addition of CaCl₂, the control) or presence of various [Ca²⁺], (3–1,000 nM). Current density (closed circles) and relative length of smooth muscle cells (open circles) were plotted against the [Ca²⁺]. Each point indicates mean ± SD (n = 7–20). Values obtained above 30 nM [Ca²⁺], for the I_c, and above 100 nM [Ca²⁺], for the shortening are statistically significant. Continuous curve of the I_c was drawn by the least-squares fit to the equation described in the text (see "Materials and Methods"), and the relation line for changes in the cell length was drawn by eye.

![Diagram](image)

**Figure 7.** Effects of [Ca²⁺], on the amplitude of I_c and the shortening of smooth muscle cells. To normalize amplitudes of I_c recorded in individual cells, the amplitude of I_c was expressed as a current density (see "Materials and Methods"). The shortening of the single smooth muscle was expressed as a relative cell length evoked by the test pulse (Figure 6B). To clarify the relation between the Ca²⁺ influx and the inhibition of I_c, the amount of Ca²⁺ influx induced by the conditioning pulse was estimated from a total area of the inward current and was plotted against the relative amplitude of I_c evoked by the test pulse (Figure 6C). The amplitude of I_c, evoked by the test pulse and the amount of Ca²⁺ influx were inversely related. However, when the same amount of Ca²⁺ influx was obtained at two different conditioning potentials, the higher conditioning pulse inhibited the I_c to a greater extent. Increases in the concentration of EGTA from 0.1 to 1 mM shifted the relation between the I_c and the influx of Ca²⁺ to the right and upward.

**Relation Between [Ca²⁺], and Inhibition of I_c or Shortening of the Cell**

To investigate whether or not the level of [Ca²⁺], required to produce the contraction was sufficient to inhibit the I_c, the amount of [Ca²⁺], required to produce mechanical responses was estimated from shortening of the single cells. In the Ca²⁺-free bath solution containing Mg²⁺, lengths of single cells of the rabbit portal vein ranged from 150 to 250 μm. The introduction of Ca²⁺ into the cell by passive diffusion through the pipette led to cell contraction. After measuring the cell shortening, the bath solution was replaced with solution containing Ca²⁺, and the I_c was recorded. The amplitude of I_c was expressed (μA/μF) as the current density. Figure 7 shows amplitudes of the I_c and relative lengths of cells plotted against the [Ca²⁺]. These results clearly show that increases in the [Ca²⁺] inhibited the I_c, and the amplitude of I_c, was expressed as a current density. The minimum concentration of the [Ca²⁺], required to inhibit the single cell was estimated to be about 100 nM, whereas the threshold concentration of [Ca²⁺], required to inhibit the I_c, was 30 nM. The K_0 and Hill's coefficient (n) for the inhibition of the amplitude of I_c were calculated to be 65 nM and 0.98, respectively.

**Discussion**

Increases in the [Ca²⁺], induced by an intracellular perfusion technique and by Ca²⁺ influx during application of a conditioning pulse, inhibited the amplitude of I_c, dose dependently, in single smooth muscle cells of the rabbit portal vein. Such a correlation between the [Ca²⁺], and I_c is considered to relate to the Ca²⁺-dependent inhibition of I_c, channel, as noted in other excitable cell membranes.6,8,9,3,3

Contaminating Ca²⁺-dependent and/or voltage-dependent outward currents at high [Ca²⁺], such as K⁺, H⁺, and Cl⁻ currents, were negligible because the inhibition of I_c has usually been investigated during application of the command potential of 0 mV (close to the equilibrium potentials for the H⁺ and Cl⁻ currents) and because K⁺ has not been included in the solutions.12-22 The blockers of these outward currents, TEA and 4-aminopyridine,6,12,24,25 have had little effect on the amplitude of I_c (authors' unpublished observations). Thus, increases in these contaminating currents are unlikely to contribute to the inhibition of I_c observed at high [Ca²⁺]. The irreversible run-down phenomenon of the I_c also could not account for inhibitions of the I_c at high [Ca²⁺], because the amplitude of I_c was restored by reduction in the [Ca²⁺], (Figure 2).

As the [Ca²⁺], did not modify the steady-state inactivation curve, inhibitions of the I_c at high [Ca²⁺] were not due to the negative shift of the inactivation curve. Lack of change in the voltage-dependent inactivation kinetics by alterations of the [Ca²⁺], has also been noted in guinea pig ventricular cells.3 Furthermore, similar I-V curves could be drawn in different [Ca²⁺], (Figure 4). These results suggest that changes in the surface potential at the internal membrane do not contribute to the inhibition of I_c.

Decreases in the driving force were unlikely to account for the inhibition of I_c induced by increases in [Ca²⁺]. Because the calculated fraction of inhibition of the I_c was below 0.1% of the total current, even when the [Ca²⁺], was increased from 3 nM to 1 μM, as estimated from the amplitude of I_c (I_c), the following equation of the constant field theory can be used, under the assumption that other variables remained the same:

\[
I_c = P_c(4VF^2/RT)\{[Ca^{2+}]_i \text{exp}(2VF/RT) - [Ca^{2+}]_i \text{exp}(2VF/RT) - 1\}
\]
where $V$ is membrane potential ($-30 \text{ mV} - 50 \text{ mV}$); $P_c$ is the Ca$^{2+}$ permeability coefficient; and $R$, $T$, and $F$ have their standard meanings.

Increases in the [Ca$^{2+}$], inhibited both the $I_c$ and $I_b$ to much the same extent. Therefore, the inhibitory effects of [Ca$^{2+}$], seemed to work on the Ca$^{2+}$ channel. From the relation between the inhibition of $I_c$ and increases in the [Ca$^{2+}$], (Figure 7), the $K_i$ value was 65 nM, and the Hill’s coefficient was about 1.0. Plant et al$^{11}$ proposed that a Ca$^{2+}$ binding site linked to the inactivation mechanism is located near the inside mouth of the Ca$^{2+}$ channel in neurons. The present results also suggest the presence of a Ca$^{2+}$ binding site on the inside of the plasma membrane, and the stoichiometry of Ca$^{2+}$ to the binding site seems to be 1:1. When we assumed that the Hill’s coefficient was 1.0 and the $i_{c_{\alpha}}$ was the same as $i_{c_{\beta}}$ measured in the presence of 3 nM [Ca$^{2+}$], the $K_i$ value of 106 nM could be obtained from the data of Figure 1, and this value was roughly the same as estimated from the data shown in Figure 7. This $K_i$ value was slightly smaller than that observed in Helix neurons (1 $\mu$M),$^{38}$ but was much the same as observed in snail and rat neurons.$^{33,34}$

In the present experiments, the calcium concentration in the pipette was assumed to be [Ca$^{2+}$], and this value may differ from that obtained by direct measurement using a calcium-sensitive microelectrode.$^{14}$ We think that internal perfusion could achieve complete replacement of the intracellular fluid within 10 minutes as the amplitude of $I_c$ did not change subsequently and a good dose-response relation was observed.$^{11}$ Although the calculated [Ca$^{2+}$], may not be accurate, direct measurements by a calcium-sensitive electrode are also limited in their ability to estimate the real [Ca$^{2+}$], especially at concentrations lower than 0.1 $\mu$M, due to a poor seal around the electrode and to alterations in electrode responsiveness during measurement.$^{33}$ In any case, if 10 mM EGTA used here was insufficient to reduce [Ca$^{2+}$], the value of $K_i$ may be underestimated.

Low concentrations of [Ca$^{2+}$], (30 nM–1 $\mu$M) inhibited the Ca$^{2+}$ channel of smooth muscle membrane in the rabbit portal vein, in a dose-dependent manner, but even high concentrations of Ba$^{2+}$ (up to 100 $\mu$M) did not do so. Therefore, the inhibitory effects of [Ca$^{2+}$], on the Ca$^{2+}$ channel seem to be specific. Brown et al$^{35}$ found no inhibitory effect of increased [Ba$^{2+}$], (up to 1 mM) on the Ca$^{2+}$ channel in neurons.

One explanation for the mechanisms related to [Ca$^{2+}$],-dependent inactivation of the Ca$^{2+}$ channel was introduced by Chad and Eckert.$^{35}$ They proposed that inactivation of the $I_c$ may occur during dephosphorylation of a Ca$^{2+}$ channel-related protein and that the Ca$^{2+}$-dependent inactivation was the result of activation of a Ca$^{2+}$-dependent phosphatase. In such cases, the binding of Ca$^{2+}$ corresponds to the enzymatic reaction that occurs in certain biochemical processes. Ohya et al$^{38}$ reported that intracellular applications of ATP enhanced the $I_c$ with slight prolongation of the decay of the current in intestinal smooth muscle cells. Studies are underway to clarify the inactivation of the Ca$^{2+}$ channel in smooth muscle cells in relation to enzymatic processes.

Two types of Ca$^{2+}$ channels have been reported,$^{36-38}$ and in heart and dorsal root ganglion cells, only one of these is inactivated by a Ca$^{2+}$-dependent mechanism.$^{39,40}$ In the present study, as we did not find any evidence that suggested two types of Ca$^{2+}$ channels in the smooth muscle cells of rabbit portal vein, the possible contribution of two such Ca$^{2+}$ channels was not taken into consideration for measurement of the peak $I_c$.

In smooth muscle cells, the inactivation curve obtained from the double pulse study with a short conditioning pulse was U shaped,$^{33,34}$ and the inhibition of $I_c$ was closely related to the influx of Ca$^{2+}$ during the conditioning pulse. In the present experiments, increases in the concentration of EGTA suppressed the inhibitory effect induced by the conditioning pulse. These results are in good accord with a Ca$^{2+}$-dependent inactivation mechanism of the Ca$^{2+}$ channel. Increases in the concentration of EGTA in the cell may suppress the elevation of [Ca$^{2+}$], induced by conditioning pulses. The enhancement of the $I_c$ induced by increases in the concentration of EGTA in the pipette solution observed in the present study is probably due to decreases in the steady level of [Ca$^{2+}$].

The voltage-dependent inactivation mechanism of the Ca$^{2+}$ channel in smooth muscle cells was also elucidated from the following evidence: 1) The steady-state inactivation curve was initiated at the command potential where the $I_c$ was not generated (Figure 4). 2) When the conditioning pulse was sufficiently long, turn-up of the inactivation curve observed at high command-potential levels was suppressed in the double pulse study.$^{33}$ Inhibitions of the $I_c$ could not solely be explained quantitatively by the amount of influx of Ca$^{2+}$ induced by the conditioning in the double pulse study with a short conditioning pulse; that is, higher conditioning depolarization inhibited the $I_c$ to a greater extent even when the amount of Ca$^{2+}$ influx induced by the conditioning pulses was the same with higher and lower conditioning depolarizing pulses (Figure 5).

Single smooth muscle cells of the rabbit portal vein contracted with application of over 100 nM [Ca$^{2+}$], and the maximum shortening was observed at 1 $\mu$M. This Ca$^{2+}$ sensitivity of the mechanical response was much the same as that observed in skinned muscle tissues of the rabbit mesenteric artery determined using the isometric tension recording methods.$^{23,41}$ Therefore, the perfusion of Ca$^{2+}$, in a passive manner, into the cytosol from the pipette was considered to be adequate.

The [Ca$^{2+}$], at the resting state in vascular smooth muscle cells was about 100 nM, as estimated from experiments using Ca$^{2+}$-sensitive dyes, quin2 and fura2,$^{42,43}$ and also from the Ca$^{2+}$ sensitivity of skinned smooth muscle tissues.$^{23,40}$ Therefore, about 50% of the Ca$^{2+}$ channel may be inactivated during the resting state. The role of these inactivated Ca$^{2+}$ channels at the resting state, in relation to the contraction-relaxation cycle in the smooth muscle cells, has yet to be clearly defined. However, together with other factors, such as
the Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel,\textsuperscript{16} the intracellular Ca\textsuperscript{2+}-dependent inactivation may well function to prevent Ca\textsuperscript{2+} overload in the cytosol.

We conclude that Ca\textsuperscript{2+}-dependent inactivation of the Ca\textsuperscript{2+} channel contributes to physiological alterations of [Ca\textsuperscript{2+}], in smooth muscle cells of the rabbit portal vein.

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References


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20. Ohya Y, Terada K, Kitamura K, Kuriyama H: D600 blocks the Ca\textsuperscript{2+} channel from the outer surface of smooth muscle membrane of the rabbit intestine and portal vein. Pfugers Arch 1987;408:80–83
29. Harafujii H, Ogawa Y: Re-examination of the apparent binding constant of ethylene glycol bis (β-aminoethyl ether)–N,N,N',N'-tetraacetic acid with calcium around neutral pH. J Biochem (Tokyo) 1980;87:1305–1312
Ohya et al. (Ca⁺), Inactivates Iᵥ, in Vascular Muscles


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Regulation of calcium current by intracellular calcium in smooth muscle cells of rabbit portal vein.

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