Intracellular Ca^{2+} Inhibits Smooth Muscle L-Type Ca^{2+} Channels by Activation of Protein Phosphatase Type 2B and by Direct Interaction with the Channel

Klaus Schuhmann,* Christoph Romanin,‡ Werner Baumgartner,‡ and Klaus Groschner*

From the *Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, A-8010, Graz, Austria; and ‡Institut für Biophysik, Universität Linz, A-4010 Linz, Austria

ABSTRACT Modulation of L-type Ca^{2+} channels by tonic elevation of cytoplasmic Ca^{2+} was investigated in intact cells and inside-out patches from human umbilical vein smooth muscle. Ba^{2+} was used as charge carrier, and run down of Ca^{2+} channel activity in inside-out patches was prevented with calpastatin plus ATP. Increasing cytoplasmic Ca^{2+} in intact cells by elevation of extracellular Ca^{2+} in the presence of the ionophore A23187 inhibited the activity of L-type Ca^{2+} channels in cell-attached patches. Measurement of the actual level of intracellular free Ca^{2+} with fura-2 revealed a 50% inhibitory concentration (IC_{50}) of 260 nM and a Hill coefficient close to 4 for Ca^{2+}-dependent inhibition. Ca^{2+}-induced inhibition of Ca^{2+} channel activity in intact cells was due to a reduction of channel open probability and availability. Ca^{2+}-induced inhibition was not affected by the protein kinase inhibitor H-7 (10 μM) or the cytoskeleton disruptive agent cytochalasin B (20 μM), but prevented by cyclosporin A (1 μg/ml), an inhibitor of protein phosphatase 2B (calcineurin). Elevation of Ca^{2+} at the cytoplasmic side of inside-out patches inhibited Ca^{2+} channels with an IC_{50} of 2 μM and a Hill coefficient close to unity. Direct Ca^{2+}-dependent inhibition in cell-free patches was due to a reduction of open probability, whereas availability was barely affected. Application of purified protein phosphatase 2B (12 U/ml) to the cytoplasmic side of inside-out patches at a free Ca^{2+} concentration of 1 μM inhibited Ca^{2+} channel open probability and availability. Elevation of cytoplasmic Ca^{2+} in the presence of PP2B, suppressed channel activity in inside-out patches with an IC_{50} of ~380 nM and a Hill coefficient of ~3; i.e., characteristics reminiscent of the Ca^{2+} sensitivity of Ca^{2+} channels in intact cells. Our results suggest that L-type Ca^{2+} channels of smooth muscle are controlled by two Ca^{2+}-dependent negative feedback mechanisms. These mechanisms are based on (*a*) a protein phosphatase 2B-mediated dephosphorylation process, and (*b*) the interaction of intracellular Ca^{2+} with a single membrane-associated site that may reside on the channel protein itself.

KEY WORDS: L-type Ca^{2+} channels • gating • protein phosphatase 2B • vascular smooth muscle • patch clamp

INTRODUCTION

The function of L-type Ca^{2+} channels is controlled by voltage as well as by the Ca^{2+} influx through the channel (Kass and Sanguinetti, 1984; Eckert and Chad, 1984; Lee et al., 1985). An inactivation process is induced by the charge carrier Ca^{2+} itself, providing a negative feedback mechanism that is considered crucial for Ca^{2+} homeostasis in a variety of cell types, including smooth muscle cells (Jmari et al., 1986; Granitkevich et al., 1987; Granitkevich and Isenberg, 1991). There is an ongoing debate as to whether Ca^{2+} induces channel inactivation via direct binding to proteins of the channel complex or via a more remote mechanism such as Ca^{2+}-dependent dephosphorylation (Chad and Eckert, 1986; Armstrong, 1989) or disruption of the cells' cytoskeleton (Johnson and Byerly, 1993). Inhibition of L-type channels by cytosolic Ca^{2+} has been demonstrated in cell-free membranes, supporting the view of a rather direct inhibitory mechanism such as interaction of Ca^{2+} with the channel (Romanin et al., 1992; Haack and Rosenberg, 1994; Schmid et al., 1995). Recent evidence suggests that Ca^{2+}-induced inactivation is a property of the pore-forming α1 subunit of the Ca^{2+} channel (Zong and Hofmann, 1996), and an EF-hand Ca^{2+} binding motif in the α1 subunit has recently been identified as a structure that is essential for Ca^{2+}-dependent inactivation (De Leon et al., 1995). Nonetheless, different lines of investigations (Ohya et al., 1988; Hirano and Hiraoka, 1994; You et al., 1995) support the hypothesis of a more complex Ca^{2+}-dependent control of L-type Ca^{2+} channels, involving Ca^{2+}-dependent enzymatic mechanisms in addition to direct binding of Ca^{2+}

Address correspondence to Dr. K. Groschner, Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010, Graz, Austria. Fax: 316-380-9890; E-mail: Klaus.Groschner@kfunigraz.ac.at

to the channel. Both stimulatory (Hirano and Hiraoka, 1994) and inhibitory modulation (Ohya et al., 1988; Hirano and Hiraoka, 1994; You et al., 1995) of Ca^{2+} channels has been observed in response to increases in bulk cytoplasmic Ca^{2+} to levels lower than the concentrations reported for inhibition of L-type Ca^{2+} channels in cell-free membranes (Romanin et al., 1992; Haack and Rosenberg, 1994; Schmid et al., 1995). Candidate mechanisms for a remote control of L-type Ca^{2+} channels in cardiac and smooth muscle are Ca^{2+}-dependent phosphorylation and dephosphorylation (Chad and Eckert, 1986; Armstrong, 1989; Hirano and Hiraoka, 1994), as well as Ca^{2+}-dependent disruption of the cytoskeleton (Johnson and Byerly, 1993, 1994).

The present study was aimed at comparing Ca^{2+}-dependent modulation of smooth muscle L-type Ca^{2+} channels in intact cells and cell-free patches and to test for a possible role of phosphorylation/dephosphorylation and modification of the cytoskeleton in intact cells. We present evidence for the control of L-type Ca^{2+} channels in vascular smooth muscle cells by two Ca^{2+}-dependent mechanisms that comprise both dephosphorylation by protein phosphatase 2B and direct binding of Ca^{2+} to a membrane-associated site.

MATERIALS AND METHODS

Cell Preparation

The media of human umbilical veins was enzymatically disaggregated to obtain single smooth muscle cells as described previously (Schuhmann and Groschner, 1994). In brief, endothelial cells were removed with dispase (type II; Boehringer Mannheim, Mannheim, Germany), and smooth muscle tissue was subsequently dissociated by filling the vessels with low Ca^{2+} (0.1 mM) Hanks buffer (Sera Lab Ltd., Sussex, UK), supplemented with 0.5 mg/ml collagenase (type II; Worthington Biochemical Corp., Freehold, NJ), 0.5 mg/ml trypsin inhibitor (Worthington Biochemical Corp.), and 1 mg/ml fatty acid–free bovine serum albumin. Vessels filled with collagenase containing Hanks solution were incubated at 37°C. After incubation for 10 min, the Hanks buffer contained single, mostly relaxed, elongated smooth muscle cells that were harvested by centrifugation (5 min, 250 *g*). The cells were resuspended and stored in a solution containing (mM): 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA (pCa = 7, see below) at 4°C, and used for experimentation within 36 h.

Measurement of Single-channel Currents

Cell potentials were set to approximately zero by use of a high K^+ low Cl^- extracellular solution that contained (mM): 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA, pH was adjusted to 7.4 with *N*-methyl-D-glucamine, and pCa was adjusted using a Ca^{2+}-sensitive electrode. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK), and had resistances of 5–10 MOhm. For recording of Ba^{2+} currents through single Ca^{2+} channels, the pipettes were filled with a solution containing (mM): 10 $BaCl_2$, 100 NaCl, 30 TEA-Cl, and 15 HEPES, pH adjusted to 7.4. The dihydropyridine-Ca^{2+} channel activator S(−)-BayK 8644 (0.5 μM) was included in the pipette solution to facilitate stabilization of channel activity in inside-out patches. Run down of Ca^{2+} channel activity in inside-out experiments was prevented by addition of calpastatin (2 U/ml) plus 1 mM ATP/Na_2 to the bath solution before patch excision (Romanin et al., 1992). In experiments recording Ca^{2+}-activated K^+ channels, the pipette solution contained (mM): 137 NaCl, 5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH was adjusted to 7.4. All experiments were performed at room temperature. Exchange of bath solutions and administration of drugs was performed during constant flow perfusion.

Voltage clamp and current amplification was performed with a patch-clamp amplifier (EPC/7; List, Darmstadt, Germany). Current records were filtered at 1 kHz (−3 dB) and digitized at a rate of 5 kHz. Experiments were controlled using pClamp software (Axon Instruments, Foster City, CA). For idealization of current records, a custom-made level detection software was employed (Pastushenko and Schindler, 1997). Ca^{2+} channel activity was calculated as the mean number of open channels during depolarizing pulses. The dependency of channel activity on cytoplasmic free Ca^{2+} concentration was characterized in terms of 50% inhibitory concentration (IC_{50} values)[1] and Hill coefficients by fitting the data with a four-parameter logistic function (De Lean et al., 1978). The gating properties of single channels were analyzed in terms of channel availability P_s; i.e., the probability that a channel will open upon depolarization, as well as the open probability of available channels as outlined below.

Determination of Availability and Open Probability

Recently, a method was developed (Schmid et al., 1995; Baumgartner et al., 1997) to allow for an estimation of the number of channels, their availability P_s and open probability P_o from idealized channel traces with $n < 9$ channels based on the following assumptions: (*a*) the channels are identical and behave independently; (*b*) the total number of channels remains constant within the duration of the recording; and (*c*) the process regulating channels' gating within a sweep is distribution ergodic with respect to the number of sampling points in each conductance level. Hence, the probability of finding a sampling point in the k^{th} conductance level can be described by a binomial distribution with the parameters n_A and P_o.

$$p(k) = \binom{n_A}{k} \cdot P_o^{\,k} \cdot (1 - P_o)^{n_A - k} \quad (1)$$

Eq. 1 and assumption *c* allow us to determine the probability for one sweep (Eq. 2) at given n_A with the following variables defined as: n, maximum number of channels in a patch; n_{Ai}, available channels in the i^{th} sweep; t_{ik}, number of sampling points in the k^{th} conductance level in the i^{th} sweep; T_i, vector of t_{ik} for the i^{th} sweep; T, matrix of all t_{ik}; T_{ges}, number of sampling points in a sweep; P_s, availability; P_o, open probability; M, total number of sweeps; K_i, set of conductance levels that occur in the i^{th} sweep.

The elements of T_i that describe the i^{th} sweep obey a multinomial distribution (Weiß, 1987) and the probability of T_i at given n_{Ai} and P_o is

$$p(T_i \mid n_{Ai}, P_o) = \left\{ \prod_{k=0}^{n_{Ai}} \left[\binom{n_{Ai}}{k} \cdot P_o^{\,k} \cdot (1 - P_o)^{n_{Ai} - k} \right]^{t_{ik}} \right\} \frac{T_{ges}!}{\prod_{k \in K_i} t_{ik}!} \quad (2)$$

if n_{Ai} is higher than or equal to the highest conductance level in T_i. If n_{Ai} is smaller than the highest conductance level, then

[1] *Abbreviations used in this paper:* IC_{50}, 50% inhibitory concentration; pNPP, *p*-nitrophenyl phosphate.

$$p(T_i|n_{Ai}, P_o) = 0 \quad (3)$$

Because of assumptions *a* and *b*, the n_{Ai} are binomially distributed, yielding the probability for T_i at given n, P_S, and P_o to be

$$p(T_i|n, P_S, p) = \sum_{n_{Ai}=0}^{n} P_o(T_i|n_{Ai}, P_o) \cdot \binom{n}{n_{Ai}} \cdot P_S^{n_{Ai}} \cdot (1-P_S)^{n-n_{Ai}} \quad (4)$$

leading to the probability for T comprising all sweeps

$$p(T|n, P_S, p) = \prod_{i=1}^{M} P_o(T_i|n, P_S, P_o) \quad (5)$$

Using Eq. 5, a maximum likelihood estimator for n, P_S, and P_o was constructed by maximizing the probability for T.

Measurement of Intracellular Concentrations of Ca^{2+} and H^+

Cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) and intracellular pH (pH_i) were determined using the fluorescent Ca^{2+} and pH indicators fura-2 and BCECF ((2′,7′)-bis(carboxyethyl)-(5,6)-carboxyfluorescein), as described by Wakabayashi and Groschner (1996). In brief, cells were suspended in 5 ml of physiological solution containing (mM): 137 NaCl, 5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4, and loaded with fura-2 or BCECF for 60 min at 37°C. After loading, the cells were once washed and resuspended in high K^+ low Cl^- extracellular solution (see above). Fluorescence measurements were carried out using a dual wavelength spectrophotofluorometer (F2000; Hitachi Ltd., Tokyo, Japan). The cell suspension (2 ml) was stirred and maintained at room temperature. For $[Ca^{2+}]_i$ measurement, excitation wavelengths were 340 and 380 nm, and emission was collected at 510 nm. For pH_i measurement, excitation wavelengths were 506 and 455 nm, and emission was collected at 530 nm. Using the ratios (R) of fluorescence intensity (F), F_{340}/F_{380} and F_{506}/F_{455}, the fractional changes in $[Ca^{2+}]_i$ and pH_i were determined, respectively. Fluorescence after sequential addition of 0.1% Triton X-100 and EGTA to the cell suspension provided the respective maximum fluorescence ratio (R_{max}) and minimum fluorescence ratio (R_{min}). $[Ca^{2+}]_i$ was calculated as described (Wakabayashi and Groschner, 1996). Calibration of pH_i measurements were performed using nigericin (7 μM)-containing high K^+ solution at various extracellular pH_i values (pH_o).

Assay of p-Nitrophenyl Phosphate Phosphatase Activity

The activity of protein phosphatase 2B was measured employing *p*-nitrophenyl phosphate (pNPP) as substrate as described (Takai and Mieskes, 1991). 1 U phosphatase activity was the amount that catalyzed dephosphorylation of 1 mmol pNPP/min.

Statistics

Averaged data are given as mean ± SEM from the indicated number of experiments. Statistical analysis was performed using Student's *t* test for unpaired values. Differences were considered statistically significant at $P < 0.05$.

Materials

PP2B was obtained from Upstate Biotechnology Inc. (Lake Placid, NY), collagenase, type CLS II, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., dispase type II was from Boehringer Mannheim, fatty acid–free bovine serum albumin was from Behring (Marburg, Germany), Hanks balanced salt solution was from Sera Lab Ltd., H-7, cytochalasin B, and cyclosporin A were from Research Biochemicals, Inc. (Natic, MA), and calpastatin and all other chemicals were from Sigma Chemical Co. (Deisenhofen, Germany). Calpastatin was dialyzed overnight against bath solutions (high K^+ low Cl^- solutions, see above).

RESULTS

Ca^{2+}-dependent Inhibition of L-Type Ca^{2+} Channels in Intact Cells

Ca^{2+}-dependent modulation of L-type Ca^{2+} channels in intact cells was studied by raising intracelluar Ca^{2+} of the cells via elevation of extracellular Ca^{2+} in the presence of the Ca^{2+} ionophore A23187 (1 μM). A typical experiment is illustrated in Fig. 1. The cell was initially bathed in a solution containing ~10 nM free Ca^{2+} (pCa 8). A23187 by itself did not affect channel activity in the cell-attached patch under these conditions. Extracellular Ca^{2+} was increased in the presence of A23187 to 10 μM, and subsequently to 100 μM. Channel activity was barely effected at 10 μM extracellular Ca^{2+}, but clearly suppressed when Ca^{2+} of the bath solution was raised to 100 μM, and activity recovered partially during a following period of reduction of extracellular Ca^{2+}. The actual level of average cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) obtained during elevation of extracellular Ca^{2+} was measured in parallel experiments using the Ca^{2+}-sensitive fluorescent dye fura-2. As shown in Fig. 2 *A*, $[Ca^{2+}]_i$ increased barely upon application of the Ca^{2+} ionophore at 10 nM free extracellular Ca^{2+}; i.e., an extracellular pCa (pCa_o) of 8. As expected from the ability of A23187 to deplete intracellular Ca^{2+} stores, a small and transient rise in $[Ca^{2+}]_i$ was observed occasionally. During elevation of extracellular Ca^{2+} to 10 μM, $[Ca^{2+}]_i$ increased rapidly from a resting level of ~50 nM to 216 ± 19 nM ($n = 7$). Upon further elevation of extracellular Ca^{2+} to 100 μM, $[Ca^{2+}]_i$ increased to a level of 326 ± 14 nM (~pCa_i 6.5, $n = 7$). These values of $[Ca^{2+}]_i$ did not change significantly within a period of 2–4 min after elevation of extracellular Ca^{2+}. To obtain additional information on the actual levels of $[Ca^{2+}]_i$ at the cytoplasmic face of the plasma membrane of single cells, we measured the activity of large conductance Ca^{2+}-activated ("maxi") K^+ channels, which are known to exhibit a typical Ca^{2+} dependence in the low micromolar range. Fig. 2 *B* shows a representative recording of maxi-K^+ channel activity under conditions corresponding to those of the Ca^{2+} channel recordings illustrated in Fig. 1. Fig. 2 *B* (*left*) illustrates K^+ channel activity in a cell-attached patch recorded at pCa_o 8 (*top*) and pCa_o 4 (*bottom*) in the presence of 1 μM A23187. It is clearly evident that channel activity increased only slightly when extracellular Ca^{2+} was raised to 100 μM (pCa_o 4). Fig. 2 *B* (*right*) illustrates the Ca^{2+} sensitivity of this K^+ channel in the inside-out configu-

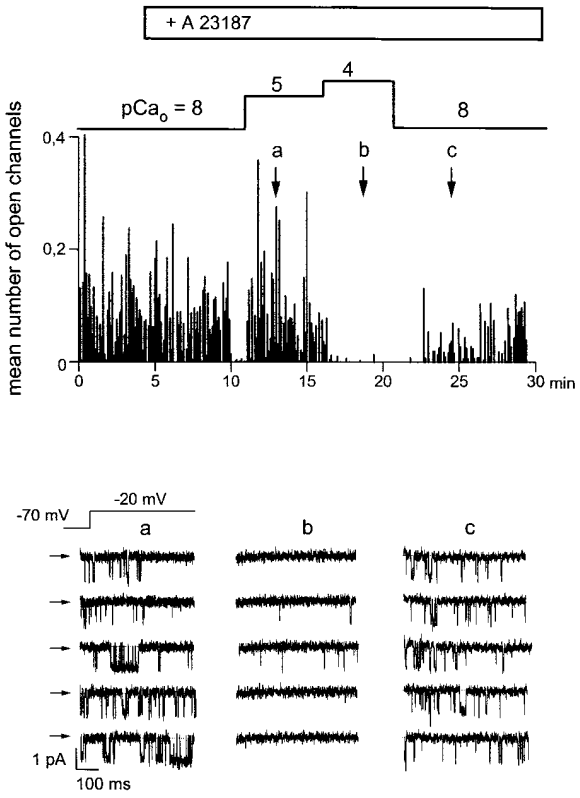

FIGURE 1. Elevation of extracellular Ca^{2+} in the presence of A23187 inhibits the activity of L-type Ca^{2+} channels in cell-attached patches. Time course of Ca^{2+} channel activity recorded in a cell-attached patch given as the mean number of open channels during individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). Administration of 1 μM A23187 and changes in the extracellular Ca^{2+} concentration, given as pCa_o values, are indicated. (*bottom*) Individual current responses to depolarizing voltage pulses at the times indicated. Records were filtered at 500 Hz and digitized at 2 kHz. Closed state is indicated by arrows.

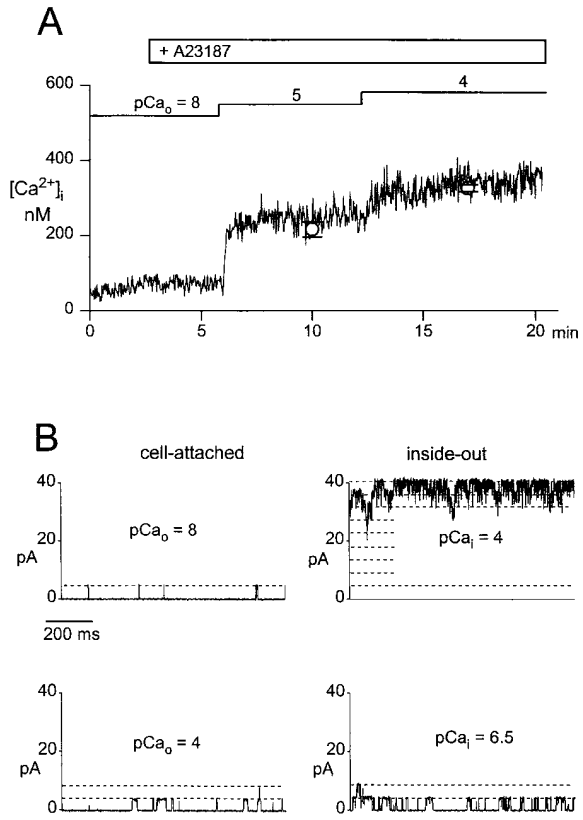

FIGURE 2. Determination of intracellular Ca^{2+} levels during elevation of extracellular Ca^{2+} in the presence of A23187. (*A*) Time course of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) during elevation of extracellular Ca^{2+} in the presence of 1 μM A23187 measured by fura-2 fluorescence. Administration of 1 μM A23187 and changes in the extracellular Ca^{2+} concentration, given as pCa_o values, are indicated. Mean values ± SEM (n = 7) of $[Ca^{2+}]_i$ determined for the period of 2–4 min after elevation of extracellular Ca^{2+} to the respective value, are shown for pCa_o 5 and 4. (*B, left*) Activity of Ca^{2+}-activated K^+ channels in a cell-attached patch (test potential = 0 mV) during elevation of extracellular Ca^{2+}, given as pCa_o values, in the presence of 1 μM A23187. (*right*) Channel activity in the same patch after excision (inside-out configuration) at pCa 4 (100 μM) and pCa 6.5 (300 nM). Records were filtered at 500 Hz and digitized at 2 kHz. Unitary current levels are indicated by dashed lines.

ration. Channel activity is shown after excision of the patch into the bath solution of pCa 4. At the cytoplasmic side, this Ca^{2+} concentration caused full activation of the K^+ channels. Reduction of the free Ca^{2+} concentration at the cytoplasmic side to 300 nM (pCa_i 6.5) diminished channel activity to a level comparable with that recorded in the cell-attached configuration at elevated extracellular Ca^{2+} (pCa_o 4, Fig. 2 *B, left*). In all of three experiments, the activity of maxi-K^+ channels recorded at pCa_i 6.5 in inside-out patches was equal to or even somewhat higher than the activity observed in intact cells during a challenge with elevated Ca^{2+} (pCa_o 4) in the presence of A23187. Thus, the measurement of maxi-K^+ channel activity in single cells was consistent with our determination of Ca_i by measurement of fura-2 fluorescence, confirming that Ca^{2+}-dependent inhibition of L-type Ca^{2+} channels in intact cells indeed occurred at a level of $[Ca^{2+}]_i$ as low as ~300 nM. Fig. 3 *A* shows the concentration dependence obtained for Ca^{2+}-induced inhibition of L-type Ca^{2+} channels in intact cells using the $[Ca^{2+}]_i$ values determined with fura-2. The IC_{50} value was 260 nM and a Hill coefficient (n_H) of ~4 (3.9) was calculated.

Since Ca^{2+} channel activity in smooth muscle is determined by intracellular pH (Klöckner and Isenberg, 1994), we first tested whether changes in intracellular pH might account for the inhibitory effects observed in intact cells. Using BCECF as a pH indicator, we found that pH_i did not change significantly upon administration of A23187 and subsequent elevation of extracellular Ca^{2+}. A pH_i of 7.41 ± 0.16 (n = 9) in controls and 7.40 ± 0.11 (n = 4) in the presence of A23187 plus 100 μM Ca^{2+} was measured.

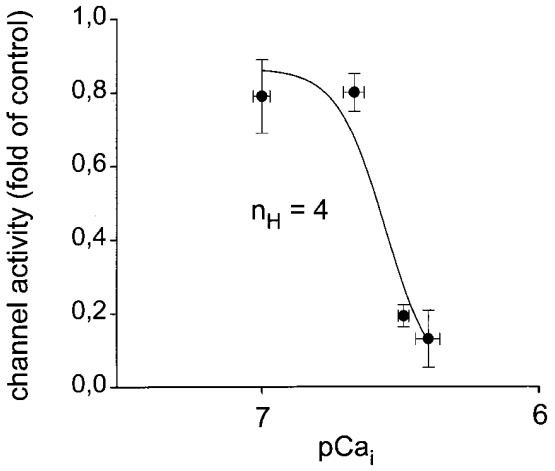

FIGURE 3. Concentration dependence of Ca^{2+}-induced inhibition of Ca^{2+} channel activity in cell-attached patches. Mean values ± SEM of channel activity obtained from two to four experiments. Data were fitted by a four-parameter logistic function, and the derived Hill coefficient (n_H) is given.

We have recently reported on a mechanism of Ca^{2+} channel inhibition that involves Ca^{2+}-phospholipid-dependent protein kinases (Schuhmann and Groschner, 1994); thus, we tested whether the protein kinase inhibitor H-7 is able to blunt Ca^{2+}-dependent inhibition of channels in intact cells. H-7 (10 μM) did not affect the inhibitory modulation in intact cells ($n = 4$). Similarly, cytochalasin B, a cytoskeletal disrupter, failed to suppress Ca^{2+}-dependent inhibition of L-type channels ($n = 3$).

Cyclosporin A Prevents Ca^{2+}-dependent Inhibition of Ca^{2+} Channels in Intact Cells

A role of Ca^{2+}-dependent dephosphorylation has previously been implicated in Ca^{2+}-induced negative feedback mechanisms (Armstrong, 1989). Thus, we tested cyclosporin A as an inhibitor of the Ca^{2+}-dependent protein phosphatase 2B (calcineurin). Elevation of extracellular Ca^{2+} from 10 nM to 100 μM (pCa_o 4) in the presence of 1 μM A23187 resulted in an increase in intracellular free Ca^{2+} to ~300 nM (pCa_i 6.5, Fig. 2) and suppressed channel activity substantially within 1 min (Fig. 4 *A*). However, when cells were pretreated with cyclosporin A (1 μg/ml), channel activity remained constant even during prolonged elevation of intracellular Ca^{2+} (Fig. 4 *B*; $n = 3$). Removal of cyclosporin A resulted in the expected inhibition of Ca^{2+} channel activity, which recovered upon reduction of extracellular Ca^{2+} to pCa 8 (Fig. 4 *B*). Cyclosporin A by itself did not affect channel activity significantly under basal conditions ($n = 3$). Experiments with fura-2 confirmed that elevation of $[Ca^{2+}]_i$ induced by extracellular Ca^{2+} in the presence of A23187 remained unchanged by cyclosporin A ($n = 3$). Thus, cyclosporin A specifically antagonized Ca^{2+}-induced suppression of channel activity. These results demonstrate that cyclosporin A interferes with Ca^{2+}-dependent negative feedback control of

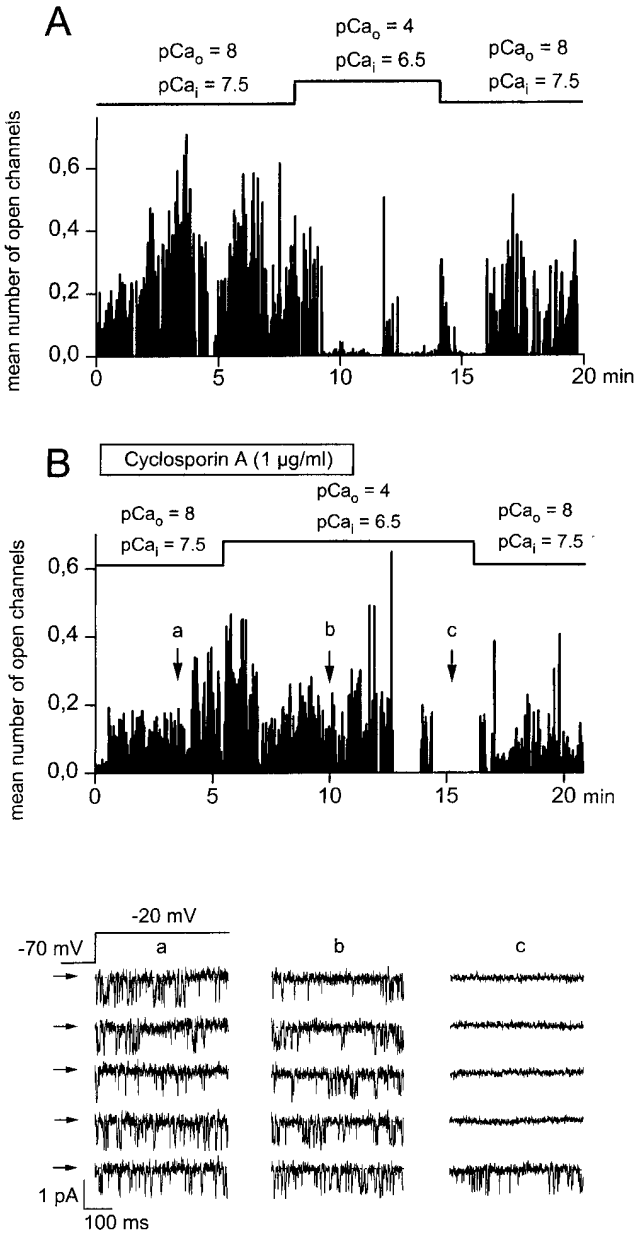

FIGURE 4. Cyclosporin A prevents Ca^{2+}-induced inhibition of Ca^{2+} channels in intact cells. Time courses of Ca^{2+} channel activity recorded in cell-attached patches. A23187 (1 μM) is present throughout the experiments. Changes in the extra- and intracellular Ca^{2+} concentration are given as pCa values (pCa_o and pCa_i). (*A*) Control experiment. (*B*) Experiment in the presence of cyclosporin A (1 μg/ml; 10 min). Channel activity is given as the mean number of open channels during individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). (*bottom*) Individual current responses to depolarizing voltage pulses at the times indicated. Records were filtered at 500 Hz and digitized at 2 kHz. Closed state is indicated by arrows.

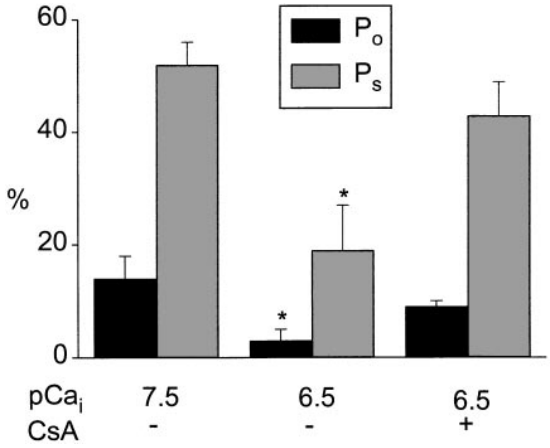

FIGURE 5. Cyclosporin A prevents Ca^{2+}-induced inhibition of open probability (P_o) and availability (P_s) of channels in intact cells. P_o and P_s of channels are shown under control conditions (pCa_i 8.5) and at elevated cytoplasmic Ca^{2+} (pCa_i 6.5) in the absence and presence of cyclosporin A (1 μg/ml). Mean values ± SEM ($n = 4$) are given. *Significant difference versus control values.

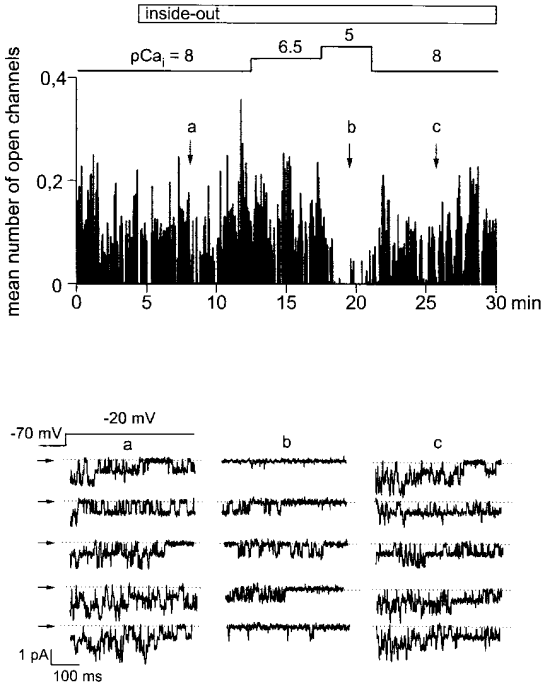

FIGURE 6. Ca^{2+}-induced inhibition of Ca^{2+} channels in cell-free patches. Time course of Ca^{2+} channel activity in the cell-attached and inside-out configuration, given as the mean number of open channels during individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). Patch excision and subsequent changes in the Ca^{2+} concentration at the cytoplasmic side (pCa_i) are indicated. (*bottom*) Individual current responses to depolarizing voltage pulses at the times indicated. Records were filtered at 500 Hz and digitized at 2 kHz. Closed state is indicated by arrows.

Ca^{2+} channels in intact smooth muscle cells, and indicate a role of protein phosphatase 2B, the classical target of cyclosporin A.

In an attempt to further characterize the cyclosporin A–sensitive effect of cytoplasmic Ca^{2+}, we evaluated the effects of Ca^{2+} on open probability (P_o) and availability (P_s); i.e., the probability for a channel to open once during a depolarization. Using a recently developed procedure that allows for determination of these parameters from multichannel records (Schmid et al., 1995), we found that Ca^{2+}-induced suppression of channel activity in intact cells was associated with a reduction of P_o and in addition a marked reduction of P_s. These effects were clearly antagonized by cyclosporin A (Fig. 5).

Inhibition of L-Type Ca^{2+} Channels in Cell-free Patches by Ca^{2+} and Protein Phosphatase 2B

Ca^{2+} channel activity in excised, inside-out patches remained stable over more than 30 min when the cytoplasmic side was exposed to a solution containing calpastatin (2 U/ml) and ATP (1 mM) (Romanin et al., 1992; Schmid et al., 1995). This experimental protocol allowed for investigation of the sensitivity of smooth muscle L-type Ca^{2+} channels to cytoplasmic Ca^{2+} as well as to dephosphorylation by protein phosphatase 2B. As a first step, we studied channel inhibition by Ca^{2+} itself. As illustrated in Fig. 6, Ca^{2+} channels recorded in excised patches were apparently not affected when the Ca^{2+} concentration of the solution facing the cytoplasmic side was increased to 300 nM (pCa_i 6.5), but significantly inhibited at 10 μM free Ca^{2+} (pCa_i 5). This effect was rapidly reversible upon subsequent reduction of the free Ca^{2+} concentration to 10 nM (pCa_i 8). Fig. 7 depicts the concentration dependence obtained for Ca^{2+}-induced inhibition of L-type Ca^{2+} channels in excised patches. Inhibition was characterized by an IC_{50} value of 2 μM and a Hill coefficient (n_H) close to unity (1.1).

Since our experiments with cyclosporin A indicated a role of PP2B in Ca^{2+}-dependent downregulation of Ca^{2+} channels in intact cells, the next step was to test whether purified PP2B is able to suppress Ca^{2+} channel activity in excised patches. The effects of PP2B were studied in the presence of 1 μM calmodulin. Fig. 8 illustrates that administration of purified PP2B (1 μg/ml) to the cyctoplasmic side of Ca^{2+} channels in inside-out patches failed to affect channel activity at pCa 8, but inhibited channels at pCa 6 within 1–2 min to a level below 10% of control ($n = 3$). Administration of 1 μM calmodulin alone at pCa 6 did not affect channel activity ($n = 3$). The concentration dependence of Ca^{2+}-induced channel inhibition in the presence of PP2B (1 μg/ml) is illustrated in Fig. 9. Elevation of cytoplasmic Ca^{2+} in the presence of PP2B inhibited Ca^{2+} channel activity in inside-out patches with an IC_{50} of 379 nM and a Hill coefficient (n_H) of ~3 (3.1). Consis-

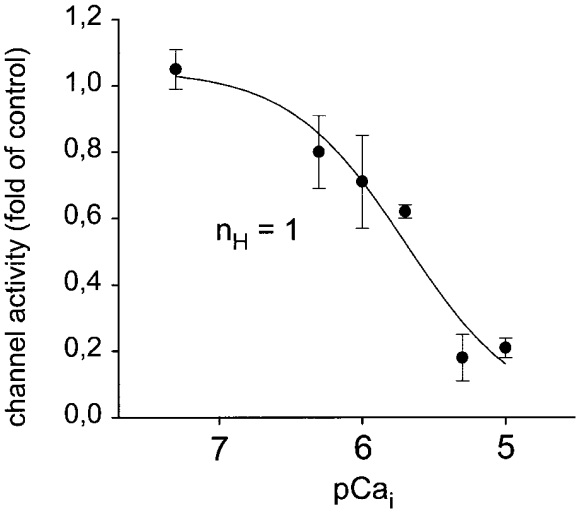

FIGURE 7. Concentration dependence of Ca^{2+}-induced inhibition of Ca^{2+} channel activity in excised inside-out patches. Mean values ± SEM of channel activity obtained from two to four experiments. Data were fitted by a four parameter logistic function, and the derived Hill coefficient (n_H) is given.

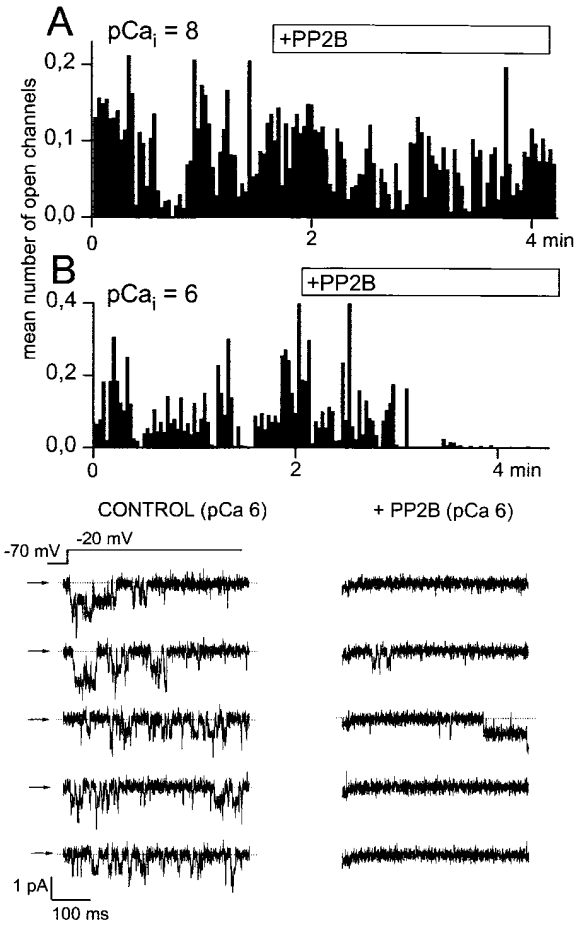

FIGURE 8. PP2B-induced inhibition of Ca^{2+} channels in cell-free patches. Time courses of Ca^{2+} channel activity recorded in inside-out patches at a pCa_i of 8 (*A*) and 6 (*B*). Addition of PP2B (1 μg/ml) to the cytoplasmic side is indicated. Channel activity is given as the mean number of open channels during individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). (*B, bottom*) Individual current responses to depolarizing voltage pulses in the absence and presence of PP2B (12 U/ml). Records were filtered at 500 Hz and digitized at 2 kHz. Closed state is indicated by arrows.

tent with the idea of PP2B effects being mediated by protein dephosphorylation, PP2B phosphatase activity was enhanced by Ca^{2+} in the same range of concentrations, with half-maximal stimulation at 430 nM free Ca^{2+} and a Hill coefficient of 4.5 ($n = 4$). To test whether downregulation of Ca^{2+} channels by PP2B is due to an impairment of Ca^{2+} channel stabilization by calpastatin, we preincubated calpastatin for 10 min with PP2B (1 μg/ml) plus calmodulin (1 μM) at pCa 6. This preincubation did not affect the ability of calpastatin to stabilize Ca^{2+} channel activity in excised patches ($n = 2$).

To further characterize Ca^{2+}- and PP2B-mediated modulation of channels in excised patches, we analyzed channel function in terms of P_o and P_s. As shown in Fig. 10, elevation of cytoplasmic Ca^{2+} to 1 μM (pCa_i 6) barely reduced P_o and P_s in the absence of PP2B. Further elevation of cytoplasmic Ca^{2+} to 10 μM (pCa_i 5) inhibited channel activity mainly by suppression of P_o. Thus, direct inhibition of Ca^{2+} channels in excised patches by cytoplasmic Ca^{2+} concentrations >1 μM was different from Ca^{2+}-dependent inhibition in intact cells, which was associated with a reduction of both P_o and P_s. The Ca^{2+}-dependent effect of PP2B in excised patches was characterized by a substantial reduction of P_o and P_s. Thus, channel modulation by PP2B mimicked Ca^{2+}-dependent inhibition in intact smooth muscle cells. These results demonstrate two types of Ca^{2+}-dependent downregulation of L-type Ca^{2+} channels in smooth muscle. Elevation of intracellular Ca^{2+} by itself inhibits specifically P_o of the channel, whereas in the presence of calmodulin plus PP2B, elevation of Ca^{2+} induces suppression of both P_o and P_s.

DISCUSSION

The present study provides evidence for the existence of two distinct mechanisms by which intracellular Ca^{2+} governs L-type Ca^{2+} channel function in smooth muscle. These Ca^{2+}-mediated mechanisms exhibit different concentration dependencies and are based on different changes in single-channel properties. Our results suggest that intracellular Ca^{2+} inhibits smooth muscle L-type Ca^{2+} channels by induction of a dephosphorylation process mediated by PP2B and by direct binding to a membrane-associated regulatory site.

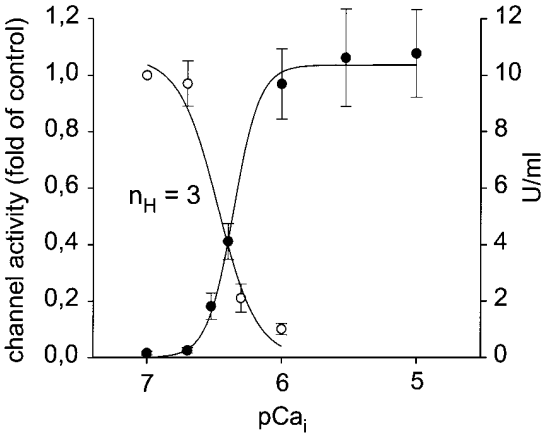

FIGURE 9. Concentration dependence of Ca^{2+}-induced inhibition of Ca^{2+} channel activity in excised inside-out patches and Ca^{2+}-induced stimulation of PP2B phosphatase activity. Mean values ± SEM of channel activity (○; n = 3–4) and of PP2B phosphatase activity (●; n = 5) measured in parallel with pNPP as substrate. Data were fitted by four-parameter logistic functions and the derived Hill coefficient (n_H) of Ca^{2+}-dependent inhibition of channel activity is given.

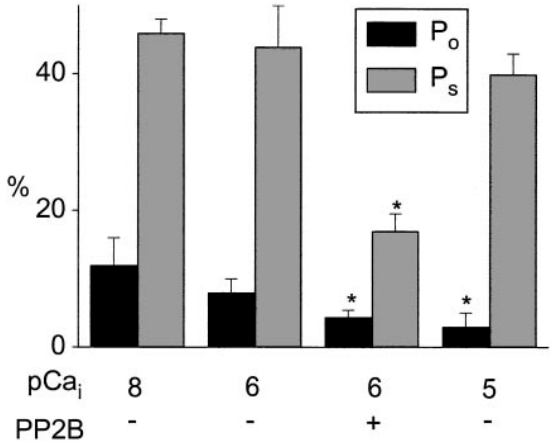

FIGURE 10. PP2B inhibits open probability (P_o) and availability (P_s) of Ca^{2+} channels in cell-free patches. P_o and P_s of channels in inside-out patches are shown at different cytoplasmic Ca^{2+} (given as pCa) in the absence or presence of PP2B (12 U/ml). Mean values ± SEM (n = 3–4) are given. *Significant difference versus control values.

Ca^{2+} Sensitivity of Smooth Muscle L-Type Ca^{2+} Channels in Intact Cells and Cell-free Patches

In the present study, Ca^{2+} channel function was measured with the charge carrier Ba^{2+}, which fails to mimic the negative feedback inhibition mediated by Ca^{2+}. Consequently, the use of Ba^{2+} as charge carrier allows us to determine the dependency of channel inhibition on steady state Ca^{2+} concentrations at the cytoplasmic side. It is of note that this approach does not allow us to evaluate the effects of Ca^{2+} permeation through the pore on channel function. Nonetheless, this approach allows for a detailed characterization of regulatory Ca^{2+} interaction sites at the cytoplasmic face of the membrane and of Ca^{2+}-dependent intracellular regulatory mechanisms. We demonstrate that L-type Ca^{2+} channels exhibit different Ca^{2+} sensitivities in intact cells and cell-free patches. The observed Ca^{2+} sensitivity of smooth muscle channels in inside-out patches (IC_{50} = 2 μM) is in accordance with reports on the Ca^{2+} sensitivity of cardiac L-type channels in excised patches (Romanin et al., 1992) and in planar lipid bilayers (Haack and Rosenberg, 1994). In a previous study on the Ca^{2+} sensitivity of L-type channels in excised patches of rat mesenteric artery, inhibition of Ca^{2+} channels required millimolar concentrations of cytoplasmic Ca^{2+}, suggesting a lower Ca^{2+} sensitivity of the channel (Huang et al., 1989). The reason for this discrepancy is unclear.

In the present study, a Hill coefficient close to 1 was calculated for the inhibition observed in cell-free patches, indicating interaction of Ca^{2+} with a single target site. This finding is in line with the idea that inhibition in excised patches is due to a direct interaction of Ca^{2+} with a binding site on the α1 subunit of the channel, which is a single EF-hand motif present both in cardiac and in smooth muscle channels (De Leon et al., 1995). In intact cells, accurate determination of the actual intracellular concentration of Ca^{2+}, in particular at the cell membrane, is difficult. We have therefore employed two independent methods to estimate the level of intracellular free Ca^{2+}; i.e., fura-2 fluorescence and open probability of Ca^{2+}-activated K^+ channels. Both methods yielded consistent results. When intracellular Ca^{2+} was elevated by exposure of the cells to A23187 plus 100 μM extracellular Ca^{2+}, an intracellular Ca^{2+} concentration of ~300 nM was obtained and Ca^{2+} channel activity was significantly suppressed. This result is in agreement with a previous study demonstrating that whole-cell Ba^{2+} currents in smooth muscle cells are inhibited when the cells' cytoplasmic Ca^{2+} concentration is elevated in the high nanomolar range (Ohya et al., 1988). Similarly, in cardiac muscle, a mechanism of Ca^{2+}-dependent inhibition of L-type channels was detected that apparently required a rather modest rise in intracellular Ca^{2+}; i.e., to levels below 1 μM (Ohya et al., 1988). These reports on inhibition of L-type channels in intact cells at submicromolar concentrations of cytoplasmic Ca^{2+} are in clear contrast to the observation that Ca^{2+}-dependent inhibition of Ca^{2+} channels in excised patches was found to require concentrations above 1 μM (Romanin et al., 1992; Schmid et al., 1995). Thus, in intact cells, Ca^{2+}-dependent inhibition occurred at about one order of magnitude lower concentrations with a substantially steeper ($n_H \cong 4$) concentration dependence than in excised patches. These data suggest that elevation of bulk cytoplasmic Ca^{2+} up

to the low micromolar range is able to suppress Ca^{2+} entry through L-type Ca^{2+} channels in intact smooth muscle cells via a rather complex cellular mechanism, while local elevation of Ca^{2+} at the cytoplasmic face of the channel above 1 μM induces inactivation via interaction with a single target site.

Role of Protein Phosphatase Type 2B

In an attempt to identify the Ca^{2+}-dependent mechanism that suppresses Ca^{2+} channel activity in intact cells, we tested the involvement of putative indirect pathways. Up to now, several Ca^{2+}-dependent signaling pathways have been implicated in the control of Ca^{2+} channel function. Our measurements of intracellular pH demonstrated that pH remained constant during the observed Ca^{2+}-dependent suppression of channel activity.

L-type channel function is known to be governed via Ca^{2+}-dependent protein kinase activity (Schuhmann and Groschner, 1994) as well as by Ca^{2+}-dependent changes in the cells cytoskeleton (Johnson and Byerly, 1994). Thus, we tested the kinase inhibitor H-7 and the cytoskeleton destabilizer cytochalasin B for its ability to interfere with Ca^{2+}-dependent channel inhibition. Neither agent affected Ca^{2+}-induced inhibition of L-type Ca^{2+} channel activity, arguing against a role of kinases and/or the cells cytoskeleton.

Since it has been proposed previously that functionality of Ca^{2+} channels requires basal phosphorylation of channel proteins, and that Ca^{2+}-dependent dephosphorylation represents an important mechanism of downregulation of cellular Ca^{2+} channel activity (Chad and Eckert, 1986; Armstrong, 1989), we tested cyclosporin A, an inhibitor of the Ca^{2+}-dependent phosphatase type 2B (calcineurin). Cyclosporin A by itself did not affect basal channel function, indicating that PP2B-dependent dephosphorylation is not involved in the control of Ca^{2+} channel function at basal, resting conditions. This is not surprising since PP2B is not expected to be active at the low level of intracellular free Ca^{2+} (50 nM) measured in our cell preparation under control conditions (Klee et al., 1979). Nonetheless, Ca^{2+}-dependent inhibition of channel activity was completely occluded in cells pretreated with a relatively low concentration (1 μg/ml) of cylosporin A. Based on the known potency and specificity of cyclosporin A as an inhibitor of PP2B, this result suggests that PP2B may be involved in Ca^{2+}-mediated negative feedback control of L-type Ca^{2+} channels in smooth muscle. Consequently, we tested whether PP2B is able to inhibit Ca^{2+} channel activity and to promote Ca^{2+}-dependent inhibition of smooth muscle Ca^{2+} channels. Addition of purified PP2B in the presence of 1 μM calmodulin and 1 μM free Ca^{2+} resulted in a profound suppression of channel activity. Addition of calmodulin (1 μM) to the cytoplasmic side of excised patches failed to inhibit channel activity at 1 μM free Ca^{2+} in the absence of exogenous PP2B, arguing against the presence of endogenous PP2B in the patches of membrane. In the presence of PP2B, Ca^{2+}-dependent inhibition of Ca^{2+} channel activity in excised patches exhibited a concentration dependence corresponding to that of Ca^{2+}-dependent stimulation of PP2B phosphatase activity, indicating that the effects of PP2B are due to downregulation of Ca^{2+} channels by a Ca^{2+}-dependent dephosphorylation process. The characteristics of this concentration dependence (i.e., IC_{50} value and Hill coefficient) resembled those observed for Ca^{2+}-dependent inhibition of Ca^{2+} channels in intact smooth muscle cells, supporting the idea that PP2B is involved in Ca^{2+}-dependent control of L-type Ca^{2+} channel function in smooth muscle.

Modulation of Single Channel Properties by Cytoplasmic Ca^{2+} and PP2B

Analysis of the effects of cytoplasmic Ca^{2+} on single channel properties revealed a striking difference between Ca^{2+}-dependent modulation of channels in intact cells and in cell-free patches. In contrast to the inhibition of channels in inside-out patches, which was mainly due to a reduction in P_o, inhibition of Ca^{2+} channels in intact cells was based on a reduction of both P_o and P_s. The observation of a Ca^{2+}-induced low P_o gating mode in cell-free membranes is consistent with the recently proposed model of direct binding of Ca^{2+} ions to an EF-hand motif of the channels' α1 subunit, which results in typically low P_o gating ("mode Ca") of the channel (Imredy and Yue, 1994; De Leon et al., 1995). Nonetheless, a specific effect of cytoplasmic Ca^{2+} on channels in intact smooth muscle cells was reduction of P_s. This reduction of P_s was not induced by elevation of Ca^{2+} at the cytoplasmic side of cell-free patches, but mimicked when Ca^{2+} was elevated in the low micromolar range in the presence of PP2B. These results of the present study demonstrate for the first time Ca^{2+}-induced suppression of the availability of Ca^{2+} channels in intact smooth muscle cells and in cell-free membranes exposed to PP2B. We have recently reported on the downregulation of smooth muscle L-type Ca^{2+} channels by PP2A-induced dephosphorylation (Groschner et al., 1996). In contrast to channel modulation by PP2B, PP2A did not suppress channel availability but affected specifically fast gating properties (P_o) of the channels by suppression of long lasting channel openings (mode 2 gating). Interestingly, the reduction of P_o observed in the presence of PP2B was not based on suppression of mode 2 gating, as evident from two experiments that allowed analysis of open time distributions (our unpublished observations). Taken together, these data strongly support the idea of multiple regulatory phosphorylation sites that are dephosphorylated in a rather selec-

tive manner by specific phosphatases and control different kinetic properties of the channel.

Physiological and Pharmacological Implications

Fine adjustment of Ca^{2+} entry according to the actual Ca^{2+} concentration in the cytosol is an important cellular mechanism. The results of the present study suggest that in vascular smooth muscle, this negative feedback control involves at least two sensors for Ca^{2+} within the cell. One of these sensors appears to be located close to the Ca^{2+} entry pathway, while another more remote sensor detects changes in the bulk cytosolic Ca^{2+} concentration. Albeit the present study does not provide information as to how Ca^{2+} affects channel function by its permeation through the channel pore, it is clearly demonstrated that the cytoplasmic Ca^{2+} controls channel function through two distinct intracellular mechanisms. The existence of multiple feedback mechanisms is not unexpected since cellular Ca^{2+} homeostasis is based on a variety of mechanisms, including Ca^{2+} transport across the plasma membrane and across the membrane of intracellular Ca^{2+} stores. It is already well established that Ca^{2+} signaling within a cell involves Ca^{2+} gradients (van Breemen et al., 1995). According to the present results, elevation of intracellular Ca^{2+} within the smooth muscle cell causes a reduction of Ca^{2+} entry due to PP2B-mediated dephosphorylation that may take place even at low or moderate rates of Ca^{2+} entry. On the other hand, the rate of Ca^{2+} entry through the L-type channel is unequivocally subject to a tight, local feedback mechanism. This local mechanism is likely to serve rapid, short term modulation of Ca^{2+} entry. Nonetheless, both the local and the remote mechanism may be of particular importance for smooth muscle Ca^{2+} homeostasis. Suppression of one of these mechanisms may produce severe disturbances in smooth muscle Ca^{2+} homeostasis. In the present study, we demonstrate that cyclosporin A, which is widely used as an immunosuppressant drug, effectively inhibits the remote Ca^{2+}-dependent control of L-type Ca^{2+} channels in human vascular smooth muscle. The interference of cyclosporin A with the negative feedback control of Ca^{2+} entry may contribute significantly to the severe vascular side effects (Sturrock et al., 1992) (i.e., promotion of vasoconstriction and hypertension) observed during cyclosporin A treatment.

In summary, our results demonstrate that in smooth muscle cells, the cytoplasmic Ca^{2+} concentration governs the function of L-type Ca^{2+} channels via two distinct negative feedback systems involving both Ca^{2+}-dependent dephosphorylation and direct Ca^{2+} binding.

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