A type 2A phosphatase-sensitive phosphorylation site controls modal gating of L-type Ca^{2+} channels in human vascular smooth-muscle cells

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The patch-clamp technique was employed to investigate phosphorylation/dephosphorylation-dependent modulation of L-type Ca^{2+} channels in smooth-muscle cells isolated from human umbilical vein. Okadaic acid, an inhibitor of phosphoprotein phosphatases type 1 (PP1) and 2A (PP2A), increased the probability of channels being in the open state (P_o) in intact cells. This increase in P_o was due mainly to promotion of long-lasting channel openings, i.e. promotion of 'mode 2' gating behaviour. Exposure of the cytoplasmic side of excised patches of membrane to the purified catalytic subunit of PP2A ($PP2A_c$) resulted in the opposite modulation of channel function. $PP2A_c$ (0.2 unit/ml) reduced the P_o of Ca^{2+} channels mainly via suppression of 'mode 2' gating. This effect of $PP2A_c$ was completely prevented by 1 μM okadaic acid. The catalytic subunit of PP1 (0.2 unit/ml), however, barely affected channel activity. Our results provide evidence for a PP2A-sensitive regulatory site that controls modal gating of L-type Ca^{2+} channels in smooth muscle.

INTRODUCTION

Phosphorylation/dephosphorylation processes play a key role in cellular regulation of Ca^{2+} channel function. Both the pore-forming ($\alpha 1$) and the β subunit of the L-type Ca^{2+} channel are substrates for various protein kinases that phosphorylate multiple sites on these proteins [1–3]. Channel proteins are in turn dephosphorylated in a rather selective manner by protein phosphatases type 1 and 2A [4], and inhibition of cellular protein phosphatases was found to result in substantial changes in the functional properties of the L-type Ca^{2+} channel.

The current through L-type Ca^{2+} channels is determined by several properties of the channel proteins. The Ca^{2+} current measured in a cell membrane (I_{Ca}) is proportional to the current flow through a single open channel (i_{Ca}), the total number of channels, N_T, of which a fraction (P_s) is available to open on membrane depolarization, and the probability of available channels to stay in an open state (P_o) during the depolarization. The relationship between I_{Ca} and i_{Ca} is thus given by:

$$I_{Ca} = i_{Ca} \times N_T \times P_s \times P_o$$

Cellular regulation of L-type Ca^{2+} channels involves mainly the modulation of the probability of being open (P_o) and the availability (P_s). Consistently, two types of gating, i.e. transitions between functional states of the channel protein, can be distinguished: a slow gating between unavailable and available states [5,6] and a fast gating of available channels between open and closed states. Initially three distinct patterns of gating were classified as 'gating modes' [5]. The situation when a channel is 'sleeping', i.e. fails to open on membrane depolarization, has been termed 'mode 0'. Once a channel is available, two discrete levels of P_o are usually observed. These activity states have been designated 'mode 1' (low P_o), characterized by brief openings, and 'mode 2' (high P_o), displaying typically long-lasting openings [5].

Both slow gating between available and unavailable states and also fast gating between open and closed states are thought to be controlled via phosphorylation/dephosphorylation. This hypothesis is supported by the finding that inhibitors of protein phosphatases affect P_s as well as P_o of Ca^{2+} channels [7,8]. Recent studies demonstrated that potent inhibitors of phosphatase type 1 induce predominantly a change in slow gating (P_s) [8,9], whereas inhibitors that preferentially suppress phosphatase type 2A activity exert effects mainly on fast gating (P_o) of Ca^{2+} channels [8]. The widely used phosphatase inhibitor okadaic acid is known to inhibit PP2A at lower concentrations than PP1 [10] and was reported to enhance Ca^{2+} channel activity in both cardiac and smooth muscle [10,11]. In cardiac myocytes, the stimulatory effect of okadaic acid was found to be due in large part to promotion of mode 2 gating [7,8]. These results have led to the hypothesis that fast gating of cardiac Ca^{2+} channels is controlled via a type 2A phosphatase-sensitive site. The present study was designed to test this hypothesis for Ca^{2+} channels of human smooth muscle. We characterized okadaic acid-induced changes in the gating behaviour of Ca^{2+} channels in intact cells, and tested whether a specific phosphatase is able to induce opposite effects when applied to the intracellular side of channels under cell-free conditions. Our results provide clear evidence that type 2A phosphatase dephosphorylates a regulatory site that determines fast (modal) gating behaviour of the smooth-muscle Ca^{2+} channel.

MATERIALS AND METHODS

Materials

Purified type 2A protein phosphatase catalytic subunit ($PP2A_c$) was prepared as previously described [12] and purified type 1 protein phosphatase catalytic subunit ($PP1_c$) was obtained from

Abbreviations used: P_s, probability of finding a channel available to open on depolarization; P_o, probability of finding an available channel in the open state; $PP1_c$, type 1 protein phosphatase catalytic subunit; $PP2A_c$, type 2A protein phosphatase catalytic subunit; NP_o, mean number of open channels given by the product of N (number of active channels in a patch) times P_o

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Upstate Biotechnology (Lake Placid, NY, U.S.A.); collagenase type CLS II and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.); dispase type II was from Boehringer Mannheim (Mannheim, Germany); fatty acid-free BSA was from Behring (Marburg, Germany); *S*(−)-BayK 8644 was from Research Biochemicals Incorporated (Natic, MA, U.S.A.); okadaic acid was purchased from Calbiochem (San Diego, CA, U.S.A.); and all other chemicals were from Sigma Chemical Co. (Deisenhofen, Germany).

Preparation of smooth-muscle cells

Smooth-muscle cells were isolated from the media of human umbilical veins as described previously [13]. The isolated cells were stored in a high K^+/low Cl^- extracellular solution (see below) at 4 °C, and used for experimentation within 36 h.

Single-channel recording

Ba^{2+} currents through single Ca^{2+} channels were recorded in the membrane of intact smooth muscle cells (cell-attached configuration) and in inside-out patches. Cell potential was set to approximately zero by use of a high K^+/low Cl^- extracellular solution that contained (in mM): 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 Hepes, 2 EGTA, pH adjusted to 7.4 with *N*-methyl-D-glucamine, and pCa adjusted to 7.5. To prevent run-down of channel activity in the inside-out configuration, 1 mM ATP and 2 units/ml calpastatin [14] were added to the bath solution immediately before patch excision. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments, Pangbourne, Berkshire, U.K.), and had resistances of 5–10 MΩ when filled with a solution containing (in mM): 10 $BaCl_2$, 100 NaCl, 30 triethanolamine/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 excised patches [14]. All experiments were performed at room temperature. Okadaic acid or protein phosphatases were applied with a perfusion system that allowed exchange of the bath solution within approx. 15 s.

Voltage clamp and current amplification were performed with a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany). Current records were filtered at 1 kHz (−3 dB) and digitized at 5 kHz. Control of voltage protocols and idealization of current records was performed with pClamp software (Axon Instruments, Foster City, CA, U.S.A.) as well as with custom-made software. Events were compiled in open-time histograms constructed with variable bins (logarithmic scale) and fitted with biphasic functions. The total number of functional channels (N) in multi-channel patches as well as the channels' mean P_s and mean P_o were determined as described previously [15]. The stability of single-channel characteristics was tested in control experiments that demonstrated the stability of open-time distributions, unitary current amplitude and the mean number of open channels (NP_o) in the absence of pharmacological interventions over a period of 20 min. Averaged results are given as means ± S.E.M. for the indicated number of experiments. Statistical analysis was performed with Student's t test for paired values and differences were considered statistically significant at $P < 0.05$.

Assay of *p*-nitrophenyl phosphate phosphatase activity

The activity of protein phosphatases, used in the electrophysiological experiments, was adjusted to 0.2 unit/ml by using *p*-nitrophenyl phosphate as substrate as described previously [16]. One unit of phosphatase activity was the amount that catalysed dephosphorylation of 1 mmol of *p*-nitrophenyl phosphate in 1 min.

RESULTS

Okadaic acid enhances Ca^{2+} channel activity by promotion of long-lasting openings

Figure 1 illustrates the stimulation of Ca^{2+} channel activity in an intact smooth-muscle cell by okadaic acid. Channel activity was characterized in terms of the number of functional channels, the probability of the channels opening on a depolarizing stimulus (P_s) and the probability of available channels' being in an open state (P_o). Although overlapping channel openings were barely

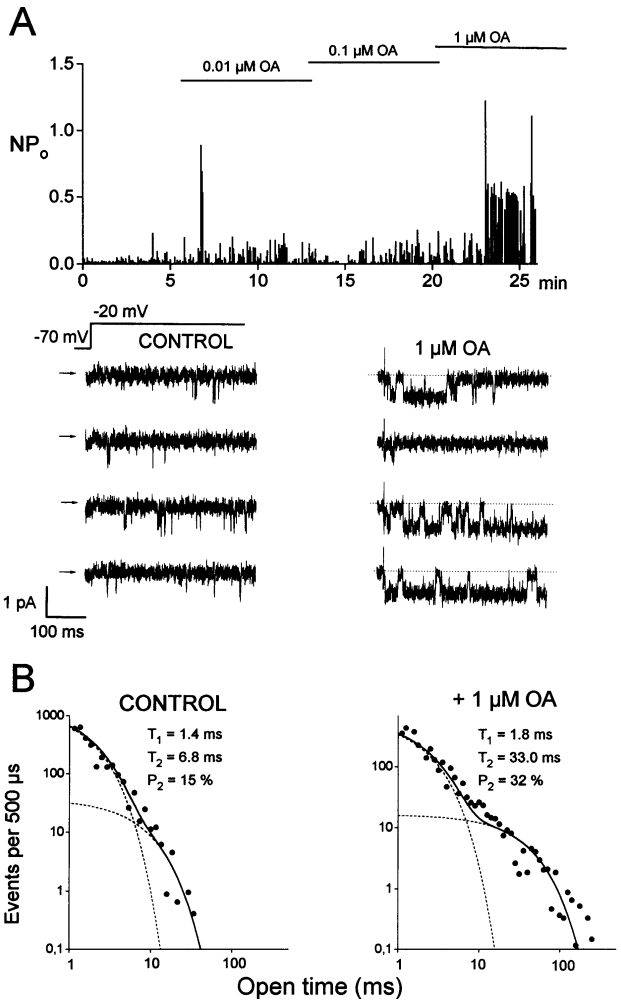

Figure 1 Okadaic acid stimulates smooth-muscle Ca^{2+} channel activity by promotion of long-lasting openings

(**A**) Upper panel: time course of channel activity recorded in a cell-attached patch. Columns represent the mean number of open channels (NP_o) calculated for individual depolarizing pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). Exposure of the cell to increasing concentrations of okadaic acid (OA) is indicated. Lower panel: individual current responses in the absence (control) and presence of 1 μM okadaic acid. Records are filtered at 1 kHz and digitized at 5 kHz. Zero current levels are indicated by arrows or dotted lines. (**B**) Open-time histograms derived in the absence (control) or presence of 1 μM okadaic acid. Histograms were constructed by using 180 and 145 sweeps respectively, and fitted with biphasic functions. Individual components of the fit (broken lines) as well as time constants (T_1, T_2) and the proportion of long-lasting openings (P_2) are indicated.

Table 1 Effects of okadaic acid and $PP2A_c$ on P_o and P_s of single L-type Ca^{2+} channels

Results are means ± S.E.M. ($n = 4$) of P_o and P_s of channels recorded in cell-attached patches in the absence (control) or presence of 1 μM okadaic acid, and in inside-out patches in the absence (control) or presence of 0.2 unit/ml $PP2A_c$ (2–4 min after application to inside-out patches). Values are calculated from 80–120 depolarizing sweeps. $^*P < 0.05$ compared with control.

	P_o	P_s
Control (cell-attached)	3.1 ± 0.8	30.5 ± 8.7
+ okadaic acid	14.3 ± 5.0*	26.5 ± 10.0
Control (inside-out)	12.0 ± 5.3	28.5 ± 9.2
+ $PP2A_c$	3.0 ± 1.0*	22.5 ± 5.3

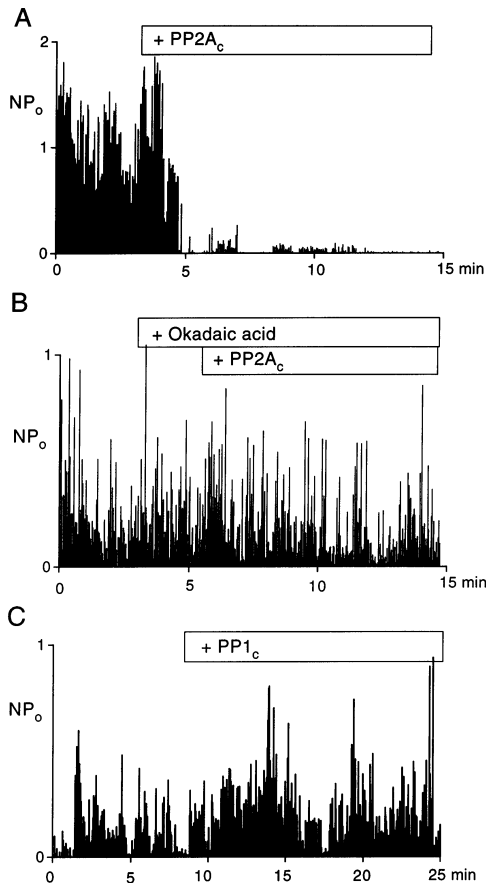

Figure 2 $PP2A_c$ inhibits smooth-muscle Ca^{2+} channels but $PP1_c$ does not

Time courses of channel activity recorded in inside-out patches are shown. Columns represent the mean number of open channels (NP_o) calculated for individual depolarizing pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). (**A**) Effect of 0.2 unit/ml $PP2A_c$. (**B**) Prevention of the effect of 0.2 unit/ml $PP2A_c$ by 1 μM okadaic acid (OA). (**C**) Effect of 0.2 unit/ml $PP1_c$.

detectable, the patch was found to contain two functional channels exhibiting a mean P_s of 31 % and a mean P_o of 2.2 % in the control. Channel activity increased on administration of 1 μM okadaic acid, with a marked elevation of mean P_o to 28 % (Figure 1A), whereas P_s remained almost unchanged (39 %). Open-time distributions were best fitted by a biphasic function yielding characteristic open times of 1.4 and 6.8 ms and a

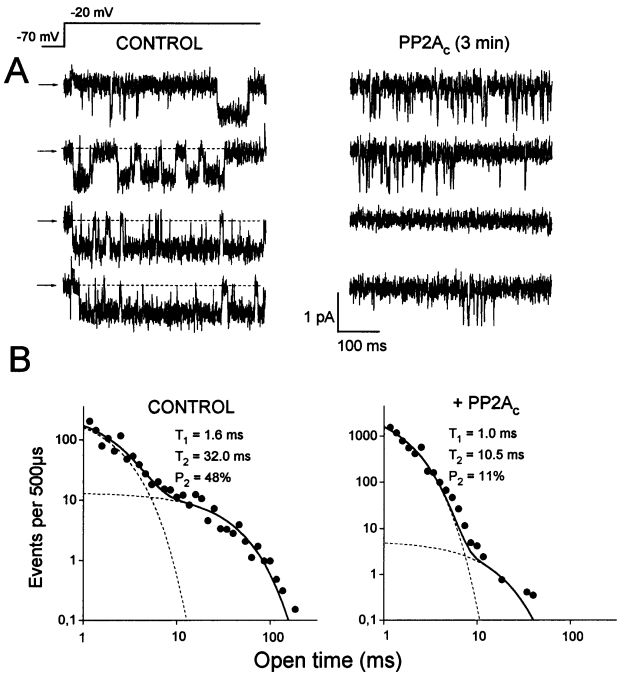

Figure 3 $PP2A_c$ inhibits smooth-muscle Ca^{2+} channels by suppressing long-lasting openings

(**A**) Individual current responses to consecutive depolarizations (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz) in the absence (control) or presence of 0.2 unit/ml $PP2A_c$. Records were filtered at 1 kHz and digitized at 5 kHz. Zero-current levels are indicated by arrows and broken lines. (**B**) Open-time histograms derived in the absence (control) or presence of 0.2 unit/ml $PP2A_c$. The effect of $PP2A_c$ was analysed by using the period 2–4 min after application. Histograms were constructed by using 100 and 120 sweeps respectively, and fitted with biphasic functions. Individual components of the fit (broken lines) as well as time constants (T_1, T_2) and the proportion of long-lasting openings (P_2) are indicated.

proportion of long openings of 15 % for the control (Figure 1B). The okadaic acid-induced increase in P_o was due to the promotion of long-lasting channel openings. A proportion of 32 % of long openings with a characteristic open time of 33 ms was calculated in the presence of the phosphatase inhibitor (Figure 1B). The effects of okadaic acid on P_o and P_s are given in Table 1. P_o increased significantly in the presence of 1 μM okadaic acid whereas P_s remained unchanged. Thus okadaic acid induced predominantly a change in fast gating of Ca^{2+} channels in that the occurrence of long-lasting channel openings (mode 2 sweeps) was increased. Because okadaic acid is known to inhibit PP2A preferentially, the observed promotion of mode 2 gating may reflect stabilization of a PP2A-sensitive regulatory phosphorylation. One approach to clarifying the role of a specific phosphatase in cellular regulation of channel function is to study channel modulation by the purified enzyme by using the inside-out configuration of the patch-clamp technique, which allows the administration of enzymes to the cytoplasmic side of membranes.

PP2A inhibits Ca^{2+} channel activity by suppression of long-lasting openings

Figure 2(A) illustrates the effect of 0.2 unit/ml $PP2A_c$ on channel activity in an inside-out patch. The patch contained six functional channels that exhibited a mean P_s of 35 % and a mean P_o of 17.5 % in controls. On exposure of the cytoplasmic side of the patch to 0.2 unit/ml $PP2A_c$, channel activity declined rapidly (within 2 min) mainly owing to a decrease in P_o to 4.8 %, whereas

P_s decreased only moderately (21 %). Prolonged exposure of the patch to $PP2A_c$ abolished channel activity within 10–15 min ($n = 4$). The effects of $PP2A_c$ were completely prevented by 1 μM okadaic acid ($n = 3$; Figure 2B), suggesting that the observed inhibition was due specifically to phosphatase activity. In contrast, exposure of inside-out patches to 0.2 unit/ml $PP1_c$ failed to inhibit channel activity within 10 min ($n = 3$) as illustrated in Figure 2(C), indicating that the observed inhibitory modulation was a specific effect of $PP2A_c$. The effects of $PP2A_c$ on Ca^{2+} channel P_o and P_s calculated for the fairly stable period of 2–4 min after addition of $PP2A_c$ are given in Table 1. The phosphatase clearly decreased P_o but changes in availability were not statistically significant. Thus PP2A preferentially affected the fast gating of Ca^{2+} channels. Figure 3 illustrates the effects on fast gating at the single-channel level. In this experiment one conductance level was mostly observed (Figure 3A), which enabled the analysis of channel open-time distributions (Figure 3B). Channel activity was relatively high in the control, and mode 2 gating was clearly evident. $PP2A_c$ decreased P_o mainly by suppression of mode 2 gating, i.e. suppression of long-lasting openings. The proportion of long-lasting openings decreased from 48 % in the control to 11 % in the presence of $PP2A_c$. It is of note that $PP2A_c$ increased the frequency of short openings, as evident from the marked (about 10-fold) increase in the frequency of short openings compared with the control.

DISCUSSION

This study demonstrates that the phosphatase inhibitor okadaic acid stimulates Ca^{2+} channel activity in smooth muscle by promoting a high-activity gating mode (mode 2) that is in turn specifically suppressed by direct application of the catalytic subunit of PP2A to the intracellular side of the channel. Thus we show for the first time the modulation of single Ca^{2+} channels by a purified protein phosphatase.

The role of phosphatases in the cellular regulation of smooth-muscle Ca^{2+} channels is complex, in that both inhibition and stimulation have been observed in experiments with phosphatase inhibitors [6,7,9]. We have recently demonstrated that the inhibitory effect of the potent PP1 inhibitor tautomycin is based on changes in slow gating of smooth-muscle Ca^{2+} channels between available and unavailable states [7]. With the present study we provide evidence that stimulation of smooth-muscle Ca^{2+} channel activity by okadaic acid is mainly due to the modulation of fast gating in terms of a shift towards high-activity gating mode 2. In gastric smooth muscle, okadaic acid was found to exert a concentration-dependent dual effect on whole-cell Ca^{2+} channels [9]. Because the inhibitory component of the action was observed at low concentrations, which are known to inhibit type 2A phosphatases preferentially, the inhibitory effects have been explained by stabilization of a PP2A-sensitive phosphorylation site. In the present study okadaic acid exerted substantial stimulatory effects at micromolar concentrations, and even at low nanomolar concentrations the phosphatase inhibitor induced a slight increase rather than a decrease in channel activity (Figure 1A). A possible explanation for the divergent results obtained in different smooth-muscle tissues is that basal phosphatase activity might vary considerably between different tissues, comprising several okadaic acid-sensitive phosphatase subtypes that may play diverse roles in channel regulation.

To clarify the role of type 2A and type 1 phosphatases in Ca^{2+} channel regulation we used the strategy of applying purified phosphatases to the cytoplasmic side of channels in excised inside-out patches. We observed that $PP2A_c$ markedly suppressed channel activity in inside-out patches. $PP2A_c$-induced down-regulation was not mimicked by $PP1_c$ and completely antagonized by okadaic acid, suggesting the involvement of a specific PP2A-induced dephosphorylation process. $PP2A_c$-induced modulation of channels in inside-out patches was inverse to the modulation induced by okadaic acid in intact cells. Thus okadaic acid might well stimulate Ca^{2+} channel activity in intact smooth-muscle cells by preventing PP2A-mediated dephosphorylation. An analysis of channel open times clearly revealed that $PP2A_c$ induced a substantial change in fast gating, in that it suppressed long-lasting channel openings. It is thus suggested that PP2A activity is an important determinant of fast gating of L-type Ca^{2+} channels in smooth-muscle cells. Interestingly, $PP1_c$ failed to inhibit, and even slightly stimulated, channel activity in excised patches. Different functions for PP1 and PP2A in Ca^{2+} channel gating are not surprising in view of biochemical results demonstrating divergent susceptibilities of specific phosphorylation sites on the Ca^{2+} channel to dephosphorylation by PP1 and PP2A [4]. Although modulation of Ca^{2+} channels in inside-out patches by $PP2A_c$ might be explained in principle by dephosphorylation of any regulatory protein present in the membrane, it seems reasonable to speculate that dephosphorylation of a protein of the channel complex itself mediates the effect of PP2A. Thus our results can be interpreted in terms of fine adjustment of channel function via selective phosphorylation and dephosphorylation of Ca^{2+} channel subunits. Channel function was analysed in the present study in terms of (1) the ability of channels to open on membrane depolarization (availability, P_s), as well as (2) the probability of finding an available channel in the open state (open probability, P_o). Our results strongly support the hypothesis that phosphorylation of a PP2A-sensitive site is required for high-P_o (mode 2) gating of the Ca^{2+} channel. Because $PP2A_c$ decreased P_o without significant changes in P_s, it is concluded that PP2A mediates conversion of high-P_o gating (mode 2) to low-P_o gating (e.g. mode 1) rather than into an unavailable state (mode 0). Prolonged exposure to $PP2A_c$ resulted in the complete loss of channel activity, indicating that $PP2A_c$ is in principle able to dephosphorylate additional sites, which results ultimately in conversion into an unavailable state (mode 0). The possible role of PP2A in the transition between gating modes is illustrated in Figure 4, which depicts a hypothetical scheme assuming phosphorylation/dephosphorylation of the Ca^{2+} channel itself as the regulatory mechanism.

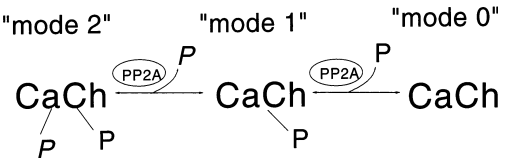

Figure 4 Model of PP2A-mediated transitions between gating modes of the smooth-muscle Ca^{2+} channel

L-type Ca^{2+} channels are subject to complex cellular regulation involving phosphorylation/dephosphorylation processes. The results of the present study support the view that PP2A-mediated selective dephosphorylation of the channel or of regulatory proteins serves as an effective mechanism for down-regulation of smooth-muscle Ca^{2+} channels.

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