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Ser^{1901} of α_{1C} subunit is required for the PKA-mediated enhancement of L-type Ca^{2+} channel currents but not for the negative shift of activation

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Abstract Cardiac L-type Ca^{2+} channel is facilitated by protein kinase A (PKA)-mediated phosphorylation. Here, we investigated the role of Ser^{1901}, a putative phosphorylation site in the carboxy-terminal of rat brain type-II α_{1C} subunit (rbCII), in the PKA-mediated regulation. Forskolin (3 μM) enhanced Ca^{2+} channel currents (I_{Ca}) and shifted the activation curve to negative voltages, which were abolished by protein kinase inhibitor. Replacement of Ser^{1901} of rbCII by Ala abolished the enhancement of I_{Ca} by forskolin but not the shift of the activation curve. These results indicate that Ser^{1901} is required for the PKA-mediated enhancement of I_{Ca}, and that the voltage-dependence of the activation of I_{Ca} appears to be modulated via another PKA phosphorylation site.

Key words: Calcium channel; Protein kinase A; Forskolin; Subunit; Mutation; Modulation

1. Introduction

PKA-mediated phosphorylation is the crucial step in the positive regulation of cardiac L-type Ca^{2+} channels through β-adrenergic receptor pathway [1]. Cardiac L-type Ca^{2+} channel is composed of at least three subunits, pore-forming α_{1C} subunit, β subunit, and α_2/δ subunit. Target amino acids in α_{1C} and β subunits for PKA phosphorylation have been identified [2–6]. Ser^{1928} in the carboxy-terminal of rabbit cardiac α_{1C} subunit appears to be the only substrate within α_{1C} subunit for the PKA phosphorylation [3,7,8] and this Ser is highly conserved among various species including human (see Fig. 1). However, the functional link between the PKA-mediated modulation of Ca^{2+} channel function and the PKA-mediated phosphorylation of Ser^{1928} has been controversial [8,9]. Because of the difficulty in reconstituting the PKA-mediated facilitation of L-type Ca^{2+} channels in the expression system, there are few successful reports dealing with the functional role of phosphorylation sites [7–9]. The carboxy-terminal domain of α_{1C} subunit has been shown to be involved in the regulation of gating properties of the Ca^{2+} channel [10]. Thus, in the present study, aiming at clarifying the role of the phosphorylation site in the PKA-mediated modulation of L-type Ca^{2+} channels, we mutated Ser^{1901} of rat brain α_{1C} subunit (rbCII) that corresponds to Ser^{1928} in rabbit cardiac α_{1C} subunit. The approximate locations of putative phosphorylation sites and Ser^{1901} are illustrated in Fig. 1A.

2. Materials and methods

2.1. Point mutation in rbCII

A point mutation was introduced into rbCII (kindly supplied by Dr. T.P. Snutch) by replacing Ser^{1901} with Ala (S1901A). The mutation was introduced into the *Xba*I (5933)–*Sac*I (6391) fragment of rbCII by use of a QuickChange Kit (Qiagen). The fragment was amplified by polymerase chain reaction using *Pfu* polymerase (Stratagene) and verified by sequence analysis. The α_{1C} constructs were subcloned into pcDNAIII vector.

2.2. Cell culture and transfection

BHK6 cells (baby hamster kidney cells) stably expressing rabbit skeletal muscle β_{1a} and α_2/δ subunits [11] were cultured as has been described [12]. Wild-type α_{1C} subunit (rbCII) or the mutated S1901A were transiently expressed in BHK6 cells using SuperFect transfection reagent (Qiagen). The transfected cells were identified by a green fluorescent protein (GFP) signal coexpressed with α_{1C} subunit (pEGFP-C2, Invitrogen). Ca^{2+} channel currents were detected in 60–80% of the GFP-positive cells.

2.3. Electrophysiological recordings

L-type Ca^{2+} channel currents (I_{Ca}) were measured in the whole-cell configuration of patch-clamp technique as has been described [12]. The external solution contained (in mM): NaCl, 137; KCl, 5.4; $MgCl_2$, 1; HEPES, 10; glucose, 10 (pH adjusted to 7.4 with NaOH). The resistance of the recording pipettes was between 1 and 4 MΩ when filled with the internal solution containing (in mM): CsCl, 120; TEACl, 20; EGTA, 14; Mg-ATP, 5; Na_2 creatine phosphate, 5; GTP, 0.2; HEPES, 10 (pH 7.3 adjusted with CsOH). In some experiments, protein kinase inhibitor (PKI) was included in the patch pipette at a final concentration of 20 μM.

I_{Ca} was measured using a patch/whole cell clamp amplifier (Nihon Koden, Tokyo, Japan) or Axopatch 1D (Axon Instruments, Foster City, CA, USA) via an A/D converter (Digidata 1200, Axon Instruments, Foster City, CA, USA). Voltage-clamp protocols and data acquisition were performed using pCLAMP6 software (Axon Instruments, Foster City, CA, USA). Current signals were sampled at 2.5 kHz, filtered at 5 kHz, then digitized and stored. Capacitative currents were electrically compensated, and leak current was subtracted by P/-4 protocol. The I_{Ca} density ranged between 6 and 86 pA/pF. All the experiments were performed at room temperature.

The half activation potential (V_{50}) for the current–voltage relationships (*I–V* curves) were determined by an interactive non-linear regression fitting procedure to:

$$I = (V_m - V_{rev})G_{max}[1/\{1+\exp((V_m - V_{50})/k\}] \quad (1)$$

where V_m is the membrane potential, V_{50} is the half activation potential, V_{rev} is the reversal potential, k is the slope factor and G_{max} is the maximum of conductance.

Results are expressed as the mean ± S.E.M. Statistical analysis was performed with Student's paired *t*-test or when appropriate with Student–Welch's unpaired *t*-test.

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2.4. Drugs

Forskolin (Wako, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C as a stock solution (3 mM). The final concentration of DMSO in the bath solution was below 0.1% which, we confirmed, had no direct effect on I_{Ca} (data not shown). PKI (Sigma, St. Louis, MO, USA) was dissolved in double distilled water at 0.4 mM with 0.1 mg/ml of bovine albumin (essentially fatty acid free) (Sigma, St. Louis, MO, USA) as a stock solution and stored at −20°C.

3. Results

Fig. 2B shows typical Ca^{2+} channel currents (I_{Ca}) recorded with 2 mM Ca^{2+} as a charge carrier. The individual *I–V* curve exhibited typical properties as I_{Ca}. Because of the large variation of I_{Ca} density (from 6 to 67 pA/pF at 0 mV test potential), *I–V* curves were normalized to the maximum value of peak currents, typically measured at −10 or 0 mV, and shown in Fig. 2D. Forskolin at 3 μM augmented I_{Ca}, as is observed in native cardiac myocytes, within 7 min after switching the external solution [1] (Fig. 2A). The relative increment of I_{Ca} by forskolin varied widely from cell to cell (1.00–2.50-fold, average 1.53 ± 0.15-fold, $n = 12$, Fig. 4). However, the application of forskolin at higher concentration, such as 10 μM, rather inhibited I_{Ca}, which could be due to the direct effects of forskolin on Ca^{2+} channels [13,14] (data not shown, $n = 3$). Forskolin induced dual effects similar to those observed in native cardiac myocytes upon β-adrenergic receptor stimulation [1]: the enhancement of the peak amplitude of I_{Ca} at all test potentials and the hyperpolarizing shift of the *I–V* curve (Figs. 2B–D and 5A).

To verify that the forskolin-induced effects were mediated by PKA, we applied 20 μM of PKI into cells through the patch pipette. Current recordings were started at least 10 min after membrane rupture in order to give enough time for the diffusion of PKI into the cell. PKI by itself did not affect the amplitude of I_{Ca}, suggesting that the basal PKA activity is not high enough to modify I_{Ca}. Therefore, I_{Ca} and its *I–V* curve measured in the presence of PKI was indistinguishable from those recorded in the absence of PKI (Fig. 2E,G). However, in the presence of PKI, the subsequent application of forskolin showed neither the enhancement of the peak I_{Ca} amplitude nor the shift of *I–V* relationships (Fig. 2G). Peak I_{Ca} amplitude gradually decreased during the application of forskolin to 0.89 ± 0.03-fold of control ($n = 5$) (Fig. 4), in a way similar to that of vehicle control measured with 0.1% DMSO (0.85 ± 0.06, $n = 3$, data not shown). V_{50} values determined in control experiments were −10.6 ± 1.3 and −15.6 ± 1.2 mV before and after application of forskolin, respectively ($n = 11$, $P < 0.05$, Fig. 5A). In contrast, in the presence of PKI, V_{50} values were −13.5 ± 1.8 and

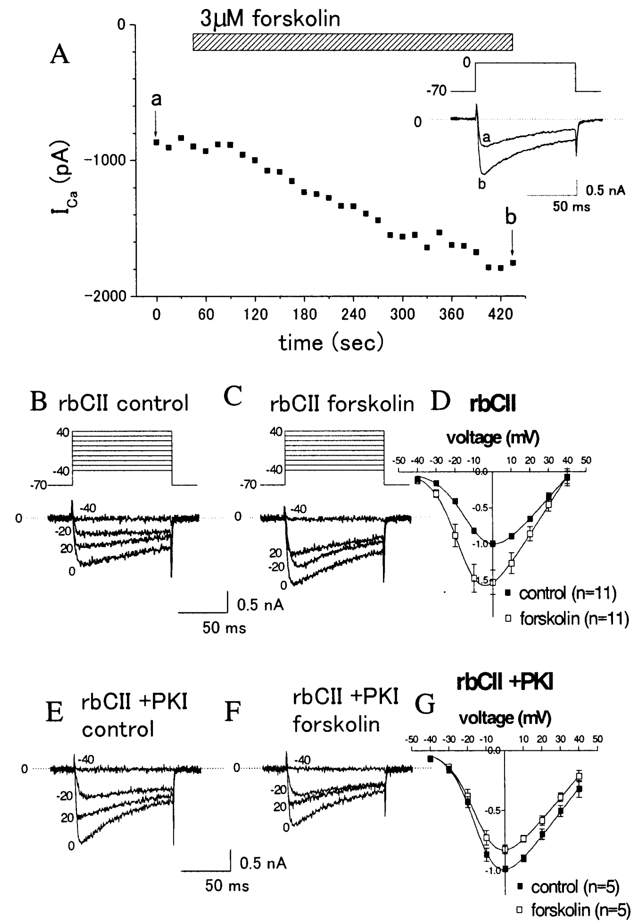
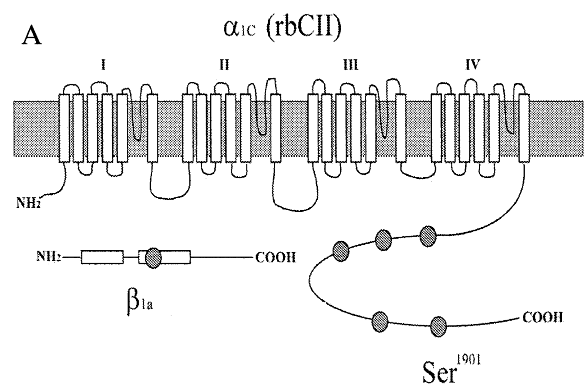


Fig. 2. The effect of forskolin on I_{Ca} recorded in rbCII expressed in BHK6 cells. A: Representative time course of enhancement of peak I_{Ca} during application of forskolin to rbCII. I_{Ca} was elicited by pulses to 0 mV from a holding potential of −70 mV every 15 s. Inset shows current traces recorded at time points indicated by arrows a and b. B: Current traces elicited by depolarization to −40, −20, 0 and +20 for 100 ms from a holding potential of −70 mV. C: Current traces in the presence of forskolin (3 μM) recorded from the same cell as shown in B. D: Current–voltage relationships (*I–V* curves) measured in the absence (■) and the presence (□) of forskolin (3 μM) are superimposed. The peak amplitude of I_{Ca} was normalized to the maximum peak I_{Ca} of control recordings. E and F: Current traces recorded under the same condition as used in B and C, respectively, with the intracellular application of PKI (20 μM) through the patch pipette. Traces in E and F were recorded from the same cell. G: *I–V* curves measured after the intracellular application of PKI are superimposed.



B

Ser^{1901}

Rat brain type II α_{1C} (rbCII)	1895: SLGRRASFHLECLKRQKDQGG :1915
Rat aorta α_{1C}	1921: SLGRRASFHLECLKRQKDQGG :1941
Mouse brain α_{1C}	1891: SLGRRASFHLECLKRQKDQGG :1911
Rabbit heart α_{1C}	1922: SLGRRASFHLECLKRQKNQGG :1942
Guinea pig heart α_{1C}	1921: SLGRRASFHLECLKRQKNHGG :1941
Human heart α_{1C}	1934: SLGRRASFHLECLKRQKDRGG :1954

Fig. 1. Schematic drawing of the voltage-gated L-type Ca^{2+} channel. A: Putative phosphorylation sites, including Ser^{1901}, are shown as gray circle. B: Amino acid sequences around Ser^{1901} are highly conserved in various species and organs. All of α_{1C} variants contain consensus sequences of PKA substrate such as RRAS (amino acids 1898–1901 in rbCII).

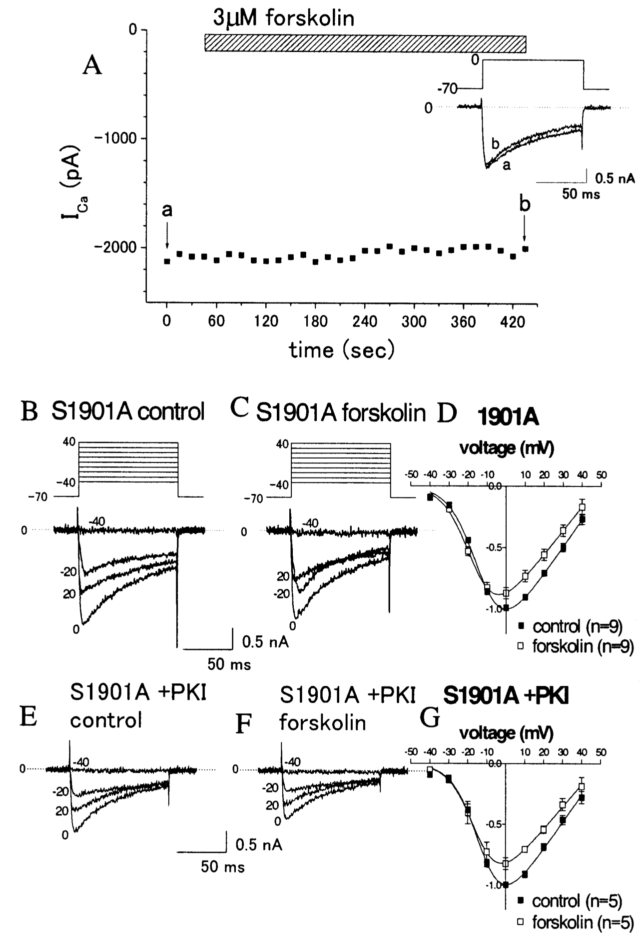


Fig. 3. The effect of forskolin on I_{Ca} recorded from S1901A expressed in BHK6 cells. A: A representative time course of peak I_{Ca} during the application of forskolin to S1901A. The procedures were the same as those of Fig. 2A. Inset shows the current traces recorded at time points indicated by arrows a and b. B and C: Current traces recorded from S1901A in the absence (B) and the presence (C) of forskolin (3 μM). D: I–V curves are shown after the peak amplitude of I_{Ca} was normalized to the maximum I_{Ca} of control recording. E and F: Current traces recorded from the same cell in the absence and the presence of forskolin after the intracellular dialysis of PKI (20 μM) through the patch pipette. G: I–V curves measured in the presence of PKI (20 μM) are superimposed.

-13.1 ± 2.4 mV before and after application of forskolin, respectively ($n = 5$, Fig. 5B). These results demonstrate that the dual effects of forskolin on Ca^{2+} channel currents were mediated by PKA-dependent phosphorylation.

The mutant α_{1C} subunit (S1901A) produced Ca^{2+} channel currents with the current density ranging between 13 and 86 pA/pF at 0 mV test potential, which was not statistically different from that of rbCII. We confirmed that the molecular size of the mutated α_{1C} subunit expressed in BHK6 cells is identical to that of rbCII (~220 kDa) by Western blotting (data not shown). Neither gating kinetics nor I–V curves differed from those recorded in the wild-type α_{1C} (rbCII) (Fig. 3B,D), indicating that the point mutation did not cause gross misfolding of α_{1C} subunit protein or the change of gating properties. In S1901A, however, forskolin (3 μM) failed to enhance I_{Ca} even after more than 7 min of application (Fig. 3A). The peak I_{Ca} amplitude gradually decreased to 0.93 ± 0.04-fold of initial amplitude ($n = 10$) during the application of forskolin, which was almost identical to the results obtained in the presence of PKI, 0.93 ± 0.05-fold of control ($n = 5$, Fig. 4). These results indicate that the PKA-mediated enhancement of I_{Ca} requires Ser[1901] of rbCII.

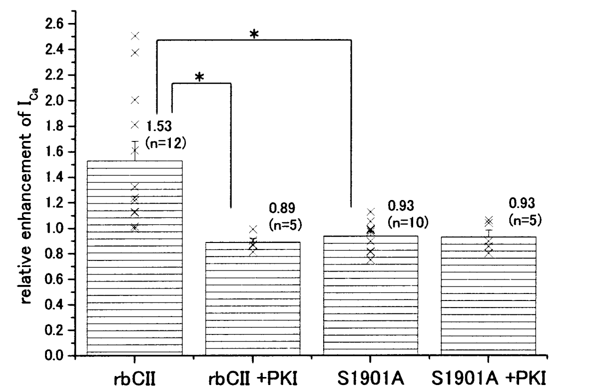


Fig. 4. Enhancement of I_{Ca} amplitude by forskolin was abolished in S1901A. Relative amplitudes of peak I_{Ca} in the presence of forskolin (3 μM) are summarized. Each symbol (×) represents the individual relative I_{Ca} amplitude measured in the presence of forskolin. The voltage protocols for recording I_{Ca} were as shown in Fig. 2. A: On top of each column, the mean value and the number of experiments are indicated. *, $P < 0.05$.

Interestingly, in S1901A, forskolin continued to produce the hyperpolarizing shift of the activation curve (Figs. 3D and 5C). V_{50} values determined in S1901A were -12.6 ± 1.1 and -16.3 ± 1.2 mV, before and after application of forskolin, respectively ($n = 9$, $P < 0.05$). This effect of forskolin was mostly absent in the presence of PKI (Figs. 3G and 5D). V_{50} values were -12.4 ± 0.9 and -14.1 ± 2.0 mV, before and after application of forskolin, respectively ($n = 5$, not significant). These results indicate that the forskolin-induced hyperpolarizing shift of the I–V curve is indeed due to the PKA-

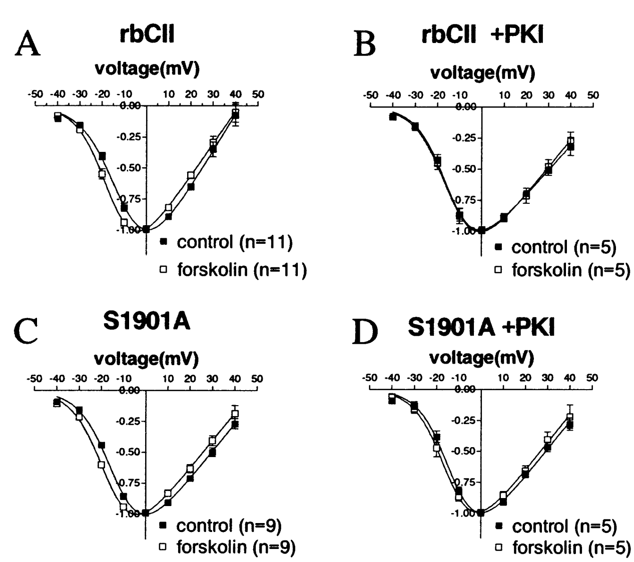


Fig. 5. The effect of forskolin on activation potentials of I_{Ca} measured in rbCII and S1901A. To compare the effect of forskolin on activation potential, I–V curves measured in the absence (■) and the presence (□) of forskolin (3 μM) were normalized to the respective maximum peak I_{Ca} amplitude and superimposed so that the negative shift of the activation curves can be compared. The data as shown in Figs. 2D,G and 3D,G were re-drawn.

dependent phosphorylation of a target protein that is distinct from Ser[1901] of α_{1C} subunit.

4. Discussion

In native cardiac myocyte, the stimulation of β-adrenergic receptor causes the activation of PKA and then modulation of L-type Ca^{2+} channels characterized by the enhancement of I_{Ca} density and the negative shift of *I–V* relationships [1]. In the present study, we could reconstitute these effects in BHK6 cells expressing the L-type Ca^{2+} channel by the application of forskolin. BHK cells may be equipped with the intracellular environment and endogenous kinases required for the modulation of L-type Ca^{2+} channels as reported before [15]. Taking advantage of this expression system, we showed that Ser[1901] plays a critical role in the PKA-mediated enhancement of Ca^{2+} channel current, but not in the PKA-mediated negative shift of the activation curve.

In the present study, we coexpressed α_{1C} and α_2/δ subunits together with β_{1a} subunit that has been shown to associate with α_{1C} subunit and to modulate its function [16]. It has been reported that cardiac myocytes express β_2 subunits as an accessory subunit of L-type Ca^{2+} channels [17,18]. However, for instance, β_{2a} subunit co-expressed with α_{1C} and α_2/δ subunits failed to reconstitute the Ca^{2+} channel current that shows fast inactivation kinetics in cardiac myocytes [19]. In human heart, β_{1a}, β_{1b} and β_{1c} subunits have been detected, while other subtypes of β subunits are detected in other species [20]. Thus, the real β subunit associated with α_{1C} subunit in heart has not been identified.

In this study, we showed that Ser[1901] plays a critical role in the PKA-mediated enhancement of I_{Ca}. However, it has been reported that the distal portion of carboxy-terminus of α_{1C} subunit may be cleaved off by proteolytic processing in the native cardiac myocytes [3], which suggests that Ser[1901] may not be involved in the PKA-mediated regulation [9]. However, the cleaved carboxy-terminal portion may be associated with the membrane near the Ca^{2+} channel even after the proteolytic cleavage [21], and that portion appears to be colocalized with Ca^{2+} channels in cardiac myocytes at t-tubular membranes [18]. Thus, it is possible that Ser[1901] continues to participate in the PKA-dependent modulation of Ca^{2+} channels even after the proteolytic truncation.

What could be the molecular mechanism underlying the up-regulation of Ca^{2+} channel activity subsequent to the phosphorylation of Ser[1901]? β-adrenergic receptor stimulation increases the open probability of L-type Ca^{2+} channels without changing the number of Ca^{2+} channels [22]. The deletion of α_{1C} subunit carboxy-terminal resulted in the enhancement of current density without significant effect on gating charge movement [10]. Therefore, the carboxy-terminal of α_{1C} subunit may serve as an inhibitory domain of Ca^{2+} channel gating, which may be removed on the PKA-dependent phosphorylation of Ser[1901].

We showed that the negative shift of *I–V* relationships induced by PKA did not result from the phosphorylation of Ser[1901]. These results suggest that another PKA substrate is responsible for the modulation of the voltage-dependence of activation of L-type Ca^{2+} channels. Single channel studies on cardiac Ca^{2+} channels have predicted the multiple phosphorylation sites involved in the regulation of distinct properties of the Ca^{2+} channel [23,24]. In the present study, we showed that the enhancement of I_{Ca} and the negative shift of current–voltage relationships appear to be independently regulated via distinct phosphorylation sites. We predict that the possible target could be β subunit based on several reasons: (1) it is unlikely that there are substrates for PKA other than Ser[1901] within Ca^{2+} channel α_{1C} subunits [3], (2) Ca^{2+} channel β_{1a} subunit used in this study is a substrate of PKA phosphorylation [2], (3) β subunit modulates the current–voltage relationships when co-expressed with α_{1C} [16]. It is also possible that the PKA phosphorylation of other proteins associated with Ca^{2+} channels mediate the negative shift of *I–V* relationships.

In summary, we have shown that Ser[1901] in the carboxy-terminal of α_{1C} subunit of Ca^{2+} channel is required for the PKA-mediated enhancement of I_{Ca}, and that *I–V* curves of S1901A as well as rbCII were shifted to hyperpolarized potentials by PKA stimulation.

We conclude that PKA-mediated modulation of L-type Ca^{2+} channel involves at least two independent mechanisms, enhancement of I_{Ca} density via phosphorylation of Ser[1901], and negative shift of activation curve via phosphorylation of another site that is to be elucidated in future studies.

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