PKA and phosphatases attached to the \( \text{CaV1.2} \) channel regulate channel activity in cell-free patches

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^1Department of Physiology, Graduate School of Medical & Dental Sciences, Kagoshima University, Kagoshima, Japan; ^2Department of Pharmaceutical Toxicology, School of Pharmacy, China Medical University, Shenyang, China; ^3Department of Ethnopharmacology, School of Pharmacy, China Medical University, Shenyang, China; and ^4Laboratory of Environmental Biology, Northeastern University, Shenyang, China

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Xu J, Yu L, Minobe E, Lu L, Lei M, Kameyama M. PKA and phosphatases attached to the \( \text{CaV1.2} \) channel regulate channel activity in cell-free patches. Am J Physiol Cell Physiol 310: C136–C141, 2016. First published November 11, 2015; doi:10.1152/ajpcell.00157.2015.—Calcium channel activation in cell-free patches is attenuated in a time-dependent manner. After rundown of the channels for 10 min or longer, CaM can only minimally reactivate the \( \text{CaV1.2} \) channel (47). This indicates that additional regulatory factor(s) might be required for an effective interaction of CaM with the channel for the regulation of basal \( \text{CaV1.2} \) channel activity.

It has been well established that activity of the \( \text{CaV1.2} \) \( \text{Ca}^{2+} \) channel is enhanced by PKA-mediated phosphorylation (19, 24, 46). In inside-out patches, the rundown of the \( \text{L-type} \) \( \text{Ca}^{2+} \) channel is delayed by the catalytic subunit of PKA (PKAc) + ATP in cardiac myocytes (33, 51) and noncardiac cells (2). Although it has been reported that PKAc + ATP can restore channel activity in inside-out patch to full activity (33), another report suggests that PKA regulates \( \text{CaV1.2} \) channel activity, not directly, but indirectly, by modulating the channel sensitivity to other cytoplasmic regulatory factors (48, 49). The reason for this discrepancy on the effect of PKA-mediated phosphorylation is not clear. Thus a study focused on better understanding the relationship between the effect of CaM in the inside-out mode and PKA-mediated phosphorylation of the channels seems to be important to understand the mechanism underlying \( \text{CaV1.2} \) channel activity.

In the present study, we examined the effect of PKA-mediated phosphorylation on the CaM interaction with the channels in the regulation of basal channel activity. We have found that CaM, together with PKAc and ATP, produces no rundown of \( \text{CaV1.2} \) channels in inside-out patches for more than 30 min. This indicated that maintaining \( \text{CaV1.2} \) channels in a phosphorylated state is required for the channel to be available for the CaM interaction to repress the channels for activation.

MATERIALS AND METHODS

Materials. BAY K 8644 (\( \text{Ca}^{2+} \) channel agonist) was purchased from Wako (Osaka, Japan). PKAc, okadaic acid (OA), MgATP, and 3',5'-cytidine adenosine monophosphate (cAMP) were purchased from Sigma-Aldrich (St. Louis, MO).

Solutions. The pipette solution contained the following (mM): \( \text{BaCl}_2, 50; \text{TEA-Cl}, 70; \text{EGTA}, 0.5; \) BAY K 8644, 0.003; and HEPES-CsOH buffer, 10 (pH 7.4). The basic internal solution (IO solution) consisted of the following (mM): potassium aspartate, 90; KCl, 30; \( \text{KH}_2\text{PO}_4, 10; \) EGTA, 1; MgCl\(_2, 0.5; \) CaCl\(_2, 0.5; \) and HEPES-KOH buffer, 10 (pH 7.4 and calculated free \( \text{Ca}^{2+} \) was 80 nM). PKAc was stored in a solution containing 200 mM DTT and 20

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mM HEPES (pH 7.4 by KOH), and OA was dissolved in 1 mM DMSO. CaM, MgATP, and cAMP were dissolved in basic internal solution, unless otherwise indicated. PKAc (1:10,000) and OA (1:100) were diluted in basic internal solutions before use.

**Electrophysiology.** Ventricular myocytes were obtained from adult guinea pig hearts by collagenase and protease dissociation, as described previously (52). Ca$^{2+}$ channel activity in the myocytes, perfused with the basic internal solution at 31–35°C, was monitored by the patch-clamp technique. Ca$^{2+}$ channel current was elicited by depolarizing pulses from −70 to 0 mV for 200 ms at a rate of 0.5 Hz using a patch pipette (2–4 MΩ), recorded with a patch-clamp amplifier (EPC-7, List, Darmstadt, Germany), and fed to a computer at a sampling rate of 3.3 kHz. The capacity and leakage currents in the current traces were digitally subtracted. The mean current during the period of 5–105 ms after the onset of the test pulses (i) was measured and divided by the unitary current amplitude (i) to yield $\Delta P_0$, (since $I = N \times P_0 \times i$), where $N$ is the number of channels in the patch, and $P_0$ is the time-averaged open-state probability of the channels. The channel open probability ($P_0$) value was used to represent the channel availability. Channel availability was defined as the ratio of active traces (i.e., traces containing one or more openings during the test pulse) out of the total number of recorded traces. In each experiment, the basal channel activity was recorded in the cell-attached mode for 1–2 min before the membrane patch was excised from the cell to establish the inside-out patch configuration. The channel activity ($P_{0\text{IO}}$) in the inside-out configuration was normalized by the basal activity recorded in the cell-attached mode. In the inside-out patch mode, 0.75 μM CaM, together with 3 mM ATP, was applied routinely to prevent the rundown of channels (47), unless otherwise indicated.

The experiments were done with ethical approval of the Committee of Animal Experimentation, Kagoshima University.

**Purification of CaM protein.** The cDNA of human CaM was cloned into the pGEX6P-3 vector (GE Healthcare Bioscience, Uppsala, Sweden) and transformed into the pGEX6P-3 vector (GE Healthcare, Uppsala, Sweden) and transformed in BL21 to express glutathione S-transferase fusion protein which was purified using glutathione-Sepharose 4B (GE Healthcare). The glutathione S-transferase region was cleaved by PreScission Protease (GE Healthcare). The purity of CaM was confirmed by SDS-PAGE and quantified by the Bradford method (Thermo Fisher Scientific, Rockford, IL) using an authentic CaM that had a molar extinction coefficient of 0.18 M$^{-1}$ cm$^{-1}$ at 276 nm.

**Statistical analysis.** Data are expressed as means ± SE ANOVA, followed by Dunnett’s test used for statistical evaluation. $P$ values < 0.05 were considered statistically significant.

**RESULTS**

**Phosphorylation state determines Cav1.2 channel activity induced by CaM in inside-out patches.** Although CaM + ATP reversed rundown of the Cav1.2 channel in inside-out patches soon after patch excision (13, 16, 17, 30, 41, 47), CaM + ATP only minimally recovered the channel activity after rundown for 10 min (Fig. 1A), suggesting an unavailable CaM interaction after a long-term, inside-out recording. To explore the possible modulatory effect of phosphorylation on the availability of CaM interaction with the channel, we applied the PKAc (5 nM), together with 3 mM ATP, immediately after patch excision. We observed that, although PKAc + ATP did not prevent the channel activity from the rundown, the channel treated by PKAc was dramatically reactivated by CaM (0.75 μM) + ATP 10 min after the patch excision (Fig. 1B).

Considering the possibility that activatable Cav1.2 channels were already phosphorylated in the resting condition (2, 33, 42), we examined the effect of preventing the channels from dephosphorylation during the inside-out recording. When the excised patches were perfused with the nonspecific phosphatase inhibitor OA (10 μM) for 10 min, channel activity was reactivated by CaM + ATP (Fig. 1C). See Fig. 3A for a summary of the channel activities (represented as a normalized $P_{0\text{IO}}$) in the inside-out mode after application of CaM + ATP, with patches pretreated with IO solution (control), PKAc + ATP, and OA for 10 min. The channel activities were reacti-
CaV1.2 channel was mediated by activation of a protein kinase, PKAc, with a normalized NPo of 11.4 ± 19% (P < 0.01 vs. control) in the PKAc group. The phosphorylation state renders CaV1.2 channel availability in inside-out patches. The rundown of the CaV1.2 channel in the inside-out configuration manifested as an almost complete loss of the open-state probability and availability of the channels (Fig. 1A). However, maintaining the channel phosphorylation state by application of PKAc + ATP, OA, or cAMP + ATP significantly rescued the channel availability, although the open-state probability was not greatly increased, the normalized NPo were 0.7 ± 0.2% in the control group (n = 11), 12.7 ± 3.5% in the PKAc group (n = 9, P < 0.01 vs. control), 6.7 ± 2.1% in the OA group (n = 7, P < 0.05 vs. control), 11 ± 3.4% in the cAMP group (n = 7, P < 0.05 vs. control), and 2.1 ± 0.5% in the cAMP/K252a group (n = 5, P > 0.05 vs. control) (Figs. 1 and 2, and 3A). Figure 3B summarizes the channel availabilities in the inside-out mode before and after application of CaM. The channel availabilities before application of CaM were 2.7 ± 0.6% (n = 11, control), 99.4 ± 0.1% (PKAc, n = 9, P < 0.001 vs. control), 92.3 ± 3.7% (OA, n = 7, P < 0.001 vs. control), 98.1 ± 5.3% (cAMP, n = 7, P < 0.001 vs. control), and 3.5 ± 19% (cAMP/K252a, n = 5, P > 0.05 vs. control) of that in the cell-attached mode. The channel availabilities after application of CaM were 14.7 ± 2.6% (control), 107.9 ± 4.4% (PKAc, P < 0.001 vs. control), 98.1 ± 3.2% (OA, P < 0.001 vs. control), 114.9 ± 16.5% (cAMP, P < 0.001 vs. control), and 20.8 ± 5.3% (cAMP/K252a, P > 0.05 vs. control). These

PKA and phosphatase(s) still attach on or near CaV1.2 in inside-out patches and modulate rundown of the CaV1.2 channel. Because the inhibition of phosphatase(s) by OA maintained the available CaM interaction with the CaV1.2 channel (Fig. 1C), it was reasonable to presume that active phosphatase(s) was still tethered on the channel, even in inside-out patches. We further speculated that PKA also stayed on or near the CaV1.2 channel in inside-out patches, although the kinase activity was lost because of washout of the agonist cAMP. To examine this hypothesis, we applied cAMP (10 μM) instead of PKAc during the 10-min rundown period. Similar to the result from pretreatment with PKAc, application of CaM/ATP induced CaV1.2 channel activity recovery (Fig. 1A), with a normalized NPo of 108 ± 14% (P < 0.01 vs. control) (Fig. 1A). To confirm that the effect of cAMP on the CaV1.2 channel was mediated by activation of a protein kinase, we added the protein kinase inhibitor K252a (10 μM) in the cAMP solution. We found that the effect of cAMP was greatly diminished (Fig. 2B): only 11 ± 1.4% (P > 0.05 vs. control group) was induced by CaM/ATP (Fig. 3A). These results indicated that PKA and phosphatase(s) were still attached on or near the channel in inside-out patches, and therefore, the balance between phosphorylation and dephosphorylation was shifted toward dephosphorylation, either because of a loss of kinase activity, or activation of phosphatase(s).

The phosphorylation state renders CaV1.2 channel availability in inside-out patches. The rundown of the CaV1.2 channel in the inside-out configuration manifested as an almost complete loss of the open-state probability and availability of the channels (Fig. 1A). However, maintaining the channel phosphorylation state by application of PKAc + ATP, OA, or cAMP + ATP significantly rescued the channel availability, although the open-state probability was not greatly increased, the normalized NPo were 0.7 ± 0.2% in the control group (n = 11), 12.7 ± 3.5% in the PKAc group (n = 9, P < 0.01 vs. control), 6.7 ± 2.1% in the OA group (n = 7, P < 0.05 vs. control), 11 ± 3.4% in the cAMP group (n = 7, P < 0.05 vs. control), and 2.1 ± 0.5% in the cAMP/K252a group (n = 5, P > 0.05 vs. control) (Figs. 1 and 2, and 3A). Figure 3B summarizes the channel availabilities in the inside-out mode before and after application of CaM. The channel availabilities before application of CaM were 2.7 ± 0.6% (n = 11, control), 99.4 ± 0.1% (PKAc, n = 9, P < 0.001 vs. control), 92.3 ± 3.7% (OA, n = 7, P < 0.001 vs. control), 98.1 ± 5.3% (cAMP, n = 7, P < 0.001 vs. control), and 3.5 ± 19% (cAMP/K252a, n = 5, P > 0.05 vs. control) of that in the cell-attached mode. The channel availabilities after application of CaM were 14.7 ± 2.6% (control), 107.9 ± 4.4% (PKAc, P < 0.001 vs. control), 98.1 ± 3.2% (OA, P < 0.001 vs. control), 114.9 ± 16.5% (cAMP, P < 0.001 vs. control), and 20.8 ± 5.3% (cAMP/K252a, P > 0.05 vs. control). These
of the channel to CaM could rescue the channel availability (Fig. 3A).

Because PKA-mediated phosphorylation interaction with the channel after long-time recordings in the inside-out mode. Because PKA-mediated phosphorylation decreased with time, exhibiting a slow rundown process (Fig. 4A). This result hinted of a loss of support for an effective CaM channel in inside-out patches, the channel activity gradually decreased with time, exhibiting a slow rundown process (Fig. 4A). This result hinted of a loss of support for an effective CaM channel in inside-out patches. We applied CaM and ATP, together with PKAc or cAMP or OA. Time course is shown of the channel activity (NPo) in the cell-attached and io mode incubated with CaM (0.75 μM)/ATP (3 mM) (A), PKAc (5 nM) + CaM/ATP (B), cAMP (10 μM) + CaM/ATP (C), and OA (10 μM) + CaM/ATP (D).

results implied that the phosphorylated channel retained the channel availability in the inside-out configuration for responding to depolarization and perhaps also for responding to CaM-mediated activation.

**CaM interaction with phosphorylated CaV1.2 produces persistent activity in inside-out patches.** Although CaM + ATP effectively produced a high level of activity of the CaV1.2 channel in inside-out patches, the channel activity gradually decreased with time, exhibiting a slow rundown process (Fig. 4A). This result hinted of a loss of support for an effective CaM channel interaction with the channel after long-time recordings in the inside-out mode. Because PKA-mediated phosphorylation could rescue the channel availability (Fig. 3B) and the response of the channel to CaM + ATP (Fig. 1B) in inside-out patches, we expected that PKA would prevent the slow rundown of the CaV1.2 channels in inside-out patch by maintaining the channels in a phosphorylation state. We applied CaM + ATP together with PKAc (Fig. 4B), cAMP (Fig. 4C), or OA (Fig. 4D) after patch excision. We observed that channel activity was maintained to a high level for >40 min, unless the patch dropped off from the electrode (Fig. 5). These results indicated that both the CaM/ATP interaction and phosphorylation of the channels were crucial for maintaining the basal activity of the CaV1.2 channels.

**DISCUSSION**

Dynamic Ca^2+^-free CaM interaction determines the basal activity of the CaV1.2 channels. The present study suggests that PKA-mediated phosphorylation rescues the available CaM interaction with the CaV1.2 channel, which determines the basal channel activity in the inside-out configuration. In our experiments, we used BAY K 8644, a Ca^2+^-channel agonist, to facilitate the research. Since PKA-mediated facilitation and the recovery of channel activity by cytoplasmic factors (such as CaM) have been reported to be additive to the effect of BAY K 8644 (15, 40), we think that the presence of BAY K 8644 does not affect the main conclusion of this study.

CaM, a ubiquitous and major Ca^2+^-sensing protein in all eukaryotic cells, contains four Ca^2+^-binding sites per molecule and exists in two states: Ca^2+^-free (i.e., apoCaM) and Ca^2+^-bound (i.e., Ca^2+^-CaM) states. It is generally accepted that CaM through binding Ca^2+^ regulates Ca^2+^-channel activity indirectly or directly (39). CaM can activate some regulatory factors to, in turn, influence channel activity, including activation of Ca^2+^/calmodulin-dependent protein kinase II (CaMKII), phosphodiesterase type 1, or calcineurin (PP2B, CaM-dependent protein phosphatase) (36). Additionally, CaM can directly bind to the channel to mediate Ca^2+^-dependent regulation of the Ca^2+^-channel (7, 25, 27, 35, 37, 54). The Ca^2+^-free form of CaM (apoCaM) was reported to preassociate with the channel and confer a rapid Ca^2+^-dependent feedback regulation upon increasing Ca^2+^. The tethered apoCaM in Ca^2+^-channels has been considered to be in a dormant state (7, 37). However, this has been challenged by several lines of evidence. First, truncation of the COOH-terminal tail containing the CaM binding sites (pre-IQ and IQ domains) completely loses basal CaV1.2 channel activity (45). Second, apoCaM (at Ca^2+^-concentration of 0–100 nM), together with ATP, reverses the rundown of CaV1.2 channels in inside-out patch in a dose-dependent manner (11, 13, 16, 17, 30, 41, 47). More recently, Adams et al. (1) reported that apoCaM itself enhances

Fig. 4. Long-lasting activity of CaV1.2 channels in the io mode with CaM and ATP, together with PKAc or cAMP or OA. Time course is shown of the channel activity (NPo) in the cell-attached and io mode incubated with CaM (0.75 μM)/ATP (3 mM) (A), PKAc (5 nM) + CaM/ATP (B), cAMP (10 μM) + CaM/ATP (C), and OA (10 μM) + CaM/ATP (D).

Fig. 5. Time course of CaV1.2 channels activity in io patches maintained by CaM and ATP, together with PKAc or cAMP or OA. The CaV1.2 channel activity in the io mode maintained by CaM/ATP (●, n = 7), PKAc/CaM/ATP (●, n = 6), cAMP/CaM/ATP (●, n = 4), and OA/CaM/ATP (●, n = 5) were represented by the relative NPo, value of that in the cell-attached mode. The average of each 5-min NPo is plotted against the time. Values are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. CaM/ATP group by ANOVA, followed by Dunnett’s test.

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opening of the Cav1.3 channel sevenfold. Finally, our present study revealed that phosphorylation of the channels is not enough to confer activity to the channels, but an additional CaM interaction with the Cav1.2 channel is required for basal activity. ATP is another important factor that supports basal activity of Cav1.2 channels (34, 53). It was reported that ATP directly binds to the channels and dose-dependently reprimed Cav1.2 channels (8, 28). Thus apoCaM and ATP play crucial roles in the regulation of the basal activity of Cav1.2 channels. The interaction of CaM and ATP with Cav1.2 channels may transduce the channel conformation from an inactive state to an active state, and thus any cytoplasmic factors modulating the dynamic interactions of CaM and ATP with the channels would influence the activity of the channels.

PKA-mediated phosphorylation modulates the CaM interaction with Cav1.2 channels in the basal condition. The Cav1.2 channel has long been recognized as a target of PKA. Stimulation of cAMP-PKA signaling pathway enhances the Ca2+ current three- to sevenfold in cardiac cells (5, 19, 24, 29). Multiple PKA phosphorylation sites in the COOH-terminus of α1C-subunit of Cav1.2 channel have been reported to contribute to upregulation of the Ca2+ channel activity (6, 9, 10, 31, 32, 50), although the actual phosphorylation sites are so far in issue (46). Furthermore, the mechanism by which PKA-mediated phosphorylation regulates Cav1.2 channel activity has not been unequivocally established. Recently, Catterall and co-workers have suggested that PKA-mediated phosphorylation releases the inhibition of the channel by a region in the distal COOH-terminus of α1C through an interaction with a region in the proximal COOH-terminus (9). The present study has revealed that PKA-mediated phosphorylation supports the effective CaM interaction with the channel to maintain the basal channel activity. Thus it is interesting to examine whether this mechanism can also be applied to the up-modulation of Cav1.2 channels mediated by cAMP-PKA signaling system.

Possible mechanism of rundown of Cav1.2 channels. Provided that the CaM/ATP interaction determines Cav1.2 channel activity and PKA-mediated phosphorylation ensures the effective CaM interaction, it is reasonable to speculate that CaM, ATP, and PKA activity may be enough to maintain the basal Cav1.2 channel activity, even in inside-out patches. Indeed, our results show that there is no rundown of Cav1.2 channels in the presence of CaM, ATP, and PKAc during a long-term recording (up to 40 min) in the inside-out mode. Because cAMP and the phosphatase inhibitor OA have a similar effect as that of PKAc, it is likely that inactive PKA, resulting from washout of cAMP, and active (or activated) phosphatase(s) still attach to or exist near Cav1.2 channels in inside-out patches. Thus the rundown process of the Cav1.2 channel in inside-out patches is most likely because of a loss of interaction of CaM and ATP with the channel (fast rundown), and subsequent dephosphorylation of the channel (slow rundown). Once it is CaM- and ATP-free with complete dephosphorylation, the Cav1.2 channel would enter a stable quiescent state, possibly because of a conformation change, which blocks the access of CaM, ATP, and/or PKA.

An effect of CaMKII similar to PKA in maintaining the basal activity of Cav1.2 channels has been reported (16, 17, 43, 44). This implies that CaMKII and PKA might share a common phosphorylation site responsible for the basal activity of Cav1.2 channels. Alternatively, there may be distinct phosphorylation sites for both kinases, which yield similar consequences to the basal activity. Further studies are needed to identify the phosphorylation site responsible for the basal activity of Cav1.2 channels.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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