Biochemical and Biophysical Research Communications 391 (2010) 1170–1176

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Both N- and C-lobes of calmodulin are required for Ca^{2+}-dependent regulations of $Ca_V1.2$ Ca^{2+} channels

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ARTICLE INFO

Article history:
Received 28 October 2009
Available online 16 December 2009

Keywords:
Ca^{2+} channel
Calmodulin
Channel regulation
Facilitation
Inactivation

ABSTRACT

We investigated the concentration- and Ca^{2+}-dependent effects of CaM mutants, CaM_{12} and CaM_{34}, in which Ca^{2+}-binding to its N- and C-lobes was eliminated, respectively, on the $Ca_V1.2$ Ca^{2+} channel by inside-out patch clamp in guinea-pig cardiomyocytes. Both CaM_{12} and CaM_{34} (0.7–10 μM) applied with 3 mM ATP produced channel activity after "rundown". Concentration–response curves were bell-shaped, similar to that for wild-type CaM. However, there was no obvious leftward shift of the curves by increasing $[Ca^{2+}]$, suggesting that both functional lobes of CaM were necessary for the Ca^{2+}-dependent shift. However, channel activity induced by the CaM mutants showed Ca^{2+}-dependent decrease, implying a Ca^{2+} sensor existing besides CaM. These results suggest that both N- and C-lobes of CaM are required for the Ca^{2+}-dependent regulations of $Ca_V1.2$ Ca^{2+} channels.

Introduction

Voltage-gated L-type Ca^{2+} channel has been suggested to be involved in many essential processes by the Ca^{2+}-dependent regulation, including muscle contraction, gene expression and hormone secretion [1,2]. Ca^{2+}-dependent facilitation (CDF) and inactivation (CDI) have been widely indicated in those processes [3–6].

Calmodulin (CaM) is an important regulator of ion channels, serving as a Ca^{2+} sensor for $Ca_V1.2$ channel. Several critical molecular determinants, such as IQ, preIQ within the C-terminus and a region in the N-terminus of $Ca_V1.2$, have been reported to interact with CaM [4–10]. However, the regulations of CDF and CDI by Ca^{2+} and CaM have not been fully clarified. CaM is a protein formed by two lobes, each comprising two Ca^{2+}-binding sites. CaM mutated at Ca^{2+}-binding sites 1 and 2 in the N-lobe (CaM_{12}) and at sites 3 and 4 in the C-lobe (CaM_{34}) have been used to investigate Ca^{2+}-dependent regulation of $Ca_V1.2$ channel [10–13]. In spite of these efforts, the mechanism underlying lobe-specific regulation of CaM of the $Ca_V1.2$ channel still remains to be established.

In our previous study, we found that Ca^{2+}-free CaM (apoCaM) could produce both facilitatory and inhibitory effects on channel activity with a bell-shaped concentration–response relationship, and increasing $[Ca^{2+}]$ shifted the curve toward left (lower concentrations). This shift was not observed with a Ca^{2+}-insensitive CaM mutant (CaM_{1234}) [14]. In this study, we further explored the CaM-mediated regulation of the $Ca_V1.2$ channel with the CaM mutants (CaM_{12} and CaM_{34}). It is demonstrated that both N- and C-lobes of CaM are required for the Ca^{2+}-dependent regulations on the $Ca_V1.2$ Ca^{2+} channel.

Materials and methods

Molecular biology. Human CaM was expressed as glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli* BL21, purified using glutathione–Sepharose 4B (GE Healthcare), and the GST region was cleaved by PreScission Protease (GE Healthcare). The recovered CaM was quantified by the Bradford method (Pierce) with bovine serum albumin as a standard. Point mutations in the Ca^{2+}-binding sites of CaM were introduced by Quickchange™ site-directed mutagenesis kit (QIAGEN). CaM_{12} and CaM_{34} had mutations of E31A + E67A and S101F + E140A, respectively.

Preparation of single myocytes. Single ventricular myocytes from adult guinea-pig hearts were dispersed by collagenase and protease as described previously [15,16]. Myocytes were treated with 0.05 mg/ml^{-1} protease (Nagase NK-103, Wako Pure Chemicals, Osaka, Japan) and 0.02 mg/ml^{-1} DNase I (Type IV, Sigma Aldrich, St. Louis, USA) to improve the success rate of giga-Ohm seal.

Solutions. The solutions for single-channel recording were as described previously [15,16]. The pipette solution contained (mM): $BaCl_2$ 50, tetraethylammonium (TEA) Cl 70, EGTA 0.5, Bay K 8644

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doi:10.1016/j.bbrc.2009.11.171

0.003, and Hepes–CsOH buffer 10 (pH 7.4). The basic internal solution contained (mM): K-aspartate 120, KCl 20, glucose 10, EGTA 2, and Hepes-KOH buffer 10 (pH 7.4). The [Ca^{2+}] was calculated using a modified program of Fabiato [17].

Patch-clamp experiment. Single-channel currents were recorded in the cell-attached and inside-out modes using a patch-clamp amplifier (EPC-7, List, Darmstadt, Germany). Ca^{2+}-channel currents were elicited by depolarizing pulses from −70 to 0 mV with 200 ms duration at 0.5 Hz and digitized at 3.3 kHz. The *NPo* value, where *N* was the number of channels in the patch and *Po* was the time-averaged open-state probability during the period of 5–105 ms, was calculated for each pulse. Channel activity in control was obtained by averaging *NPo* values for 2 min (60 pulses) in the cell-attached condition, and those in test conditions were

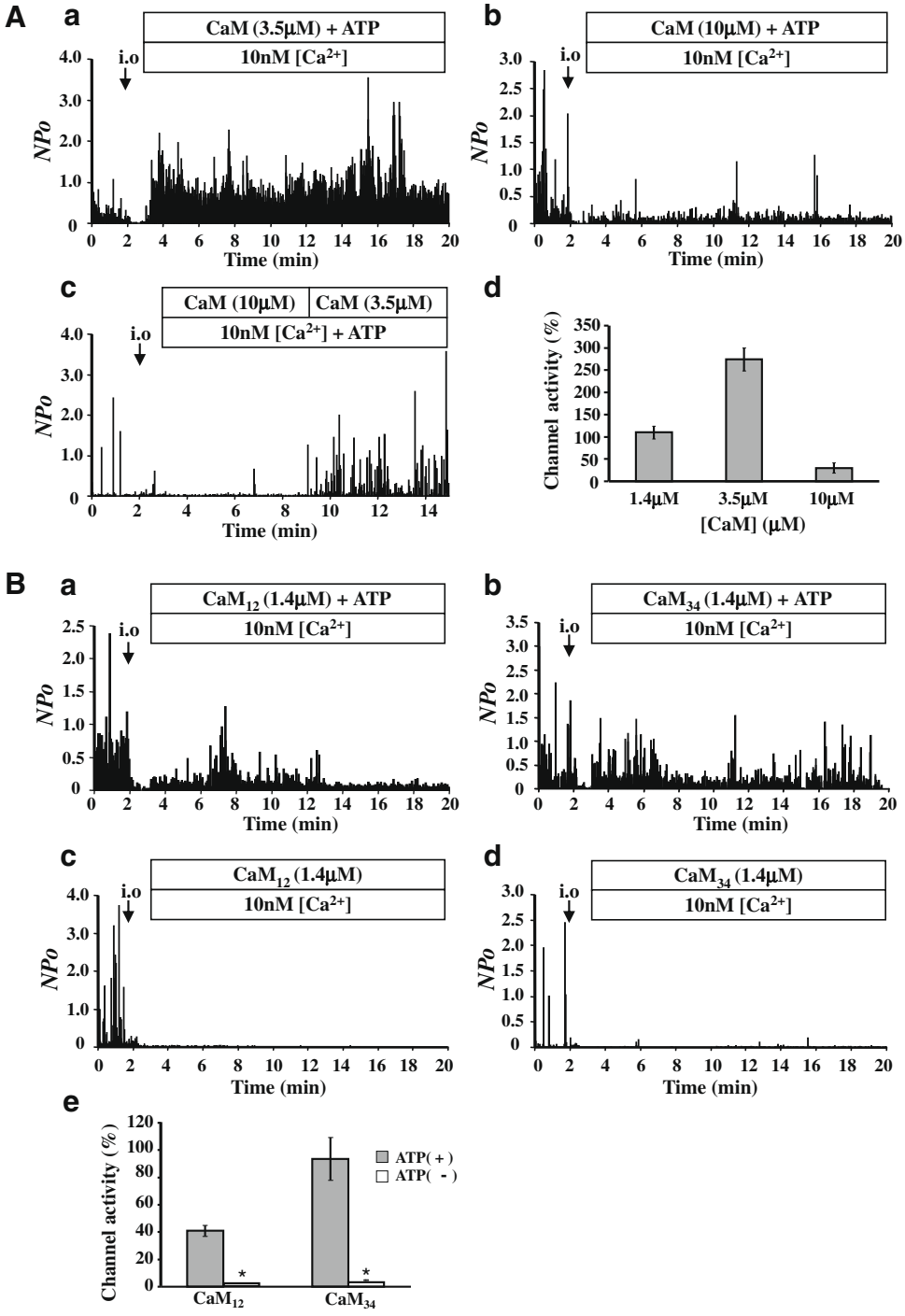

Fig. 1. Effects of CaM, CaM_{12}, and CaM_{34} on $Ca_V1.2$ channel activity. (A) Facilitatory and inhibitory effects of wild-type CaM on $Ca_V1.2$ channel activity. (a–c) Channel activity for repetitive depolarization was calculated as *NPo* and plotted against time. After 2-min recording of *NPo* in the cell-attached mode, the inside-out patch mode (i.o) was initiated as indicated by an arrow and lasted for 1-min. Then, 3.5 μM CaM (a), 10 μM CaM (b), 10 μM CaM followed by 3.5 μM CaM (c), together with 3 mM ATP at 10nM [Ca^{2+}] were applied at the times indicated by the boxes. (d) Normalized channel activity (as represented by *mNPo* relative to control value) induced by 1.4, 3.5, and 10 μM CaM. Number of myocytes for each condition was 5. (B) Effects of CaM_{12} and CaM_{34} on $Ca_V1.2$ channel activity. 1.4 μM CaM_{12} (a,c) or CaM_{34} (b,d) was applied with (a,b) or without (c,d) 3 mM ATP at 10 nM [Ca^{2+}] as indicated by the boxes. Almost no effect was seen without ATP. (e) Summary of the effects of CaM_{12} and CaM_{34} with (+) or without (−) ATP. $^*P < 0.05$ compared between ATP (+) and (−). Number of myocytes for each condition was 5.

obtained by searching the maximum of mean *NPo* values (*mNPo*) of consecutive 3-min periods (90 pulses) taken with one min interval, and normalized by the control channel activity.

Data analysis. Data were presented as means ± SEM. Student's *t*-test was used to evaluate statistical significance, and $P < 0.05$ was considered to be significant. Curve fitting for the [CaM]-channel activity relationship was performed with the software Deltagraph 5.4 according to a model, where mutually independent two CaM-binding sites, one for facilitation and the other for inhibition, was assumed in our previous study [14]. Channel activity (A) as an overall effect of CaM would be:

$$A = A_{max} \times \frac{\left(\frac{[CaM]}{Kd_f}\right)^{n_f}}{1+\left(\frac{[CaM]}{Kd_f}\right)^{n_f}} \times \frac{1}{1+\left(\frac{[CaM]}{Kd_i}\right)^{n_i}}$$

where A_{max} is the maximum effect of CaM, Kd_f and n_f represent [CaM] producing a half effect and the apparent Hill's coefficient, respectively, for facilitation, and Kd_i and n_i represent [CaM] producing a half effect and the apparent Hill's coefficient, respectively, for inhibition.

Results

Facilitatory and inhibitory effects of wild-type CaM on $Ca_V1.2$ channel activity

We first examined the effect of wild-type CaM on $Ca_V1.2$ channel activity in the inside-out patch mode. Since our previous studies suggested that ATP was required for channel activity, we applied CaM with 3 mM ATP [18–20]. As shown in Fig. 1Aa, channel activity decreased rapidly after patch excision in the basic internal solution (rundown). Then application of 3.5 μM CaM at 10 nM [Ca^{2+}]-induced channel activity to a level greater than that in the cell-attached mode (274 ± 25%). However, as shown in Fig. 1Ab, 10 μM CaM + ATP-induced channel activity to a smaller level of control (29.9 ± 11.1%). Fig. 1Ac indicated that the reduced effect of 10 μM CaM was not due to the rundown since the following application of 3.5 μM CaM induced a significant effect. Thus, the effect of CaM on $Ca_V1.2$ channel activity was biphasic as summarized in Fig. 1Ad. These results confirmed our previous finding that the concentration–response relationship of the CaM's effect

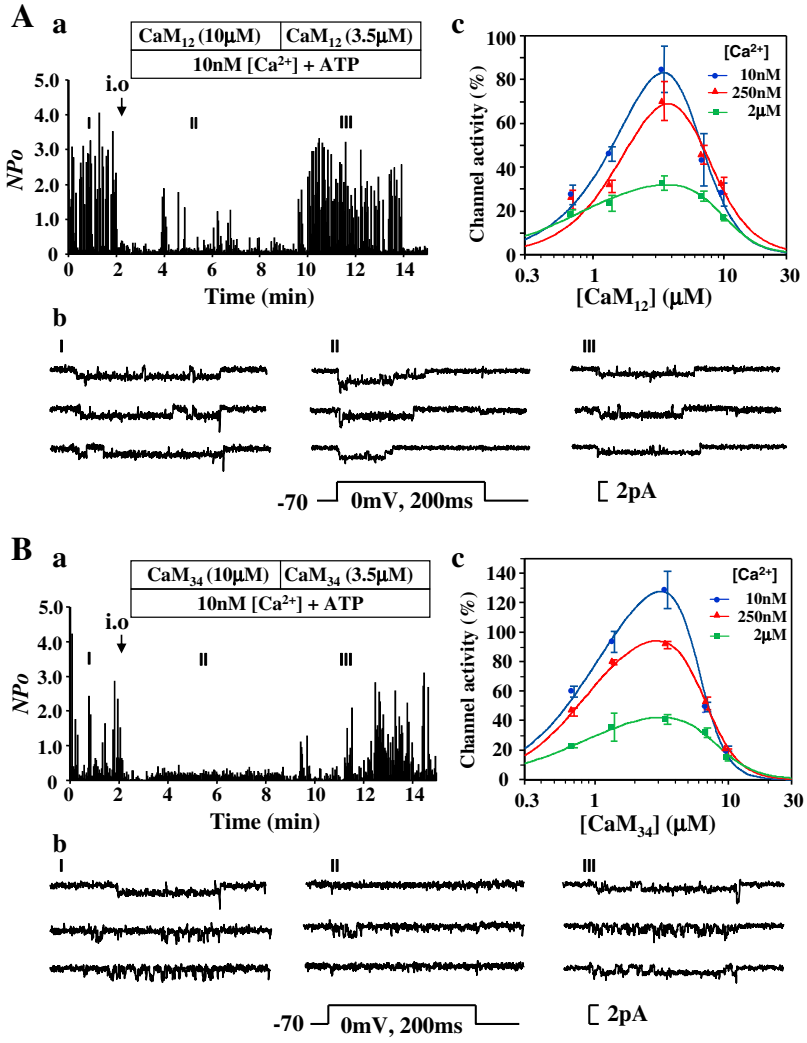

Fig. 2. Concentration-dependent effects of CaM_{12} and CaM_{34} on $Ca_V1.2$ channel activity. (A) (a) After 2-min control recording in the cell-attached mode followed by 1-min inside-out patch formation (i.o), 10 and then 3.5 μM CaM_{12} were then applied with 3 mM ATP at 10nM [Ca^{2+}] as indicated by the boxes. (b) Exemplar traces recorded at the time indicated in (a), for the control cell-attached mode (I), 10 μM (II) and 3.5 μM CaM_{12} (III). (c) Normalized channel activity (*mNPo* relative to control) induced by 0.7–10 μM CaM_{12} with ATP at fixed [Ca^{2+}] of 10 nM (blue), 250 nM (red) and 2000 nM (green). Number of myocytes for each condition was 5. Continuous lines are fitted curves with the equation in Methods. (B) (a) Effect of 10 and 3.5 μM CaM_{34} with a protocol similar to (A). (b) Exemplar traces recorded at the time indicated in (a), for control (I), 10 μM (II) and 3.5 μM CaM_{34} (III). (c) Normalized channel activity induced by 0.7–10 μM CaM_{34} with ATP at three [Ca^{2+}] as indicated. Number of myocytes for each condition was 5. Continuous lines are fitted curves with the equation. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

on the channel showed a bell-shaped facilitatory and inhibitory phase [14].

Effects of CaM_{12} and CaM_{34}

We then examined effects of the CaM mutants, CaM_{12} and CaM_{34}, on $Ca_V1.2$ channel activity. Both CaM_{12} (1.4 μM) and CaM_{34} (1.4 μM) together with 3 mM ATP at 10 nM [Ca^{2+}] produced channel activity as shown in Fig. 1Ba and Bb, respectively. These effects were abolished in the absence of ATP (Fig. 1Bc and Bd). Fig. 1Be summarized the effects of CaM_{12} and CaM_{34} on channel activity with and without ATP. It was found that the CaM mutants CaM_{12} and CaM_{34} could produce $Ca_V1.2$ channel activity in the presence of ATP at a low [Ca^{2+}], as shown for the wild-type CaM.

Concentration-dependent effects of CaM_{12} and CaM_{34}

To investigate concentration-dependent effects of the CaM mutants on $Ca_V1.2$ channel activity, 0.7–10 μM of the CaM mutants were examined with 3 mM ATP at 10 nM [Ca^{2+}]. Fig. 2Aa and Ab showed that first application of 10 μM CaM_{12} produced a relative channel activity of about 30%, and the following 3.5 μM CaM_{12} produced much higher activity of about 80% of control. Similar experiments were repeated with different concentrations of CaM_{12} (0.7–10 μM) at [Ca^{2+}] of 10, 250, and 2000 nM, and the normalized *mNPo* values were plotted in Fig. 2Ac. The curves at three different [Ca^{2+}] were bell-shaped. The effects of CaM_{34} were also biphasic (Fig. 2Ba and Bb). The normalized *mNPo* values, plotted against [CaM_{34}] at [Ca^{2+}] of 10, 250, and 2000 nM, were also bell-shaped (Fig. 2Bc). These data suggested that CaM_{12} and CaM_{34} produced both facilitatory and inhibitory effects on $Ca_V1.2$ channel activity similar to the wild-type CaM. However, it was noted that, unlike the wild-type CaM, the curves for both CaM_{12} and CaM_{34} showed no obvious shift when [Ca^{2+}] was changed from 10 to 250 nM.

Ca^{2+}-dependent effects of CaM_{12} and CaM_{34}

To further examine Ca^{2+}-dependence effects of CaM mutants on the channel, [Ca^{2+}] was changed during application of the CaM mu-

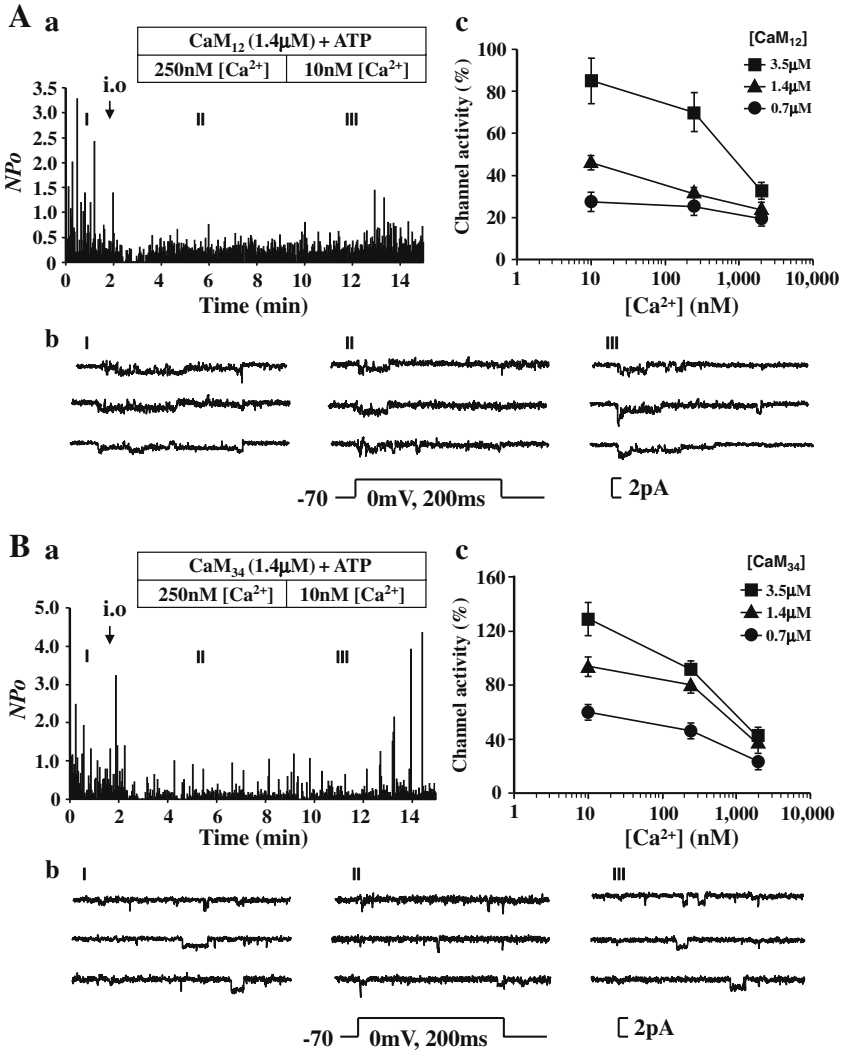

Fig. 3. Ca^{2+}-dependent effects of CaM_{12} and CaM_{34} on $Ca_V1.2$ channel activity. (A) (a) After 2-min control recording in the cell-attached mode followed by 1-min inside-out patch formation (i.o), 1.4 μM CaM_{12} with 3 mM ATP were first applied at 250 and then 10 nM [Ca^{2+}] as indicated by the boxes. (b) Exemplar traces recorded at the time indicated in (a), for control (I), CaM_{12} at 250 nM (II) and 10 nM [Ca^{2+}] (III). Normalized channel activity induced by CaM_{12} of 0.7 (●), 1.4 (▲), and 3.5 μM (■) with 3 mM ATP were plotted against [Ca^{2+}]. Number of myocytes for each condition was 5. (B) (a) Effect of 1.4 μM CaM_{34} with 3 mM ATP at 250 and then 10 nM [Ca^{2+}] as indicated by the boxes with a protocol similar to (A). (b) Exemplar traces recorded at the time indicated in (a), for control (I), CaM_{34} at 250 nM (II) and 10 nM [Ca^{2+}] (III). Normalized channel activity induced by CaM_{34} of 0.7 (●), 1.4 (▲), and 3.5 μM (■) with 3 mM ATP were plotted against [Ca^{2+}]. Number of myocytes for each condition was 5.

tants. In Fig. 3Aa and Ab, CaM_{12} (1.4 μM) was first applied at 250 nM [Ca^{2+}] and then at 10 nM, resulting in a slight increase in *mNPo*, an opposite effect seen with the wild-type CaM. Similar result was obtained also with CaM_{34} (Fig. 3Ba and Bb). The result that channel activity decreased with increasing [Ca^{2+}] was confirmed for three concentrations of CaM_{12} and CaM_{34} (Fig. 3Ac and Bc). These results suggested that, although the concentration–response curves for CaM_{12} and CaM_{34} did not show a Ca^{2+}-dependent shift, it seemed that a Ca^{2+}-dependent mechanism for the channel regulation still existed.

Ca^{2+}-dependence of the parameters for CaM_{12} and CaM_{34} effects

The maximum channel activity (A_{max}) and concentrations for the half-maximum facilitatory and inhibitory effects (Kd_f and Kd_i) of CaM_{12} and CaM_{34} (Fig. 2Ac and Bc) were estimated by curve fitting (see Methods) and were plotted against [Ca^{2+}] in Fig. 4A and B, together with those for the wild-type CaM [14]. The A_{max} values for the CaM mutants were smaller than that for the wild-type with less pronounced Ca^{2+} dependence (Fig. 4A). On the other hand, the Kd_f and Kd_i did not show a consistent change by changing [Ca^{2+}] (Fig. 4B), suggesting that Ca^{2+}-dependent change seen with the wild-type CaM required intact both lobes of CaM.

Discussion

In this study, the most intriguing finding is that exogenously applied both CaM_{12} and CaM_{34} can produce a bell-shaped concentration–response relationship. There is no obvious Ca^{2+}-dependent shift in the concentration–response curve for both the CaM mutants, in contrast to a dramatic Ca^{2+}-dependent shift in the curve for the wild-type CaM.

Concentration-dependent effects of the CaM mutants on $Ca_V1.2$ channel activity

In our previous study, CaM reactivates rundown channels in a concentration-dependent manner [19]. Furthermore, it has been

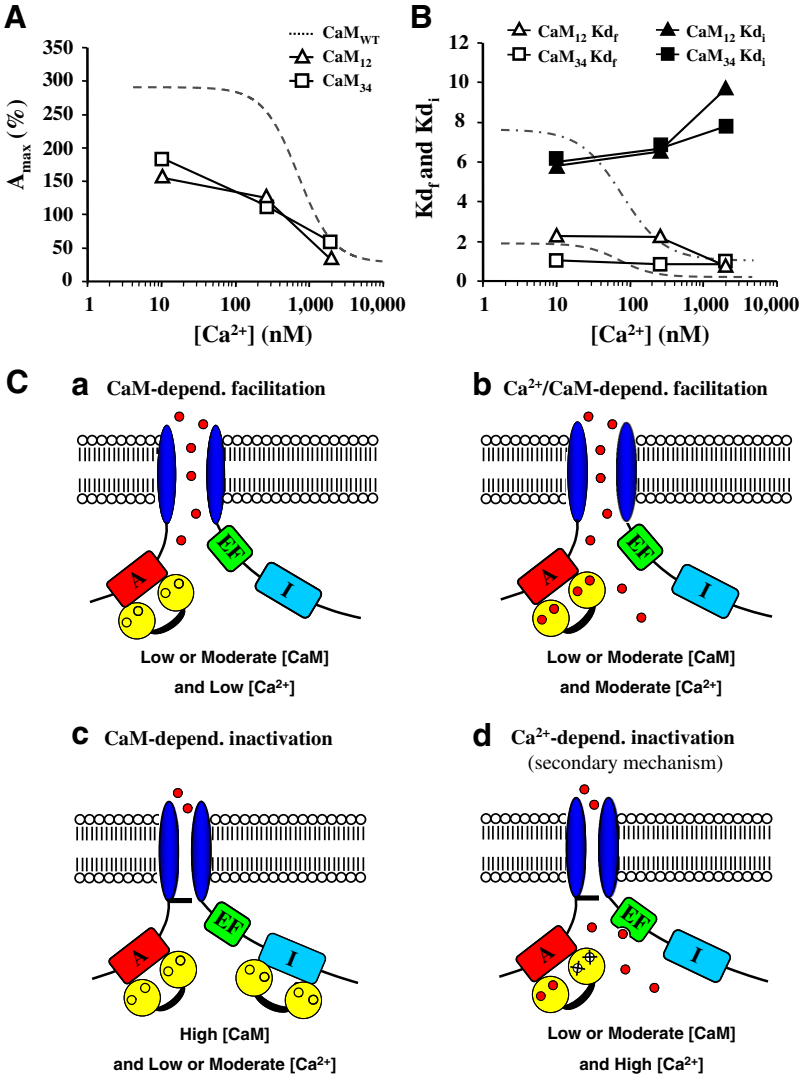

Fig. 4. Ca^{2+}-dependence of the parameters for CaM_{12} and CaM_{34} effects and a hypothetical model for CaM and Ca^{2+} regulations of $Ca_V1.2$ channel. (A) A_{max} for CaM_{12} (△) and CaM_{34} (□) plotted against [Ca^{2+}]. A_{max} for wild-type CaM taken from our previous study [14] is shown by a broken line. (B) Kd_f and Kd_i for CaM_{12} (△, ▲) and CaM_{34} (□, ■) plotted against [Ca^{2+}]. Kd_f and Kd_i for wild-type CaM taken from our previous study [14] are shown by a chain and a broken line, respectively. (C) A hypothetical model for the channel regulation. (a) At low or moderate [CaM] and at low [Ca^{2+}], the activation site (***A***) is partly bound with apoCaM, whereas the inhibitory site (***I***) is CaM-free, resulting in CaM-induced channel activity. (b) At low or moderate [CaM] and at moderate [Ca^{2+}], most of ***A*** site is bound with Ca^{2+}/CaM but ***I*** site is still free, resulting in Ca^{2+}/CaM-induced facilitation of the channel. (c) At high [CaM] and at low or moderate [Ca^{2+}], most of ***A*** and ***I*** sites are occupied by apoCaM or Ca^{2+}/CaM, resulting in apoCaM- or Ca^{2+}/CaM-induced inactivation of the channel. (d) At high [Ca^{2+}], although CaM_{12} or CaM_{34} can interact with ***A*** site but not with ***I*** site, Ca^{2+} binds directly to the extra Ca^{2+}-binding site presumably in the channel (EF), resulting in a channel inactivation (secondary mechanism).

found that CaM and CaM_{1234} show bell-shaped concentration–response relationships [14]. In the present study, CaM_{12} and CaM_{34} also exhibit bell-shaped relationships, supporting the view that the biphasic effects of CaM may be intrinsic to apoCaM. The maximum activity of the channel is produced by concentration of the CaM mutants at around 3 μM. This value is similar to that of apoCaM but not of Ca^{2+}/CaM, implying that CaM_{12} and CaM_{34} behave basically as apoCaM in the channel regulation.

The actions of CaM_{12} and CaM_{34} require ATP, which is consistent with our previous findings that ATP is necessary for basal activity of the channel [18–20]. However, a molecular mechanism underlying the action of ATP still remains to be clarified.

Ca^{2+}-dependent effect of CaM

Although a large number of studies have revealed important aspects of the CaM's role in CDF and CDI of the $Ca_V1.2$ channel [3–5,9–12], a controversy still remains in the role of each lobe of CaM. It has been showed that Ca^{2+}-binding to the C-lobe triggers a rapid CDI process whereas the N-lobe initiates a more gradual and distinct CDI mechanism in $Ca_V1.2$ [10]. However, other studies suggest that CDI of $Ca_V1.2$ channel is attributed mainly to the C-lobe [11,12]. Moreover, it is also indicated that Ca^{2+}/CaM-dependent protein kinase (CaMKII), but not a direct action of CaM, mediates CDF [21,22].

In attempt to explore the lobe-specific function of CaM in CDF and CDI of $Ca_V1.2$, we have examined the Ca^{2+}-dependent effect of CaM_{12} and CaM_{34}. Both Kd_f and Kd_i values for the biphasic action of CaM_{12} and CaM_{34} do not show an obvious Ca^{2+} dependence, which is shown with the wild-type CaM but not with CaM_{1234} [14]. This suggests that both functional lobes of CaM are necessary for manifesting the Ca^{2+}-dependent effect of CaM. Our findings may support the idea that both lobes of CaM participate in CDF and CDI in $Ca_V1.2$ channels.

CaM-independent Ca^{2+} regulation

The present study have found that CaM_{12} and CaM_{34} can decrease A_{max} in a Ca^{2+}-dependent manner similar to that observed with CaM_{1234} [14]. It is therefore hypothesized that there may be an additional Ca^{2+} sensor besides CaM. The affinity of this sensor for Ca^{2+} may be lower than that of CaM, since the decrease in A_{max} occurs at higher [Ca^{2+}] than those in Kd_f and Kd_i which are thought to reflect Ca^{2+}-binding to CaM. Although we have no evidence to identify this extra Ca^{2+}-binding site, one possible candidate may be a region having the EF-hand-like motif in the proximal C-terminus of $Ca_V1.2$ Ca^{2+} channel. Previous studies have highlighted an importance of this EF-hand region for CDI, but the conclusions remain controversial [23,24]. We speculate that CaM in the inactivation site may act as a dominant and high-affinity Ca^{2+} sensor, whereas the postulated extra Ca^{2+} sensor may act as a secondary and low-affinity sensor, constructing a prudent and sensible CDI mechanism.

A hypothetical model for Ca^{2+}-dependent regulation of $Ca_V1.2$ channel

To explain the biphasic action of CaM, we have proposed a simple functional model, in which two CaM-binding sites, one for activation and the other for inactivation, are assumed in the $Ca_V1.2$ channel [14]. The present study extends this model (Fig. 4C). To postulate two CaM-binding sites is further supported by the finding that CaM_{12} and CaM_{34} also show biphasic effects on the channel. Binding of CaM (usually apoCaM) to the activation site produces channel activity (Fig. 4Ca), and the channel activity is facilitated by Ca^{2+}-binding to CaM (Fig. 4Cb). This facilitation seems to require intact both lobes of CaM, since CaM_{12} and CaM_{34} do not show this facilitation. On the other hand, the inactivation site has a lower affinity for apoCaM, so that apoCaM can bind to this site only at [apoCaM] higher than the physiological level (Fig. 4Cc). Alternatively, when [Ca^{2+}] is elevated, Ca^{2+}/CaM would bind to the inactivation site with an increased affinity and thereby inactivate the channel. Again this effect requires intact both lobes of CaM. Besides the CaM's action, there may be a Ca^{2+}-binding site that triggers a secondary inhibitory mechanism (Fig. 4Cd). We speculate that the site may be the EF-hand region or a region that interacts with the downstream inhibitory machinery of the channel.

Conclusions

The present study reveals some novel features of the CaM-mediated regulation of $Ca_V1.2$ channel: First, The effects of CaM mutants CaM_{12} and CaM_{34} on channel activity are bell-shaped, supporting the hypothesis that CaM has both facilitatory and inhibitory effect on the channel; Second, unlike wild-type CaM, neither CaM_{12} nor CaM_{34} shows a Ca^{2+}-dependent shift in the concentration–response curve. These results suggest that both N- and C-lobes of CaM are required for the facilitatory and inhibitory effect on $Ca_V1.2$ channel activity.

Acknowledgments

We thank Ms. E. Iwasaki for secretarial work on the manuscript and Prof. Ji-qun Cai (China Medical Univ.) for continuous encouragement and support. This work was supported by research grants from the Japan Society for the Promotion of Science to L.-Y.H. and to M.K., and from the National Natural Science Foundation of China (30670761, 30870907) to L.-Y.H.

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