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Interactions of Calmodulin With the Multiple Binding Sites of Cav1.2 Ca^{2+} Channels

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Abstract. Although calmodulin binding to various sites of the Cav1.2 Ca^{2+} channel has been reported, the mechanism of the interaction is not fully understood. In this study we examined calmodulin binding to fragment channel peptides using a semi-quantitative pull-down assay. Calmodulin bound to the peptides with decreasing affinity order: IQ > preIQ > I-II loop > N-terminal peptide. A peptide containing both preIQ and IQ regions (Leu^{1599} – Leu^{1668}) bound with approximately 2 mol of calmodulin per peptide. These results support the hypothesis that two molecules of calmodulin can simultaneously bind to the C-terminus of the Cav1.2 channel and modulate its facilitatory and inhibitory activities.

Keywords: calcium channel, calmodulin, ion channel regulation, IQ motif, cardiac myocyte

Introduction

The voltage-gated L-type Ca^{2+} channel is an essential part of the signal transduction system in many cell types. The channel is a heteromeric protein complex that occurs in several subunit combinations. The Cav1.2 channel is the predominant type in the heart and consists of the pore-forming α1C subunit and auxiliary β and α2/δ subunits (1). The Cav1.2 channel is regulated by intracellular Ca^{2+} that permeates through the channel, that is, Ca^{2+}-dependent inactivation (CDI) and facilitation (CDF). These autoregulatory mechanisms are of critical importance for regulation of the electromechanical activity of the heart and other essential physiological processes (2 – 4).

Several studies have implicated that calmodulin (CaM) is tethered to the Cav1.2 channel and mediates the Ca^{2+} signals in CDI. Zühlke and Reuter have reported that deletion of the IQ region, a consensus CaM-binding motif in the C-terminal tail of Cav1.2, eliminates CDI (1). Later studies have also supported the view that the interaction of CaM with the proximal part of the C-terminal tail of the channel plays a prominent role in CDI (1 – 8). Pitt et al. (2), Pate et al. (9), Romanin et al. (10), and Erickson et al. (11) have all reported that a region between the EF-hand–like region and the IQ region, labeled peptide C (2), CB (9), or preIQ (12), binds with CaM and is also involved in CDI.

Although the IQ and preIQ regions are involved in CDI, it remains to be established how many molecules of CaM are involved in the channel regulation. Mori et al. reported that a single CaM interacts with the preIQ and IQ regions and mediates CDI (13). However, a recent crystallographic study provides evidence for multiple units of CaM binding to the preIQ–IQ region of Cav1.2 (14). Thus, although CaM binding to the Cav1.2 channel at multiple sites has been established, it is not fully understood how CaM interacts with the channel during CDI.

CaM is also suggested to be involved in CDF of the Cav1.2 channel. CaM might interact directly with the channel to produce CDF (3, 12). However, there are number of reports that suggest a critical role of channel phosphorylation mediated by Ca^{2+}/CaM-dependent protein kinase II (CaMKII) in CDF. Thus, further work needs to be done to delineate the roles CaM plays during CDF.

In this study, we have examined binding of CaM to the intracellular binding regions of the Cav1.2 channel, including the preIQ and IQ regions in the C-terminal, I-II intracellular loop, and the CaM-binding region in the N-terminus (15, 16), by using a semi-quantitative pull-

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down method. We have found that approximately 2 mol/mol CaM binds with the preIQ–IQ region with a relatively high affinity. These results are consistent with the hypothesis that more than one molecule of CaM binds to the C-terminal tail of Cav1.2 and mediates both CDF and CDI. Possible conformations of the CaM/channel complex during CDF and CDI are discussed.

Materials and Methods

Preparation of fragment peptides of Cav1.2 channel and CaM

The cDNAs of guinea-pig cardiac Cav1.2 channel (Gen Bank AB016287) corresponding to the CaM-binding region in the N-terminus (NT or the N-terminal spatial Ca^{2+} transforming element (16), Ala^{66} – Lys^{93}), an intracellular loop between repeat I and II (I-II loop, Phe^{437} – Asn^{553}), the C-terminal preIQ region (Leu^{1599} – Asp^{1639}), IQ region (Tyr^{1648} – Leu^{1668}), and a longer preIQ–IQ peptide (Leu^{1599} – Leu^{1668}) were inserted into the pGEX vector (GE Healthcare, Uppsala, Sweden). Mutant fragments of the Cav1.2 channel were constructed using the QuickChange site-directed mutagenesis and multisite-directed mutagenesis kits (Stratagene, La Jolla, CA, USA). The corresponding peptides were expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli* BL21, homogenized and solubilized with *N*-lauroylsarcosine sodium salt (1.5%; Sigma-Aldrich, St. Louis, MO, USA), and isolated using glutathione-Sepharose 4B beads (GE Healthcare). The purified peptides were checked on SDS-PAGE and quantified by densitometry using an authentic GST that had a molar extinction coefficient of 1.69 $M^{-1}\cdot cm^{-1}$ at 280 nm.

A recombinant human CaM was expressed as a GST-fusion protein in a similar way to those mentioned above. The GST region of fused CaM was removed by cleavage using PreScission Protease (GE Healthcare). The expressed CaM was checked on SDS-PAGE and quantified by the Bradford method (Pierce, Rockford, IL, USA) using an authentic CaM that had a molar extinction coefficient of 0.18 $M^{-1}\cdot cm^{-1}$ at 276 nm.

Binding assay

The interactions of CaM with the preIQ–IQ, preIQ, IQ, I-II loop, and NT of Cav1.2 channel were examined by a semi-quantitative pull-down assay. GST-fusion fragments of the Cav1.2 channel (5 μg) were immobilized on to glutathione-Sepharose 4B beads (GE Healthcare) and incubated with CaM for 3 h at 4°C in 100 μl Tris buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, and protease inhibitor cocktail (Roche, Mannheim, Germany) in the presence of 2 mM $CaCl_2$. Then the beads were gently washed two times with the same buffer containing the nonionic detergent Tween 20 (0.05%, Sigma-Aldrich), as previously described (7). Bound CaM was eluted with 1× SDS sample buffer and applied to 15% SDS-PAGE. The binding of CaM to the peptides at low [Ca^{2+}] was too weak to analyze in a quantitative manner.

Data analyses

Peptide bands in SDS-PAGE were digitized by a scanner, normalized by the densities of reference GST bands, and quantified with the Image J software (distributed by Dr. Wayne Rasband, NIH, USA) using non-linear calibration curves to calculate the mol/mol binding values of CaM to the peptides. The non-linear calibration curves were constructed with authentic CaM and GST. From the law of mass action, bound [CaM] (Y) for the one-site model is expressed by a Hill equation as follows:

$$Y = B_{max} \cdot \frac{[CaM]_F}{K_d + [CaM]_F} + NSB \quad (1)$$

, where the first term is the specific binding, denoted as B_{max} for the maximum binding, $[CaM]_F$ for the free CaM concentration, and K_d for the dissociation constant. The second term NSB, which was mainly nonspecific binding of CaM to GST and the beads, was verified to be negligible in our experiments. Since $[CaM]_F$ is not known, it is expressed by using total [CaM] (X) as:

$$Y = B_{max} \cdot \frac{(X - Y)}{K_d + (X - Y)} \quad (2)$$

Thus, Y can be expressed as:

$$Y = \frac{b - \sqrt{b^2 - 4B_{max} \cdot X}}{2} \quad (3)$$

, where $b = (K_d + B_{max} + X)$. In the case of the two binding site model, in which we assumed independent binding, the equation is as follows:

$$Y = B_{max1} \cdot \frac{(X - Y)}{K_{d1} + (X - Y)} + B_{max2} \cdot \frac{(X - Y)}{K_{d2} + (X - Y)} \quad (4)$$

, where B_{max1}, K_{d1}, B_{max2}, and K_{d2} denote B_{max} and K_d for the high and low affinity sites, respectively. Thus, Y could be obtained from the solution to the cubic equation. These equations were used to analyze the data via the Kell Ligand program (Biosoft, Cambridge, UK).

Data are presented as the mean ± S.E.M. of at least three separate experiments. Student's t test and Dunnett's test were used to estimate statistical significance and $P < 0.05$ was considered to be significant.

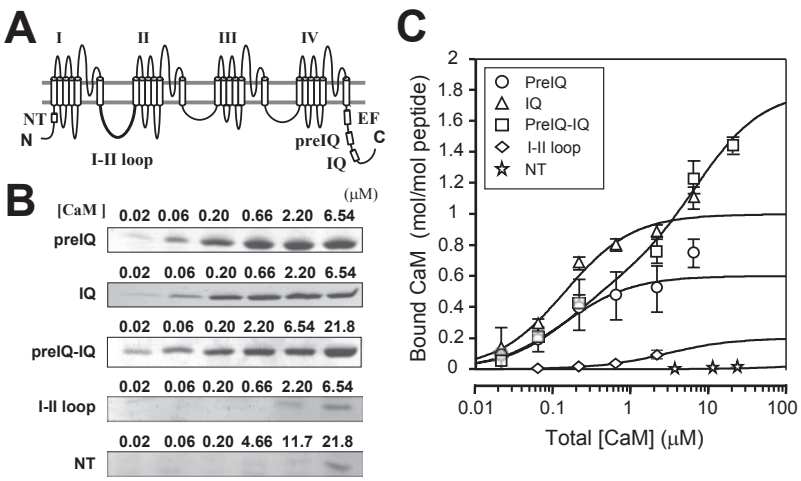

Fig. 1. CaM binding to fragment peptides of the Cav1.2 channel. A: Schematic illustration of the Cav1.2 channel. NT is the CaM-binding region in the N-terminus (ref. 16, shown as a box). EF-hand, preIQ, and IQ regions are shown as boxes in the C-terminal tail. B: GST-pull down assay for the peptides. A 5-μg sample of a GST-fusion peptide (preIQ, IQ, preIQ–IQ, I-II loop, or NT) was mixed with several concentrations of CaM (0.02 – 21.8 μM) in 2 mM Ca^{2+} buffer, and bound CaM was quantified by SDS-PAGE and densitometry. Protein bands stained with Coomassie brilliant blue (CBB) are shown. C: Bound CaM was plotted against total [CaM] on a mol/mol basis.

Results

CaM binds to the multiple binding sites in the Cav1.2 Ca^{2+} channel

It has been reported that CaM binds not only to the C-terminal tail, but also to the I-II loop and the N-terminus (Fig. 1A) (3, 14 – 16). Therefore, we first examined affinities of these regions for CaM using GST-fusion peptides encoding these portions in the semi-quantitative pull-down assay. Figure 1B shows an example of the pull-down experiment, in which various concentrations of CaM (0.02 – 21.8 μM) were incubated with a fixed concentration of GST-fusion fragment peptides at 2 mM [Ca^{2+}]. The binding of CaM to the GST-fusion preIQ or IQ peptide increased with increasing [CaM]. In the control, CaM binding to GST was negligible (not shown). CaM also bound to a longer fragment containing both the preIQ and IQ regions in a concentration-dependent manner. On the other hand, CaM binding to the I-II loop or NT (N-terminal CaM-binding region) was evident only at higher concentrations, suggesting a lower affinity of these regions for CaM.

The CaM bands in the gel was quantified by densitometry and data were analyzed by a computer program to estimate the maximum binding (B_{max}) and apparent dissociation constant (K_d) of CaM (see Materials and Methods). Figure 1C shows a plot of bound CaM against total [CaM] with fitted curves. The maximum binding (B_{max}) of CaM to the preIQ and IQ regions was approximately 0.6 and 1.0 mol/mol peptide, respectively. These data fit to the curve with the single-site model better than the two-site model. The K_d values were estimated as 0.13 and 0.07 μM, respectively. On the other hand, the data for the preIQ–IQ region were fitted with the two-site model providing a total B_{max} (B_{max1} + B_{max2}) of 1.8 mol/mol and the K_{d1} and K_{d2} values were estimated as 0.23 and 1.42 μM, respectively. It should be noted that each value of B_{max1} (0.6) and B_{max2} (1.2) might not be so reliable, although derived as the best fit, possibly due to data variation or an interaction between the presumed two sites. Nevertheless, these analyses suggested that the preIQ–IQ region bound to two molecules of CaM, whereas the preIQ and IQ regions bound to one CaM molecule.

The binding to the I-II loop and NT peptides was associated with a much lower B_{max} and could be fitted only to the one-site model, albeit with lower accuracy. The K_d values were estimated as 2.30 and >100 μM, respectively. The estimated B_{max} and K_d values are summarized in Table 1. The results suggested that CaM bound to the fragment peptides of the Cav1.2 channel in the following order of decreasing affinity: IQ > preIQ > I-II loop > NT in a one-to-one manner. On the other hand, the preIQ–IQ peptide appeared to bind two molecules of CaM, presumably to the preIQ and IQ region at the same time. There was a clear tendency that B_{max} was negatively correlated with K_d (Fig. 1C and Table 1), probably because partial dissociation of the GST-peptide–CaM complex occurred during the washing of the beads. Because of the low affinity values of the NT and I-II loop peptides, we did not further investigate the binding of the NT and I-II loop peptides with CaM.

CaM binding to the preIQ peptides

It has been reported that two regions in the C-terminal portion in the preIQ region are important for CaM interaction, that is, ^{1611}NEELRAIIKKIWKRTSMKLLDQV^{1633} (corresponding the amino acid sequence of guinea-pig Cav1.2) (10) and ^{1614}LRAIIKKIWKRTSMKLL^{1630} (9). Related to these studies, Zühlke and Reuter (1) have reported that NE (Asn^{1611} – Glu^{1612}) is important for CDI. In addition, Soldatov et al. (17) have reported that

Table 1. Dissociation constants (K_d) and maximum bindings (B_{max}) of fragment peptides of Cav1.2 channel for CaM binding

GST-peptides	Site 1		Site 2	
	K_{d1} (μM)	B_{max1} (mol/mol)	K_{d2} (μM)	B_{max2} (mol/mol)
IQ	0.07	1.00	–	–
preIQ	0.13	0.60	–	–
preIQ–IQ	0.23	0.60	1.42	1.20
I-II loop	2.30	0.20	–	–
NT	>100	0.05	–	–

Concentration-dependent binding of GST-fusion fragment peptides derived from Cav1.2 channel with CaM was examined by a semi-quantitative pull-down assay, quantified by densitometry on SDS-PAGE, and the data were analyzed with a Kell Ligand program using a model having either two (preIQ–IQ) or one (peptides other than preIQ–IQ) binding site (see Materials and Methods).

^{1629}LLDQV^{1633} also plays an important role in CDI. Based on this information, we have made several mutations in the preIQ region to explore the amino acids important for interaction with CaM.

Figure 2A shows an example of CaM binding (with total [CaM] of 6 μM at 2 mM [Ca^{2+}]) to mutated preIQ peptides: I1618D (I/D), E1612K (E/K) + I/D, and E/K + I/D + LL1629-30RR (LL/RR). The CaM binding was reduced in all mutants compared with that of wild-type, and the quantified data are summarized in Fig. 2B. These results indicated that all the mutations (E/K + I/D + LL/RR) reduced the amount of CaM binding to preIQ and that the reduction in the CaM binding reflected a decrease in the affinity of the mutated preIQ for CaM. Thus, the results supported the idea that these amino acid residues were important for CaM–preIQ interaction.

CaM binding to the IQ peptides

In contrast to the preIQ region, many studies have investigated CaM binding to the IQ region. Peterson et al. (7) reported biochemical evidence for Ca^{2+}-dependent CaM binding to the IQ motif. In addition, Zühlke et al.

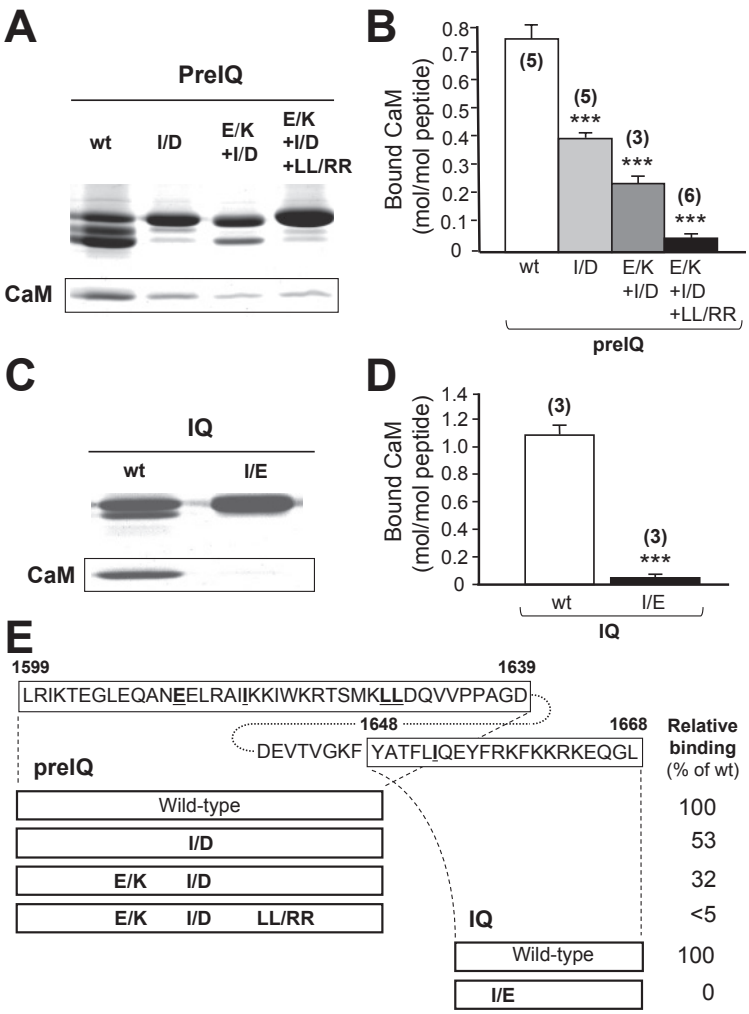

Fig. 2. CaM binding to mutated preIQ and IQ peptides. A: GST-pull down assay for preIQ peptide. GST-fusion preIQ peptide and its mutants (I/D, E/K + I/D, and E/K + I/D + LL/RR) (5 μg each) were mixed with 6 μM CaM in 2 mM Ca^{2+} buffer, and bound CaM was quantified by SDS-PAGE and densitometry. CBB-stained protein bands are shown. B: Summary of the bound CaM to the preIQ peptide and its mutants. Number of data points is shown in parentheses. ***$P < 0.001$, compared with the wild-type. C: GST-pull down assay for the IQ peptide. GST-fusion IQ peptide and its mutant (I/E) (each 5 μg) were mixed with 6 μM CaM and bound CaM was quantified as in A. CBB-stained protein bands are shown. D: Summary of the bound CaM to the IQ peptide and its mutant. Number of data points is shown in parentheses. ***$P < 0.001$, compared with the wild-type. E: Illustration of the effects of mutations in the preIQ and IQ peptides. Amino acid sequences of the preIQ and IQ peptides are shown at the top. Locations of the mutations and their residual CaM binding, relative to that of the wild-type, are also shown.

(5, 18) and Kim et al. (12) reported that a mutation of Ile[1653] to Glu (I1653E, I/E) in the IQ region inhibited CDI and reduced CaM binding.

In this study, we re-examined the effect of the I/E mutation on CaM binding to the IQ region. As shown in Fig. 2C, the I/E mutation nearly completely abolished the CaM binding (see also Fig. 2D), confirming that Ile[1653] is an important amino acid residue for the interaction between CaM and the IQ region.

The effects of mutations in the preIQ and IQ peptides are illustrated in Fig. 2E. It is shown that a single mutation in the preIQ peptide produced a significant but relatively small reduction in CaM binding, whereas the I/E mutation in the IQ peptide significantly decreased its affinity for CaM.

CaM binding to the preIQ–IQ peptides

There have been only a few reports about the binding of CaM to the preIQ–IQ peptide (Leu[1599] – Leu[1668] that covers both the preIQ and IQ regions) (14, 19). We found that the preIQ–IQ peptide could bind to more than one molecule of CaM (Fig. 1C and Table 1). To explore the binding sites of CaM in this region, we performed a pull-down assay using GST-fusion preIQ–IQ peptides containing mutations that were examined in the short preIQ and IQ peptides.

First, we examined the CaM binding with a peptide mutated at [1601]IKT[1603] since this region has been reported to be important for CDI (17). The CaM binding to the preIQ–$IQ_{IKT/AAA}$, with total [CaM] of 6 μM and 2 mM [Ca^{2+}], was 82% of that for the wild-type, which was not significantly different. In contrast, the mutation of Ile[1618] to Asp (I/D) or Ile[1653] to Glu (I/E) in the preIQ–IQ peptide resulted in a significant reduction (45% or 36%, respectively) in the CaM binding. Mutations in two or more regions, i.e., IKT/AAA + I/E, I/D + I/E, E/K + I/D + I/E, and E/K + I/D + LL/RR + I/E, resulted in further reductions in binding, except for preIQ–$IQ_{IKT/AAA+I/E}$. The results are summarized in Fig. 3A. It should be noted that although increasing the number of mutations reduced the CaM-binding in a gradual manner, even the preIQ–$IQ_{E/K+I/D+LL/RR+I/E}$ possessed a CaM-binding of about 30% of the wild-type.

As shown in Fig. 1C, a decrease in B_{max} (B_{max1} + B_{max2}) in our pull-down assay may arise from an increased K_d value, due to partial dissociation of the loosely bound GST-peptide–CaM during the washing process. To explore this possibility, we examined binding between CaM and the peptide preIQ–$IQ_{E/K,I/D,LL/RR,I/E}$ at 0.02 – 20 μM [CaM]. As shown in Fig. 4, CaM bound to the mutant peptide in a concentration-dependent manner, with a K_d of 3.23 μM. This result suggested that although the quadruple mutations of the preIQ–IQ peptide reduced B_{max}, the mutant peptide still possessed CaM-binding capability, albeit with a lowered affinity.

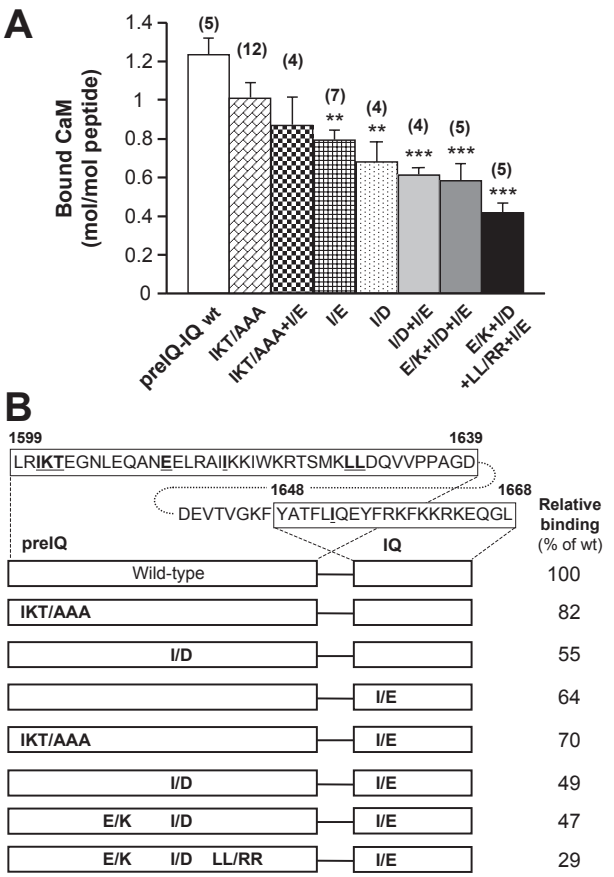

Fig. 3. CaM binding to mutated preIQ–IQ long peptides. A: Summary of CaM binding to the preIQ–IQ peptide and its mutant. Number of data points is shown in parentheses. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the wild-type. B: Illustration of the effects of mutations in the preIQ–IQ peptide. Amino acid sequences of the preIQ and IQ peptides are shown at the top. Locations of the mutations and their residual CaM binding, relative to that of the wild-type, are also shown.

Discussion

In this study, we have explored the binding of CaM to multiple binding sites in the Cav1.2 Ca^{2+} channel. The Cav1.2 channel shows CDI and CDF and CaM is thought to be involved in both (1 – 14 and see 20). Although there are many reports on the CaM-binding sites responsible for CDI or CDF, models suggesting the conformation of the CaM-channel complex are diverse and remain to be clarified.

Two molecules of CaM bind to the C-terminus of Cav1.2 channel

A number of reports suggest that CaM interacts with

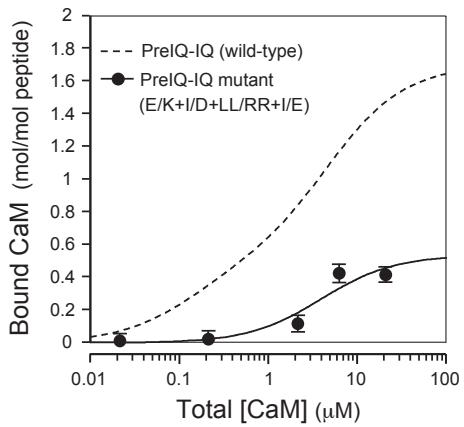

Fig. 4. Concentration-dependent binding of CaM to a multi-site mutant of the preIQ–IQ peptide. GST-fusion preIQ–$IQ_{E/K+I/D+LL/RR+I/E}$ (5 μg) was mixed with 6 μM CaM at 2 mM [Ca^{2+}] and bound CaM was quantified as in Fig. 1. CaM bound to the mutant peptide is plotted against total [CaM] (closed circle). A best-fit curve was obtained with the one-site model, rather than the two-site model, having a K_d of 3.23 μM and B_{max} of 0.50. For comparison, CaM binding to the wild-type preIQ–IQ is shown by a broken line with K_{d1} and K_{d2} of 0.23 and 1.42 μM, respectively, and B_{max1} and B_{max2} of 0.6 and 1.2, respectively.

the proximal C-terminal region including the EF-hand, preIQ, and IQ in a Ca^{2+}-dependent manner (12). The amino acid sequence of the proximal C-terminal region is nearly identical among species (21), implying an important role in the channel regulation. Pitt et al. have suggested that the C-lobe of CaM interacts with the IQ region and the N-lobe of CaM interacts with the preIQ region (2). However, Dick et al. suggest that the N-lobe of CaM does not interact with the preIQ region but interacts with a region in the N-terminus, making a bridge between the N- and C-terminal tails of the channel (16). Another line of evidence suggests that Ca^{2+}/CaM-activated CaMKII interacts directly with the C-terminal tail or mediates phosphorylation of the channel during CDF (18, 20, 22). All these hypotheses assume that only one molecule of CaM interacts directly with the channel during CDI or CDF. Recently, however, a crystallographic study has suggested that more than one molecule of CaM can simultaneously bind to the C-terminus of Cav1.2 and possibly participate in the channel regulation (14).

In this study, we employed a semi-quantitative pull-down method to examine the stoichiometric binding of CaM to the peptides derived from the Cav1.2 channel. Similar methods have been successfully employed by Pitt et al. (2) and Saud et al. (23). We have found that about two molecules of CaM can bind to the preIQ–IQ peptide of Cav1.2 (Fig. 1C). This is further supported by the finding that mutations in one region (preIQ or IQ) of the preIQ–IQ peptide only eliminate about half of the binding affinity to CaM (Fig. 3). Since short preIQ and IQ peptides can bind with one molecule each of CaM, it is reasonable to state that the longer preIQ–IQ peptide binds with two molecules of CaM at the same time. This idea is consistent with our hypothesis that CaM binding to one site plays a role in CDF and the binding of the second CaM to the other site triggers CDI (24).

Complex feature of the CaM binding

Another interesting result of this study is that although the preIQ–IQ peptide is likely to have two CaM-binding sites, the K_d value of the high affinity site for CaM (K_{d1} = 0.23 μM) is 2 – 3 times larger than that of the short IQ peptides (K_d = 0.07 μM) or the preIQ peptide (K_d = 0.13 μM). The K_d value of the low affinity site in the preIQ–IQ peptide (K_{d2} = 1.42 μM) is much higher than those for the preIQ and IQ peptides. A reason for the reduced affinity of the preIQ–IQ peptide for CaM is not clear. Nevertheless, these results indicated that the binding of CaM to the long preIQ–IQ peptide is not a linear summation of those for the individual short preIQ and IQ peptides. One possibility is that the two CaM-binding sites in the preIQ–IQ region might interact with each other in a negatively cooperative manner. A similar observation has been reported in a nuclear magnetic resonance study (19).

Mapping of the CaM-binding sites

Several important amino acid sequences have been reported to be involved in CaM binding in the preIQ and IQ regions. In the preIQ region, ^{1590}TLFALVRTAL^{1599}, ^{1601}IKTEG^{1605}, ^{1617}IIKIWKRTSMKLL^{1630}, and ^{1629}LLDQV^{1633} are suggested to be involved in the CaM binding (2, 3, 9, 10, 12, 14). In addition, Glu^{1612}, Leu^{1629}, and Leu^{1630} are reported to be important for CDI (1, 17) and are included in the presumed CaM-binding sites by Pate et al. (9). In the IQ region, ^{1643}TVGKFYATFLIQEYSRKFKKRK^{1664} has been shown to be the CaM-binding domain (14, 25, 26). In this study, we examined the effects of mutations in these domains on the CaM binding to the fragment peptides from this region.

We have confirmed that mutations in four amino acid residues, Glu^{1612}, Ile^{1618}, Leu^{1629}, and Leu^{1630}, significantly affect the CaM binding to the preIQ peptide. Total replacement of these residues reduced the CaM binding by 95%. We also confirmed that a mutation at Ile^{1653} abolishes the CaM binding to the IQ peptide. This result is consistent with reports that the mutation of Ile^{1653} diminishes CaM binding to the IQ region and CDI (5, 17). It is interesting to note that the CaM binding to the IQ peptide is abolished by a single-site mutation (at Ile^{1653}), while the binding to the preIQ peptide is only nearly abolished

by a multiple-site mutation at dispersed regions.

One of the important findings from this study is that the effects of mutations on the CaM binding in the preIQ–IQ long peptide is not simply the additive sum of the effects of the corresponding mutations in the preIQ and IQ short peptides. The preIQ–IQ peptide mutated at Glu[1612], Ile[1618], Leu[1629] – Leu[1630], and Ile[1653] still possesses a CaM-binding capability of about 30% to that of the wild-type. This remaining CaM binding may be attributed to a possible CaM-binding domain beside the mutated domains that is not detected in the experiments with the short preIQ and IQ peptides. Alternatively, the CaM–preIQ–IQ complex might have multiple conformations, for example, bridge formation between two of the CaM-binding domains named A ([1601]IKTEG[1605]) and C ([1629]LL DQV[1633]) and IQ (2). Our mutations in the preIQ–IQ peptide cannot disrupt all the possible conformations, and it is possible that one or more viable conformations of the CaM–preIQ–IQ complex are still intact. It is also possible that CaM may shift from its original binding conformation to a masked one. The present study can not exclude the possibility that there may be a CaM-binding site between the preIQ and IQ region that is not covered in our preIQ or IQ peptides. In this region, there are amino acid residues ([1643]TVGKF[1647]) that have been reported as the CaMKII binding sites in the Cav1.2 channel (4). Another limitation in this study is that we do not know the possible effect of mutations on the tertiary structure of the peptides. This effect, if any, would produce a change in the affinity for CaM of a remote site. Further studies are needed to examine these problems.

Hypothetical models for CaM binding to the preIQ–IQ region

Based on the previous and present findings, we propose hypothetical models for the CaM interaction with the preIQ–IQ region in the Cav1.2 channel. We assume that two molecules of CaM can bind to this region in multiple conformations (Fig. 5). The first model involves one molecule of CaM binding to the IQ region in a compact manner and relatively high affinity (Fig. 5Ba). The Ile[1653] plays an important role in interacting with the hydrophobic pocket of Ca^{2+}/CaM (14, 27), and its mutation produces a profound effect on CaM binding (5, 18). The second molecule of CaM binds to the preIQ region also in a compact conformation but at multiple possible sites involving Glu[1612], Ile[1618], Leu[1629], and Leu[1630] with a relatively low affinity compared with the IQ region. This weak binding will allow CaM to easily move from one site to another site within the preIQ region. The finding that single or double mutations in the preIQ region do not show a profound suppression in CaM binding is consistent with this model. One candidate for the CaM-binding

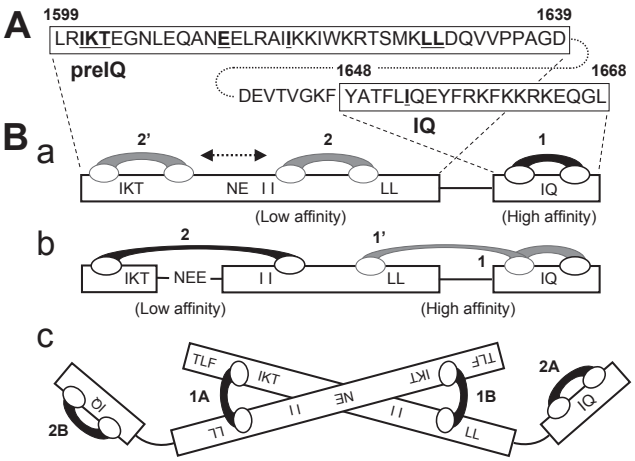

Fig. 5. Hypothetical models for CaM interaction with the preIQ and IQ regions. A: Amino acid sequences of the preIQ and IQ regions of the Cav1.2 channel. B: CaM and the preIQ and IQ regions of the channel are represented by dumb-bell forms and boxes, respectively. a: One CaM binds to IQ in a compact form (1) and the other binds to preIQ in either of the multiple non-bridging conformations (2 and 2'). b: One CaM binds to IQ in a compact form (1) or to a site bridging between preIQ and IQ (1') and the other binds to discrete sites of preIQ in a bridging conformation (2). c: A schematic illustration of the crystal structure formed by two preIQ–IQ peptides and two CaM reported by Fallon et al. (14).

site in the preIQ is a domain centered around Ile[1618], since a mutation in this residue produces about 50% reduction in the binding of CaM to the preIQ–IQ peptide (Fig. 3B). Other candidates would be domains containing Glu[1612] or Leu[1629] – Leu[1630], both of which have been reported to be important for CDI (1, 17).

The second hypothesis involves CaM binding to discrete sites in the preIQ–IQ region (Fig. 5Bb). The first molecule of CaM binds tightly to the IQ region or to both the preIQ and IQ regions, probably in a lobe-specific manner (2, 26 – 29). The second molecule of CaM may bind to a site within the preIQ region. For comparison, a crystallographic model reported by Fallon et al. (14) is shown in Fig. 5Bc. The N-lobe of CaM interacts with the proximal portion of the preIQ region of one peptide, while the C-lobe interacts with the C-terminal portion of the preIQ region of the other peptide (14, 25). However, it is hardly conceivable that a dimeric formation of the C-terminal tails bridged by CaM takes place in the membrane of intact myocytes. It is interesting to speculate that if the α-helix structure of the preIQ region was interrupted at its center (near [1611]NEE[1613]), the two CaM-binding sites would become adjacent, allowing CaM to bind to a single preIQ peptide as shown in Fig. 5Bb. Although the models are highly speculative, they would be helpful for future studies as a working hypothesis.

In this study, we have found that two molecules of

CaM bind to the preIQ–IQ peptide of the Cav1.2 channel. However, we have not evaluated the CaM binding to a full-sized channel protein nor the functional significance of the CaM binding to the channel. Therefore, further studies are required to evaluate these problems and thereby understand CaM interactions with the C-terminal tail and other regions of Cav1.2 channel during CDI and CDF.

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References

1 Zühlke RD, Reuter H. Ca^{2+}-sensitive inactivation of L-type Ca^{2+} channels depends on multiple cytoplasmic amino acid sequences of the α_{1C} subunit. Proc Natl Acad Sci U S A. 1998;95:3287–3294.

2 Pitt GS, Zühlke RD, Hudmon A, Schulman H, Reuter H, Tsien RW. Molecular basis of calmodulin tethering and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels. J Biol Chem. 2001;276:30794–30802.

3 Halling DB, Aracena-Parks P, Hamilton SL. Regulations of voltage-gated Ca^{2+} channels by calmodulin. Sci STKE. 2005;315:1–10.

4 Pitt GS. Calmodulin and CaMKII as molecular switches for cardiac ion channels. Cardiovasc Res. 2007;73:641–647.

5 Zühlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H. Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature. 1999;399:159–162.

6 Mouton J, Feltz A, Maulet Y. Interactions of calmodulin with two peptides derived from the C-terminal cytoplasmic domain of the Cav1.2 Ca^{2+} channel provide evidence for a molecular switch involved in Ca^{2+}-induced inactivation. J Biol Chem. 2001;276:22359–22367.

7 Peterson BZ, De Maria CD, Yue DT. Calmodulin is the Ca^{2+} sensor for Ca^{2+}-dependent inactivation of L-type calcium channels. Neuron. 1999;22:549–558.

8 De Leon M, Wang Y, Jones L, Perez-Reyes E, Wei X, Soong TW, et al. Essential Ca^{2+}-binding motif for Ca^{2+}-sensitive inactivation of L-type Ca^{2+} channels. Science. 1995;270:1502–1506.

9 Pate P, Mochca-Morales J, Wu Y, Zhang JZ, Rodney G, Serysheva I, et al. Determinants for calmodulin binding on voltage-dependent Ca^{2+} channels. J Biol Chem. 2000;275:39786–39792.

10 Romanin C, Gamsjaeger R, Kahr H, Schaufler D, Carlson O, Abernethy DR, et al. Ca^{2+} sensors of L-type Ca^{2+} channel. FEBS Lett. 2000;487:301–306.

11 Erickson MG, Liang H, Mori MX, Yue DT. FRET two-hybrid mapping reveals function and location of L-Type Ca^{2+} channel CaM preassociation. Neuron. 2003;39:97–107.

12 Kim J, Gosh S, Nunziato DA, Pitt GS. Identification of the components controlling inactivation of voltage-gated Ca^{2+} channels. Neuron. 2004;41:745–754.

13 Mori MX, Erickson MG, Yue DT. Functional stoichiometry and local enrichment of calmodulin interacting with Ca^{2+} channels. Science. 2004;304:432–435.

14 Fallon JL, Baker MR, Xiong L, Loy RE, Yang G, Dirksen RT, et al. Crystal structure of dimeric cardiac L-type calcium channel regulatory domains bridged by Ca^{2+}·calmodulins. Proc Natl Acad Sci U S A. 2009;106:5135–5140.

15 Zhou H, Yu K, McCoy KL, Lee A. Molecular mechanism for divergent regulation of Cav1.2 Ca^{2+} channels by calmodulin and Ca^{2+}-binding protein-1. J Biol Chem. 2005;280:29612–29619.

16 Dick IE, Tadross MR, Liang H, Tay LH, Yang W, Yue DT. A modular switch for spatial Ca^{2+} selectivity in the calmodulin regulation of Cav channels. Nature. 2008;451:830–834.

17 Soldatov NM, Oz M, O'Brien KA, Abernethy DR, Morad M. Molecular determinants of L-type Ca^{2+} channel inactivation. J Biol Chem. 1998;273:957–963.

18 Zühlke RD, Pitt GS, Tsien RW, Reuter H. Ca^{2+}-sensitive inactivation and facilitation of L-type Ca^{2+} channels both depend on specific amino acid residues in a consensus calmodulin-binding motif in the α_{1C} subunit. J Biol Chem. 2000;275:21121–21129.

19 Xiong L, Kleerekoper QK, He R, Putkey JA, Hamilton SL. Sites on calmodulin that interact with the C-terminal tail of Cav1.2 channel. J Biol Chem. 2005;280:7070–7079.

20 Dzhura I, Wu Y, Colbran RJ, Balser JR, Anderson ME. Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. Nat Cell Biol. 2000;2:173–177.

21 Ding S, Kuroki S, Kameyama A, Yoshimura A, Kameyama M. Cloning and expression of the Ca^{2+} channel α1C and β2a subunits from guinea pig heart. J Biochem. 1999;125:750–759.

22 Hudmon A, Schulman H, Kim J, Maltez JM, Tsien RW, Pitt G.S. CaMKII tethers to L-type Ca^{2+} channels, establishing a local and dedicated integrator of Ca^{2+} signals for facilitation. J Cell Biol. 2005;171:537–547.

23 Saud ZA, Minobe E, Wang WY, Han DY, Horiuchi M, Hao LY, et al. Calpastatin binds to a calmodulin-binding site of cardiac Cav1.2 Ca^{2+} channels. Biochem Biophys Res Comm. 2007;364:372–377.

24 Han DY, Minobe E, Wang WY, Fuo F, Xu JJ, Hao LY, et al. Calmodulin- and Ca^{2+}-dependent facilitation and inactivation of the $Ca_V1.2$ Ca^{2+} channels in guinea-pig ventricular myocytes. J Pharmacol Sci. 2010;112:310–319.

25 Van Petegem F, Chatelain FC, Minor DL Jr. Insights into voltage-gated calcium channel regulation from the structure of the Cav1.2 IQ domain-Ca^{2+}/calmodulin complex. Nat Struct Mol Biol. 2005;12:1108–1115.

26 Mori MX, Vander Kooi CW, Leahy DJ, Yue DT. Crystal structure of the Cav2 IQ domain in complex with Ca^{2+} calmodulin: high-resolution mechanistic implications for channel regulation by Ca^{2+}. Structure. 2008;16:607–620.

27 Fallon JL, Halling DB, Hamilton SL, Quiocho FA. Structure of Calmodulin bound to the hydrophobic IQ domain of the cardiac Cav1.2 calcium channel. Structure. 2005;13:1881–1886.

28 Tang W, Halling DB, Black DJ, Pate P, Zhang JZ, Pedersen SE, et al. Apocalmodulin and Ca^{2+} calmodulin-binding sites on the Cav1.2 channel. Biophys J. 2003;85:1538–1547.

29 Black DJ, Halling DB, Mandich DV, Pedersen SE, Altschuld RA, Hamilton SL. Calmodulin interactions with IQ peptides from voltage-dependent calcium channels. Am J Physiol Cell Physiol. 2005;288:C669–C676.