Multiple C-terminal tail Ca^{2+} /CaMs regulate $\text{Ca}_v1.2$ function but do not mediate channel dimerization

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Interactions between voltage-gated calcium channels (Ca_v s) and calmodulin (CaM) modulate Ca_v function. In this study, we report the structure of a Ca^{2+} /CaM $\text{Ca}_v1.2$ C-terminal tail complex that contains two PreIQ helices bridged by two Ca^{2+} /CaMs and two Ca^{2+} /CaM–IQ domain complexes. Sedimentation equilibrium experiments establish that the complex has a 2:1 Ca^{2+} /CaM:C-terminal tail stoichiometry and does not form higher order assemblies. Moreover, subunit-counting experiments demonstrate that in live cell membranes $\text{Ca}_v1.2$ s are monomers. Thus, contrary to previous proposals, the crystallographic dimer lacks physiological relevance. Isothermal titration calorimetry and biochemical experiments show that the two Ca^{2+} /CaMs in the complex have different properties. Ca^{2+} /CaM bound to the PreIQ C-region is labile, whereas Ca^{2+} /CaM bound to the IQ domain is not. Furthermore, neither of lobes of apo-CaM interacts strongly with the PreIQ domain. Electrophysiological studies indicate that the PreIQ C-region has a role in calcium-dependent facilitation. Together, the data show that two Ca^{2+} /CaMs can bind the $\text{Ca}_v1.2$ tail simultaneously and indicate a functional role for Ca^{2+} /CaM at the C-region site.

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Introduction

High-voltage-activated calcium channels (Ca_v1 s and Ca_v2 s) couple membrane depolarization-dependent calcium entry to excitation-contraction, gene regulation, hormone release, and synaptic transmission (Catterall, 2000; Hille, 2001). As calcium is an intracellular messenger and both Ca_v1 s and Ca_v2 s are important calcium sources (Clapham, 2007), Ca_v s are regulated by a diverse set of feedback mechanisms. These include two in which calcium–calmodulin (Ca^{2+} /CaM) has a central role (Dunlap, 2007): calcium-dependent inactivation (CDI) in which calcium influx promotes channel closing, and calcium-dependent facilitation (CDF), a process that enhances channel opening in response to elevated cytoplasmic calcium.

Ca_v1 s and Ca_v2 s are multisubunit complexes that comprise a $\text{Ca}_v\alpha_1$ pore-forming subunit, $\text{Ca}_v\beta$ and $\text{Ca}_v\alpha_2\delta$ auxiliary subunits and calmodulin (CaM; Van Petegem and Minor, 2006). The $\text{Ca}_v\alpha_1$ C-terminal cytoplasmic tail bears an IQ domain that is thought to be a central site of the CaM interactions that govern CDI and CDF (Peterson *et al*, 1999; DeMaria *et al*, 2001; Lee *et al*, 2003; Liang *et al*, 2003; Van Petegem *et al*, 2005; Kim *et al*, 2008; Mori *et al*, 2008) and that has been intensely scrutinized by crystallographic, biochemical and functional studies (Fallon *et al*, 2005; Van Petegem *et al*, 2005; Kim *et al*, 2008; Mori *et al*, 2008).

In addition to the IQ domain, biochemical studies have indicated that the $\text{Ca}_v1.2$ C-terminal tail contains other CaM-binding sites (Pate *et al*, 2000; Mouton *et al*, 2001; Pitt *et al*, 2001), generally known as the A-region and C-region (Pate *et al*, 2000; Pitt *et al*, 2001). These are located between the EF-hand motif that follows the last $\text{Ca}_v\alpha_1$ transmembrane segment, IVS6, and the IQ motif. The role of the A and C sites in Ca_v modulation, together with the issue of exactly how many CaMs bind the C-terminal tail remain unresolved. Gel shift experiments using a refolded $\text{Ca}_v1.2$ C-terminal tail construct spanning the EF-hand through IQ domain (Xiong *et al*, 2005) suggested that only single Ca^{2+} /CaM binds to the C-terminal tail, even though this construct contains three potential Ca^{2+} /CaM-binding determinants. In addition, functional studies of $\text{Ca}_v1.2$ –CaM fusions co-expressed with a dominant-negative CaM mutant have been interpreted as evidence that no more than one CaM is required for $\text{Ca}_v1.2$ CDI (Mori *et al*, 2004). In contrast, observation of a crystallographic $\text{Ca}_v1.2$ C-terminal tail dimer and a modest effect of a PreIQ E→P mutation on CDI led to the proposal that $\text{Ca}_v1.2$ channels dimerize through an anti-parallel coiled-coil inter-

action augmented by bridging interactions between Ca^{2+} /CaM and A-region- and C-region-binding sites and that this interaction is functionally relevant (Fallon *et al*, 2009).

Besides a potential role in Ca^{2+} /CaM binding, various studies have suggested that the region N-terminal to the IQ domain (the 'PreIQ' region), which overlaps with the A and C regions, may be an apo-CaM-binding site (Erickson *et al*, 2003). It has been proposed that CaM lobes move between the PreIQ and the IQ domains as part of an apo-CaM to Ca^{2+} /CaM transition that is important for function (Pate *et al*, 2000; Mouton *et al*, 2001; Pitt *et al*, 2001; Erickson *et al*, 2003; Kim *et al*, 2004b), although the details of the PreIQ-binding sites, putative transitions, and relationship to calcium-dependent Ca_V modulation remain to be elucidated.

In this study, we present a crystal structure of an *in vivo* produced Ca^{2+} /CaM- $\text{Ca}_V1.2$ PreIQ-IQ domain complex. Similar to the report by Hamilton and colleagues (Fallon *et al*, 2009) using a complex produced from refolded material, the crystallographic asymmetric unit contains an anti-parallel dimer of PreIQ helices bridged by two Ca^{2+} /CaMs. To determine whether dimerization occurs under physiological conditions, we asked whether dimers occur in solution and in full-length channels in the membranes of live cells. We find no evidence for this interaction in solution or when we directly measure the oligomeric state of $\text{Ca}_V1.2$ channels in cell membranes. Furthermore, mutations at the PreIQ helix crystallographic dimer interface cause no significant functional changes in key modulatory processes that are known to be affected by elements in the C-terminal tail: CDI, CDF, and voltage-dependent inactivation (VDI; Zühlke and Reuter, 1998; Zühlke *et al*, 1999; Kim *et al*, 2004b; Barrett and Tsien, 2008). Thus, contrary to the previous proposal (Fallon *et al*, 2009), the crystallographic dimer lacks physiological relevance.

We further show that the 2:1 Ca^{2+} /CaM- $\text{Ca}_V1.2$ tail complex has a dissociable Ca^{2+} /CaM and that there is a clear preference of the C-region site for Ca^{2+} /CaM lobes over apo-CaM lobes. Electrophysiological characterization of structure-based mutations in the PreIQ helix shows no correlation between Ca^{2+} /CaM binding and CDI, consistent with the proposal that a single CaM is required for CDI (Mori *et al*, 2004). In contrast, CDF is affected by C-region CaM-binding site mutations. Taken together, the data indicate a role for the PreIQ helix and C-region-binding site in CDF and highlight the potential for Ca^{2+} /CaM bridges to form between the PreIQ helix and other elements of the $\text{Ca}_V1.2$ complex.

Results

Crystal structure of Ca^{2+} /CaM- $\text{Ca}_V1.2$ PreIQ-IQ domain complex

We crystallized and determined the structure of Ca^{2+} /CaM- $\text{Ca}_V1.2$ C-terminal tail complex that included segments defined as the PreIQ and IQ domains (residues 1561–1637) at 2.55 Å resolution (Figure 1; Table I). The complex was made as a soluble entity by co-expression in *Escherichia coli* using a strategy used previously to make Ca^{2+} /CaM- Ca_V IQ domain complexes (Van Petegem *et al*, 2005; Kim *et al*, 2008). We denote the PreIQ segment as residues 1561–1603. This segment covers the PreIQ₂ and PreIQ₃ regions defined by Erickson *et al* (2003) and includes segments called the A-peptide (1561–1575) and C-peptide (1585–1605; Pitt *et al*, 2001; hereafter referred to as the A-region and C-region,

respectively). The structure was solved by molecular replacement using sequential searches with full-length CaM (PDB:2BE6, form A) and N-lobe models that lacked calcium ions. Appearance of difference density for the IQ helix and calcium ions indicated the veracity of the solution. Multiple rounds of building and refinement resulted in a model having R/R_{free} values of 24.1/28.8%.

The asymmetric unit contains two $\text{Ca}_V1.2$ C-terminal tails and four Ca^{2+} /CaMs (Figure 1A). The PreIQ segment forms a long (42 residue) helix separated from the Ca^{2+} /CaM-IQ complex by a disordered linker (residues 1603–1613). Two types of interactions occur in the dimer. Two Ca^{2+} /CaM molecules (denoted as CaM_{AC}) form cross-bridges in which Ca^{2+} /N-lobe_{AC} (Figure 1B) and Ca^{2+} /C-lobe_{AC} (Figure 1C) bind to sites on the A- and C-regions of symmetrically related PreIQ helices, respectively. In addition, the PreIQ helices cross and interact in a mode that SOCKET (Testa *et al*, 2009) recognizes as short anti-parallel coiled-coil that spans approximately three heptad repeats in which residues 1578, 1581, 1585 and 1588 comprise the a and d positions characteristic of coiled-coils (Lupas and Gruber, 2005; Figure 1D). Each IQ domain is located at the C-terminal end of a PreIQ helix and interacts with a single Ca^{2+} /CaM (denoted as Ca^{2+} /CaM_{IQ}) in a manner identical to previous Ca^{2+} /CaM- $\text{Ca}_V1.2$ IQ domain structures (RMSD_{C α} 1.176 and 1.126 Å for each in comparison with 2BE6 molecule 'A' (Van Petegem *et al*, 2005) and RMSD_{C α} of 1.046 and 1.066 Å with 2F3Y (Fallon *et al*, 2005; Supplementary Figure 1a).

The dimeric PreIQ helix arrangement, Ca^{2+} /CaM cross-bridges, and relative position of one of the two Ca^{2+} /CaM- $\text{Ca}_V1.2$ IQ complexes in the asymmetric unit are similar to the recent crystal structure of a Ca^{2+} /CaM- $\text{Ca}_V1.2$ tail complex made from refolded material encompassing the identical residues from a different $\text{Ca}_V1.2$ isoform (residues 1609–1685 of UniProt accession number Q13936 (Fallon *et al*, 2009)). The two complexes were crystallized from different conditions (0.2 M Li_2SO_4 , 24.5% PEG 3350, and 0.1 M bis-Tris (pH 5.5) vs 1 M LiCl, 20% PEG 6000, 3% MPD, and 0.1 M MES (pH 6.0), this structure and 3G43, respectively) and have different unit cell dimensions (39.6, 132.2, and 158.2 Å vs 39.8, 114.0, and 182.1 Å, this structure vs 3G43, respectively). Comparison of Ca^{2+} /CaM and Ca_V regions common to both yields an RMSD_{C α} = 1.319 Å (Supplementary Figure 1b). Beyond these similarities, there are a number of differences. Our structure possesses two well-defined, complete Ca^{2+} /CaM-IQ domain complexes related by two-fold symmetry (RMSD_{C α} = 0.305 Å). In contrast, 3G43 has only a single well-defined Ca^{2+} /CaM-IQ complex. Instead of a second Ca^{2+} /CaM-IQ complex, 3G43 has a single Ca^{2+} /N-lobe unrelated by symmetry to Ca^{2+} /N-lobe_{IQ} and that lacks density for the Ca^{2+} /C-lobe and IQ peptide. From our electron density, we could only build a portion of Ca^{2+} /C-lobe_{ACS} (backbone positions for 82–91, 101–110 and 115–146). These lobes are well ordered in 3G43. Nevertheless, our partial Ca^{2+} /C-lobe_{ACS} almost perfectly coincide with those in 3G43. Comparison of the structures indicates that the linker connecting the PreIQ and IQ regions has the ability to adopt varied conformations.

Given the similarities of the structures and previous description (Fallon *et al*, 2009), we briefly note key Ca^{2+} /CaM_{AC} and PreIQ helix elements that form the focus of extensive biochemical and functional analysis presented

Figure 1 Structure of the Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain complex. (A) Two Cav1.2 PreIQ helices (red and salmon) form a crystallographic dimer cross-bridged by Ca^{2+} /CaMs. Ca^{2+} /CaM N-lobe and C-lobe are coloured green and blue, respectively. N- and C-termini of PreIQ-IQ domain are indicated. Cartoon representation shows a schematic asymmetric unit. (B) Ca^{2+} /N-lobe_{AC} PreIQ A-region-binding site. (C) Ca^{2+} /C-lobe_{AC} PreIQ C-region-binding site. In (B, C), PreIQ and Ca^{2+} /CaM lobes are shown in stick and surface representation, respectively. Important contact residues are highlighted in white and labelled. Hydrophobic Ca^{2+} /CaM lobe-binding interface residues are indicated and displayed in yellow. Due to poor Ca^{2+} /C-lobe_{AC} electron density, Ca^{2+} /C-lobe from copy A of 2BE6 (Van Petegem *et al*, 2005) superimposed on the Ca^{2+} /C-lobe_{AC} model was used for the figure. (D) Schematic diagram of PreIQ anti-parallel coiled-coil interactions. a-g positions of heptad repeats and residue numbers are indicated. Right hand side shows the PreIQ coiled-coil interaction. Sidechains are shown as sticks. Interface residues targeted for mutation experiments are shown in space filling representation.

here. The groove formed by Ca^{2+} /N-lobe_{AC} residues Phe19, Met51, Met71, and Met72 interacts with the A-region through hydrophobic interactions centred around PreIQ Leu1562 and including Phe1563, Leu1565, and Val1566 (Figure 1B). The deep Ca^{2+} /C-lobe_{AC} hydrophobic pocket formed by Phe92, Leu105, Met124, Met144, and Met145 is anchored to the PreIQ helix by extensive interactions with Trp1593 (Figure 1C) in a mode commonly observed between Ca^{2+} /C-lobe and tryptophan-containing binding partners (Meador *et al*, 1992; Maximciuc *et al*, 2006).

Ca^{2+} /CaM-Cav1.2 PreIQ-IQ complex is a 2:1 complex in solution

The asymmetric unit of the Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain complex has a Ca^{2+} /CaM-to-PreIQ-IQ ratio of 4:2. Discernment of whether crystallographic interactions are meaningful from structure alone is difficult. Crystal-packing interactions, which are driven by the exact same forces that mediate physiologically relevant interactions, can have interface sizes and characteristics that are not different from validated protein-protein interactions (Bahadur *et al*,

2004; Janin *et al*, 2008). Therefore, to examine whether the Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain complex could dimerize, we used a variety of solution-based assays.

Size exclusion chromatography of the Ca^{2+} /CaM-Cav1.2 PreIQ-IQ complex using a Superdex 200 column (resolution range = 10–600 kDa) before crystallization indicated an apparent molecular weight of ~66 kDa (Supplementary Figure 2a). This did not correspond with either a monomeric complex (2:1 Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain, 42.8 kDa) or a dimeric complex (4:2 Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain complex, 85.6 kDa). Interestingly, a similar construct, the Ca^{2+} /CaM-Cav1.2 C-IQ complex (residues 1579–1644) showed essentially the same elution volume as the Ca^{2+} /CaM-Cav1.2 PreIQ-IQ domain complex (Supplementary Figure 2b). The Ca^{2+} /CaM-Cav1.2 C-IQ complex lacks the PreIQ 'A-region' and two of the six hydrophobic residues that contribute to the core of the anti-parallel coiled-coil interaction. Thus, this complex cannot form the crystallographically observed dimer. Gel filtration analysis is strongly influenced by sample hydrodynamic radius (Minor, 2007). The PreIQ-IQ complex has an elongated shape. Consideration of this

Table 1 X-Ray data collection and refinement statistics

<i>Data collection</i>	
Resolution (Å)	30.0–2.55 (2.59–2.55)
Space group	$\text{P2}_1\text{2}_1\text{2}_1$
<i>Cell dimensions</i>	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	39.6, 132.2, 158.2
α , β , γ (deg)	90, 90, 90
R_{sym}	8.5 (47.7)
Wavelength	1.11587
<i>I</i> / σ <i>I</i>	16.2 (2.7)
Completeness (%)	99.1 (97.3)
Redundancy	3.7 (3.7)
<i>Refinement</i>	
Resolution (Å)	30.0–2.55
No. of reflections	28 238
$R_{\text{work}}/R_{\text{free}}$	24.1/28.8
<i>Number of atoms</i>	
Protein	4925
Ligand/ion	14
Water	97
<i>B-factor</i>	
Protein	55.5
Ligand/ion	58.9
Water	42.7
<i>r.m.s.d.</i>	
Bond lengths (Å)	0.015
Bond angles (deg)	1.460

property together with the similar size exclusion behaviour of PrelQ –IQ and C–IQ complexes, which occurred despite the different sizes and the fact that one lacked key dimerization elements, led us to reason that gel filtration analysis was not a reliable indicator of complex size and stoichiometry. It should be noted that the previously published evidence suggesting the presence of the 4:2 complex in solution (Fallon *et al*, 2009) relies on experiments done at the resolution limit of the sizing column used in those studies (Superdex 75, resolution range = 3–70 kDa). Such conditions make discernment of an ~66 kDa complex from an ~81 kDa complex impracticable. Therefore, we used equilibrium sedimentation analysis, a method that is insensitive to the vagaries of molecular shape (Laue, 1995), to directly measure molecular mass of the complex in solution and resolve this issue.

Sedimentation equilibrium experiments using purified $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ complexes yielded data that were fit nicely by a single-species model that yielded random residuals and clearly indicated a 2:1 stoichiometry (Figure 2A). This stoichiometry was maintained over a wide concentration range (15–100 μM ; initial sample concentrations) and different centrifugation speeds (9000–13 000 r.p.m.; Figure 2B). Sedimentation equilibrium experiments using the shorter $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ C–IQ complex revealed that this construct also acted as a well-behaved 2:1 complex (Figure 2C). These results indicate that, as suspected, the gel filtration experiments were misleading regarding the true stoichiometry. Importantly, there was no evidence for a 4:2 complex. These results strongly suggest that the dimeric structure observed in the asymmetric unit is a result of crystallization, and even though there are extensive crystallographic contacts between the two Ca_v tail complexes, this association is not robust.

$\text{Ca}_v1.2$ s are monomers in live cell membranes

Pore-forming $\text{Ca}_v\alpha_1$ subunits have been thought to function as monomers (Catterall, 2000). Our biochemical and

functional data strongly suggest that the dimeric interactions in the crystallographic asymmetric unit do not represent native interactions. Nevertheless, one could imagine that the context provided by the membrane might lead to a sufficient increase in effective concentration to allow dimerization between elements that otherwise have low affinity association constants. Therefore, to determine the oligomeric state of the $\text{Ca}_v1.2$ channels in a live cell membrane, we used single-molecule subunit counting (Ulbrich and Isacoff, 2007) in which total internal reflection fluorescence microscopy (TIRFM) was used to measure discrete photobleaching steps from $\text{Ca}_v1.2$ α_1 subunits bearing a C-terminal green fluorescent protein (GFP) tag. $\text{Ca}_v1.2$ –GFP channels were functionally equivalent to untagged $\text{Ca}_v1.2$ (Figure 3A). The majority (96%) of $\text{Ca}_v1.2$ –GFP fluorescent spots (Figure 3B) bleached in a single step (Figure 3C and D), indicative of a monomeric state. Similar distributions have been observed in subunit-counting experiments of $\text{Ca}_v2.3$ (96:4%, 1:2 bleaching steps; Ulbrich and Isacoff, 2007) and Ci-VSP monomers (94:6%, 1:2 bleaching steps; Kohout *et al*, 2008). In contrast, the dimeric channel, Hv1, has a bleaching pattern (30:65:5%, 1:2:3 bleaching steps; Tombola *et al*, 2008) that is clearly different from that observed from $\text{Ca}_v1.2$ –GFP. Thus, our data clearly indicate that $\text{Ca}_v1.2$ channels are present in the membrane as monomers, not as dimers. These results confirm that the crystallographic dimer is not relevant in the context of full-length, functional channels.

$\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ complex contains a labile $\text{Ca}^{2+}/\text{CaM}$

The 2:1 $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ complex was formed during co-expression and survived multiple purification steps (metal affinity, anion exchange, and size exclusion). Nevertheless, we wondered whether either or both of the bound $\text{Ca}^{2+}/\text{CaMs}$ could be removed. Phenylsepharose chromatography is a well-established method for purifying CaM (Gopalakrishna and Anderson, 1982) and exploits differences in the abilities of $\text{Ca}^{2+}/\text{CaM}$ and apo-CaM to interact with the hydrophobic resin. Passage of the 2:1 $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ complex through a phenylsepharose column resulted in two fractions (Figure 4A), a flow-through fraction that contained CaM and the PrelQ –IQ segment, and a bound fraction that was released by EGTA and that contained only CaM (Figure 4B). Reapplication of the $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ flow-through fraction did not remove any more CaM (Supplementary Figure 3a). We obtained similar results for the $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ C–IQ complex. Size exclusion chromatography of the $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ and the $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ C–IQ complexes after phenylsepharose purification indicated an elution volume change towards lower molecular weight species (data not shown). Analytical ultracentrifugation experiments unambiguously showed that the phenylsepharose-treated $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ PrelQ –IQ and $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ C–IQ samples were 1:1 complexes (Figure 4C and D). Together with the phenylsepharose experiments, these results indicate that two forms of $\text{Ca}^{2+}/\text{CaM}$ bind to the $\text{Ca}_v1.2$ C-terminal tail simultaneously, a labile form and a non-labile form. To test which was labile, we subjected purified $\text{Ca}^{2+}/\text{CaM}$ – $\text{Ca}_v1.2$ IQ domain complexes to the phenylsepharose purification procedure. Unlike the longer PrelQ –IQ and C–IQ complexes, no CaM peak was detected at the EGTA elution step

Figure 2 Sedimentation equilibrium analysis of $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ tails. (A) Equilibrium distribution of $100 \mu\text{M}$ $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ PreIQ-IQ domain complex at 11000 r.p.m. and 4°C and measured at 293 nm. Raw data (black open circles) and single species fit (black line) are compared with predicted curves for complexes having $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ tail ratios of 1:1 (red), 2:1 (yellow), 2:2 (green), and 4:2 (blue). Inset is random distribution of residuals as a function of radial distance. (B) Comparison of the measured molecular weight of $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ PreIQ-IQ domain based on sedimentation equilibrium experiments performed at different protein concentrations and centrifugation speeds. Molecular weights for 2:1 (42.8 kDa) and 4:2 (85.6 kDa) complexes are indicated by the dotted lines. (C) Equilibrium distribution of $15 \mu\text{M}$ $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ C-IQ complex. A representative curve is shown; $15 \mu\text{M}$ of $\text{Ca}^{2+}/\text{CaM-Ca}_V1.2$ C-IQ complex at 11000 r.p.m. and 4°C and measured at 281 nm. Solid lines are calculated as in (A).

Figure 3 Subunit counting of $\text{Ca}_V1.2$ in cell membrane. (A) Inactivation properties of GFP-tagged $\text{Ca}_V1.2$ co-expressed with $\text{Ca}_V\beta_{2a}$ in *Xenopus* oocytes. Normalized Ca^{2+} and Ba^{2+} currents recorded from $\text{Ca}_V1.2\text{-GFP}$ (dark blue and light blue, respectively) are compared to those of wild type (black and grey, respectively). The pulse protocol is indicated above the graph and scale bars represent 200 nA Ca^{2+} current. (B) A representative TIRF image showing the $\text{Ca}_V1.2\text{-GFP}$ fluorescent spots in the cell surface of *X. laevis* oocytes. Bright spots are $\text{Ca}_V1.2\text{-GFP}$ single molecules when the shutter is first opened at the beginning of the bleaching experiment. Blue circles mark the selected molecules for subunit counting. (C) Time courses of photobleaching from single fluorescent spots. Two examples are shown. (D) Distribution of fluorescent spots that bleach in one or more steps. Percentile of each bleaching step is indicated.

(Supplementary Figure 3b). Together, the data suggest that the C-region is the binding site for the second $\text{Ca}^{2+}/\text{CaM}$ molecule and that $\text{Ca}^{2+}/\text{CaM}_{\text{AC}}$ is labile, whereas $\text{Ca}^{2+}/\text{CaM}_{\text{IQ}}$ is not. The ability of phenylsepharose to strip $\text{Ca}^{2+}/\text{CaM}_{\text{AC}}$ but not $\text{Ca}^{2+}/\text{CaM}_{\text{IQ}}$ from the complex suggests that $\text{Ca}^{2+}/\text{CaM}_{\text{AC}}$ has a lobe that is not engaged with the PreIQ region.

Characterization of PreIQ A-region and C-region CaM -binding sites

To obtain a detailed and quantitative understanding of the crystallographic $\text{Ca}^{2+}/\text{CaM}_{\text{AC}}$ half sites, we used a combination of isothermal titration calorimetry (ITC) and pulldown assays using individually purified CaM lobes. The peptide corresponding to the A-region (residues 1561–1580) had poor solubility and failed to yield sufficient material for ITC. Thus, we used a maltose-binding protein fusion (HMT) bearing the A-region to make material that was sufficiently soluble to study the interaction with $\text{Ca}^{2+}/\text{N-lobe}$ by ITC. Consistent with the structure, ITC experiments identified a single, weak ($K_d > 10 \mu\text{M}$) $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$ -binding site (Figure 5A; Table II) that was absent when $\text{Ca}^{2+}/\text{N-lobe}$ was tested against HMT alone (data not shown). Pulldown assays using the same components in the presence of Ca^{2+} also detected the $\text{Ca}^{2+}/\text{N-lobe-A-region}$ interaction, and were used to evaluate mutations in the crystallographically observed $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$ anchor positions (Figure 5B). Mutation at the central anchor, Leu1562Ala, and the double mutant Leu1562Ala/Phe1563Ala disrupted the $\text{Ca}^{2+}/\text{N-lobe-A-region}$ interaction (Figure 5B). In contrast, individual alanine mutations of other $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$ contact positions (Phe1563Ala, Leu1565Ala, and Val1566Ala) did not affect binding. These results indicate that, as seen in the crystal structure, Leu1562 forms the central contact residue for a low-affinity interaction between $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$ and the A-region.

Figure 4 The Ca²⁺/CaM-Ca_v1.2 PreIQ-IQ domain complex contains two types of Ca²⁺/CaM. (A) Phenylsepharose chromatograph of the Ca²⁺/CaM-Ca_v1.2 PreIQ-IQ complex. Peak 1 is the flow through fraction and Peak 2 is eluate obtained with 10 mM EGTA. Absorbance and conductivity are shown as blue and green, respectively. (B) SDS-PAGE analysis of Peak 1 and Peak 2 from (A). Peak 1 contains both CaM and Ca_v1.2 PreIQ-IQ peptide. Peak 2 contains only CaM. (C) Sedimentation equilibrium analysis of the Ca²⁺/CaM-Ca_v1.2 PreIQ-IQ complex post phenylsepharose treatment. Representative data obtained at 4°C and measured at 14 000 r.p.m. and 236 nm using 10 μM complex is shown. (D) Sedimentation equilibrium analysis of Ca²⁺/CaM-Ca_v1.2 C-IQ complex after phenylsepharose chromatography. Representative data measured at 11 000 r.p.m. and 297 nm using 90 μM protein is shown. Fits in (C) and (D) are presented as in Figure 2A and C.

As anticipated from the highly homologous nature of the N- and C-lobes (Ataman *et al*, 2007), similar experiments with Ca²⁺/C-lobe show that it can also bind the A-region. This interaction seems to be even weaker than the Ca²⁺/N-lobe-A-region interaction (Figure 5C; Table II), and is drastically weaker than the Ca²⁺/C-lobe-C-region interaction (Table II).

To evaluate C-region site interactions, we performed ITC experiments using individual CaM lobes and post-phenylsepharose 1:1 Ca²⁺/CaM-Ca_v1.2 C-IQ complexes. These reveal a single Ca²⁺/C-lobe-binding site having an affinity that is at least 20-fold stronger than the Ca²⁺/N-lobe interaction at the A-region site ($K_d = 475 \pm 150$ nM; Figure 5D; Table II). Mutation of the Trp1593 Ca²⁺/C-lobe anchor to alanine or glutamate changed the binding reaction from exothermic to endothermic and resulted in a severely reduced affinity (~17-fold) for Trp1593Ala and almost complete loss of detectable binding for Trp1593Glu (Figure 5E-G; Table II). These results validate that the measured interaction corresponds with the crystallographically observed C-region-binding site. Interestingly, Ca²⁺/N-lobe can also bind the C-region, but with lower affinity than Ca²⁺/C-lobe ($K_d = 5.6 \pm 0.5$ μM). Trp1593Ala ablated this interaction (Table II) and indicated that the Ca²⁺/N-lobe and Ca²⁺/C-lobe sites overlap, a property noted previously for Ca²⁺/CaM Ca_v IQ domain-binding sites (Van Petegem *et al*, 2005; Kim *et al*, 2008). The affinity of Ca²⁺/C-lobe for the C-region is ~100-fold weaker than Ca²⁺/C-lobe for the Ca_v1.2 IQ domain (Van Petegem *et al*, 2005) and agrees well with our observation that Ca²⁺/CaM bound to C-region is labile and our inability to strip Ca²⁺/CaM from the IQ domain by phenylsepharose treatment (Supplementary Figure 3b).

The concentration of CaM near Ca_v1.2 is suggested to be ~2.5 mM (Mori *et al*, 2004), well within the range to be relevant for the half-site interaction affinities measured here. Together, these biochemical experiments confirm that the interactions observed in the crystal structure reflect modes by which the A- and C-regions can engage the individual Ca²⁺/CaM lobes and show that the C-region site has a substantially higher binding affinity for CaM-lobe interaction.

Interactions between calcium-free CaM (apo-CaM) and the Ca_v1.2 C-terminus are thought to be crucial for channel function (Pitt *et al*, 2001; Erickson *et al*, 2003; Kim *et al*, 2004a), although the exact location of the binding site remains unresolved. As PreIQ region contains proposed apo-CaM-binding regions, we tested whether apo-CaM lobe mimics that bear mutations in the calcium-binding EF hands, Apo/N-lobe₁₂ and Apo/C-lobe₃₄ interact with the A-region and C-region using ITC. These half-apo-CaM constructs were used so the experiment could be done in the presence of calcium, which was required to maintain Ca²⁺/CaM bound to the IQ domain for the PreIQ C-IQ experiments. Circular dichroism indicates that Apo/N-lobe₁₂ and Apo/C-lobe₃₄ have similar structures to the corresponding, individual apo-lobes of wild-type CaM (Supplementary Figure 4). ITC experiments demonstrate that Apo/N-lobe₁₂ and Apo/C-lobe₃₄ bind as weakly to the A-region as the individual Ca²⁺/lobes ($K_d > 10$ μM) and have no detectable interaction with the C-region (Table II). These data establish that the C-region site has a clear preference for Ca²⁺/CaM lobes and indicate that the neither the A-region nor C-region contains a strong apo-lobe-binding motif.

Figure 5 Characterization of $\text{Ca}^{2+}/\text{CaM}$ -AC-Cav1.2 PreIQ interactions. (A) 100 μM $\text{Ca}^{2+}/\text{N-lobe}$ titrated into 10 μM HMT-tagged A-region. Panels show injections of 10 μl titrant to the target (top) and binding isotherms (bottom). (B) Pull-down assay using 20 μM HMT alone and 20 μM HMT-tagged A-region variants with 100 μM $\text{Ca}^{2+}/\text{N-lobe}$. M indicates molecular weight standards. (C) 100 μM $\text{Ca}^{2+}/\text{C-lobe}$ titrated into 10 μM HMT-tagged A-region. Panels show injections of 10 μl titrant to the target (top) and binding isotherms (bottom). (D–F) Binding isotherms for titration of 100 μM $\text{Ca}^{2+}/\text{C-lobe}$ into (D) 10 μM wild type, (E) Trp1593Ala, and (F) Trp1593Glu 1:1 $\text{Ca}^{2+}/\text{CaM}$ -Cav1.2 C-IQ complexes. (G) Overlaid binding isotherms of (D–F). Cartoon representations depict components of each experiment. A-region, C-region, and IQ domain are denoted as PreIQ A , PreIQ C , and IQ, respectively. Stars indicate mutation sites.

Electrophysiological studies identify a role for the C-region site in CDF

Although we observed no evidence for $\text{Ca}_v1.2$ tail dimerization in solution (Figure 2B), we decided to test whether mutations designed to disrupt the crystallographic anti-parallel coiled-coil affected function. Leu1585 and Ile1588 are positioned at successive a and d positions, respectively, and interact with Leu1578 and Ala1581 (Figure 1D). Because of the anti-parallel arrangement, Leu1585Ala/Ile1588Ala should disrupt interactions across two of the three turns that contribute to the crystallographic coiled-coil. Two-electrode voltage clamp experiments in *Xenopus* oocytes showed that Leu1585Ala/Ile1588Ala failed to cause a detectable change in either CDI or VDI (Figure 6A; Table III). These results together with our solution studies and subunit-counting experiments indicate that the dimerization interaction observed in the crystal structure is not relevant for function.

To investigate whether the crystallographically observed and biochemically validated $\text{Ca}^{2+}/\text{CaM}$ lobe-binding sites in the A-region and C-region represent functionally important interactions, we examined the effects of a set of structure-based mutations. Despite the detrimental effects on $\text{Ca}^{2+}/\text{N-lobe}$ binding (Figure 5B), neither Leu1562Ala nor Leu1562Ala/Phe1563Ala changes in the $\text{Ca}^{2+}/\text{N-lobe}$ A-region contact site affected CDI or VDI (Figure 6B; Table III). Previous studies reported that an A-region triple-alanine mutation Thr1561Ala/Leu1562Ala/Phe1563Ala reduced CDI (Kim *et al*, 2004b). To revisit this effect, we examined individual alanine mutations at each of these positions. Of the three, only Thr1561Ala affected $\text{Ca}_v1.2$ CDI (Figure 6B; Table III). Given the minimal contacts this position has with $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$, this CDI change does not seem to be correlated with $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$ binding to the A-region. Two of the other residues that contact $\text{Ca}^{2+}/\text{N-lobe}_{\text{AC}}$, Leu1565

Table II Titration calorimetry data

	K_a (M^{-1})	N	K_d (nM)	n	ΔH (kcal mol^{-1})	ΔS ($\text{cal mol}^{-1} \text{K}^{-1}$)	K_a/K_a (wt)	$\Delta\Delta G$ (kcal mol^{-1})
HMT-A								
Ca^{2+} /N-lobe	$< 1.00 \times 10^5$		$> 10\,000$	3				
Ca^{2+} /C-lobe	$< 1.00 \times 10^5$		$> 10\,000$	2				
Apo/N-lobe ₁₂	$< 1.00 \times 10^5$		$> 10\,000$	2				
Apo/C-lobe ₃₄	$< 1.00 \times 10^5$		$> 10\,000$	2				
C-IQ								
Ca^{2+} /N-lobe	$1.99 \times 10^5 \pm 8.84 \times 10^4$	0.83 ± 0.15	5592 ± 2490	2	-3.47 ± 0.13	12.10 ± 1.41	—	—
Ca^{2+} /C-lobe	$2.26 \times 10^6 \pm 7.26 \times 10^5$	0.77 ± 0.06	475 ± 150	2	-4.29 ± 0.44	14.13 ± 2.04	—	—
Apo/N-lobe ₁₂	$< 1.00 \times 10^5$		$> 10\,000$	1			—	—
Apo/C-lobe ₃₄	$< 1.00 \times 10^5$		$> 10\,000$	1			—	—
C-IQ W1593A								
Ca^{2+} /N-lobe	$< 1.00 \times 10^5$		$> 10\,000$	3				
Ca^{2+} /C-lobe	$1.25 \times 10^5 \pm 1.48 \times 10^4$	0.97 ± 0.16	8081 ± 1024	3	5.55 ± 0.13	42.53 ± 0.42	0.06	-1.66
Apo/N-lobe ₁₂	$< 1.00 \times 10^5$		$> 10\,000$	1				
Apo/C-lobe ₃₄	$< 1.00 \times 10^5$		$> 10\,000$	1				
C-IQ W1593E								
Ca^{2+} /C-lobe	$< 1.00 \times 10^5$		$> 10\,000$	2

Figure 6 Inactivation of $\text{Ca}_v1.2$ and $\text{Ca}_v1.2$ bearing mutations in (A) the crystallographic anti-parallel coiled-coil interface, (B) Ca^{2+} /N-lobe_{AC}-A-region binding sites, and (C) Ca^{2+} /C-lobe_{AC}-C-region binding sites. $\text{Ca}_v1.2$ s were co-expressed with $\text{Ca}_v\beta_{2a}$ and $\text{Ca}_v\alpha_{2\delta}$ in *Xenopus* oocytes. Data shows currents for 450 ms with a test pulse from -90 to $+20$ mV. Normalized Ca^{2+} and Ba^{2+} currents for wild type (black and grey, respectively) and indicated mutants (dark blue and light blue, respectively) are shown. Scale bars indicate 200 nA Ca^{2+} current.

and Val1566 are part of a second triple-alanine mutant, Leu1565Ala/Val1566Ala/Arg1567Ala, which also reduced CDI (Kim *et al*, 2004b). Individual examination of Leu1565Ala and Val1566Ala revealed that only Leu1565Ala

affected CDI (Figure 6B). Nevertheless, neither of these changes caused detectable perturbations to the Ca^{2+} /N-lobe_{AC}-A-region interaction (Figure 5B). Thus, there is little correlation between the crystallographically observed

Table III Inactivation properties of Ca_v1.2 variants

Ca _v 1.2	Ca ²⁺								Ba ²⁺	
	<i>n</i>	<i>r</i> ₃₀₀	<i>P</i>	<i>A</i> ₁	<i>τ</i> ₁ (ms)	<i>A</i> ₂	<i>τ</i> ₂ (ms)	<i>C</i>	<i>n</i>	<i>r</i> ₃₀₀
<i>A</i> -region										
WT	28	0.301 ± 0.032	—	0.557 ± 0.032	21.8 ± 2.58	0.163 ± 0.018	119.9 ± 14.7	0.280 ± 0.028	17	0.981 ± 0.009
T1561A	7	0.643 ± 0.037	***	0.229 ± 0.029	41.5 ± 6.4	0.227 ± 0.047	352.6 ± 111.5	0.543 ± 0.051	7	0.971 ± 0.013
L1562A	10	0.309 ± 0.014	NS	0.526 ± 0.016	21.3 ± 1.97	0.186 ± 0.012	134.3 ± 16.17	0.287 ± 0.016	9	0.968 ± 0.007
F1563A	9	0.295 ± 0.016	NS	0.548 ± 0.011	24.2 ± 1.269	0.177 ± 0.018	126.2 ± 16.26	0.276 ± 0.02	7	0.970 ± 0.011
L1562A/F1563A	8	0.320 ± 0.028	NS	0.524 ± 0.024	23.1 ± 2.39	0.189 ± 0.012	145.1 ± 28.0	0.287 ± 0.021	8	0.969 ± 0.009
L1565A	8	0.514 ± 0.022	***	0.297 ± 0.009	24.6 ± 1.80	0.216 ± 0.020	132.0 ± 7.1	0.487 ± 0.021	8	0.979 ± 0.008
V1566A	8	0.313 ± 0.014	NS	0.530 ± 0.017	27.2 ± 4.28	0.190 ± 0.012	143.7 ± 23.6	0.281 ± 0.012	8	0.977 ± 0.009
L1565A/V1566A	9	0.490 ± 0.029	***	0.339 ± 0.034	27.8 ± 2.52	0.210 ± 0.019	146.9 ± 13.9	0.451 ± 0.021	8	0.976 ± 0.009
<i>Dimer interface</i>										
L1585A/I1588A	9	0.279 ± 0.033	NS	0.561 ± 0.033	18.5 ± 1.51	0.193 ± 0.026	141.4 ± 15	0.246 ± 0.027	8	0.958 ± 0.011
<i>C</i> -region										
W1593A	12	0.334 ± 0.03	**	0.525 ± 0.02	26.2 ± 3.47	0.170 ± 0.025	140 ± 14.8	0.305 ± 0.032	12	0.985 ± 0.009
W1593E	7	0.290 ± 0.015	NS	0.557 ± 0.026	24.8 ± 0.94	0.180 ± 0.016	132.1 ± 11.6	0.264 ± 0.012	7	0.968 ± 0.009

NS, not significant.

Data are presented as mean ± s.d values. Values were calculated using equation of $I = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C$. *r*₃₀₀ denotes the fraction of current remaining at 300 ms to the peak current (*I*₃₀₀/*I*_{max}) measured at +20 mV. *P*-values with respect to wild-type *r*₃₀₀ were calculated using Student's *t*-test; NS, *P* > 0.05; *, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01; ***, *P* < 0.001. Error bars indicate s.d.

Figure 7 C-region mutations affect CDF. Blocking both dimer interface and Ca²⁺/C-lobe_{AC}–C-region interaction almost completely abolished Ile1624Ala CDF. Ca²⁺ currents were recorded in a 3-Hz 40-pulse train (50 ms pulse from –90 to +20 mV) and normalized to *I*_{max} of the first trace (black) for comparison. For clearer view, every fourth trace is shown in grey with an exception of the 40th trace (red).

and biochemically validated Ca²⁺/N-lobe_{AC}–A-region interactions and function.

Similar to the Ca²⁺/N-lobe_{AC} results, investigation of the consequences of Ca²⁺/C-lobe_{AC} anchor mutants, Trp1593Ala or Trp1593Glu, also failed to find functional changes of CDI or VDI (Figure 6C; Table III) even though both mutants showed significant reduction in Ca²⁺/C-lobe binding in the ITC experiments (Figure 5E–G). Thus, there is no correlation between the structure and biochemistry of Ca²⁺/CaM_{AC} interactions with the A- and C-regions and channel inactivation processes.

The interactions between the Ca_v1.2 C-terminal tail and CaM affect another calcium-dependent channel behaviour known as calcium-dependent facilitation (CDF; Pitt *et al*, 2001). To see whether any of the structural elements in the Ca²⁺/CaM–PreIQ helix complex participates in CDF, we measured the effects of the above mutations in the background of the IQ domain Ile1624Ala mutation that unmasks Ca_v1.2 CDF (Zühlke *et al*, 1999; Figure 7). Disruption of the crystallographic coiled-coil interface by Leu1585Ala/Ile1588Ala had no effect (Table IV; Supplementary Figure 5). Mutations in both the Ca²⁺/N-lobe and Ca²⁺/C-lobe anchor positions caused significant decreases in CDF (Table IV; Supplementary Figure 5). Interestingly, the CDF decreases

caused by Trp1593Ala and Trp1593Glu were augmented by introduction of Leu1585Ala/Ile1588Ala (Figure 7; Table IV; Supplementary Figure 5). The former shows a significantly ablated CDF, whereas the latter almost abolished CDF. We were unable to make similar measurements with the Ca²⁺/N-lobe mutant Leu1562Ala/Phe1563Ala, as combination with Leu1585Ala/Ile1588Ala did not yield functional channels. The observed effects are consistent with the different degrees of loss of Ca²⁺/C-lobe binding caused by Trp1593Ala and Trp1593Glu and suggest that the Ca²⁺/C-lobe_{AC}–PreIQ helix interactions and the PreIQ helix have an important role in CDF.

Discussion

Ca_v calcium-dependent modulation is highly complex and includes two processes: CDI and CDF, governed by interplay between CaM and the Ca_vα₁ subunit. Multiple segments in Ca_v1 cytoplasmic domains have been identified to interact with CaM and may have functions in calcium-dependent modulation (Peterson *et al*, 1999; Zühlke *et al*, 1999; Ivanina *et al*, 2000; Pate *et al*, 2000; Pitt *et al*, 2001; Dick *et al*, 2008). Although significance of CaM interactions with the IQ domain has been extensively studied (Peterson *et al*,

Table IV Calcium-dependent facilitation of Ca_v1.2 variants

	Ca _v 1.2	<i>n</i>	CDF (%)	<i>P</i>
A-region	I1624A	31	71.2 ± 22.5	N/A
	L1562A/F1563A/I1624A	6	38.4 ± 12.4	**
Dimer interface	L1585A/I1588A/I1624A	14	66.9 ± 25.5	NS
C-region	W1593A/I1624A	14	50.3 ± 13.3	**
	W1593E/I1624A	12	54.4 ± 13.2	*
Dimer interface/C-region	L1585A/I1588A/W1593A/I1624A	14	38.2 ± 12.9	***
	L1585A/I1588A/W1593E/I1624A	14	9.6 ± 3.8	***

NS, not significant.

CDF was calculated by $(I_{\max,40} - I_{\max,1})/I_{\max,1} \times 100$ and displayed as mean ± s.d values. *P*-values with respect to I1624A are shown and were calculated using Student's *t*-test; NS, *P* > 0.05; *, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01; ***, *P* < 0.001. Error bars indicate s.d.

1999; Pate *et al*, 2000; Pitt *et al*, 2001), much less is known about how other regions participate in CaM interactions to coordinate calcium-dependent regulation. In this study, we present the structure of Ca²⁺/CaM bound to a Ca_v C-terminal tail containing three CaM-binding motifs (A-region, C-region, and IQ domain) and use biochemical and electrophysiological approaches to characterize whether interactions observed in the crystal structure are functionally important.

One striking aspect is that the crystallographic asymmetric unit consists of a symmetric dimer that interacts through an anti-parallel coiled-coil bridged by two Ca²⁺/CaM molecules. Although some low-resolution electron microscopy studies have been interpreted as evidence for channel dimers (Wang *et al*, 2002, 2004) and infrequent multiple concerned openings of Ca_v1.2 have been recently reported (Navedo *et al*, 2010), it has not been generally thought that Ca_v channels function as dimers in cell membranes. Nevertheless, a recent structure similar to the one presented in this study was interpreted as evidence for channel dimerization (Fallon *et al*, 2009). Assessment of quaternary structure from crystallographic data alone is challenging (Ponstingl *et al*, 2000; Janin *et al*, 2008) and there are numerous examples in which two-fold related crystallographic dimers do not represent biologically relevant interactions (Bahadur *et al*, 2004). Thus, orthogonal approaches are required to validate whether crystallographically observed interactions are relevant or simply a consequence of forming the ordered array of the lattice (Kobe *et al*, 2008).

Our experimental search for evidence in favour of channel dimerization failed to find any support for the hypothesis that the crystallographic dimer represents a structural feature of the native protein. Sedimentation equilibrium experiments using purified Ca²⁺/CaM–PreIQ–IQ domain complexes find a well-behaved 2:1 complex over a wide range of concentrations (Figure 2) and do not detect higher-order complexes. It should be noted that the highest concentration of the Ca²⁺/CaM–PreIQ–IQ domain complexes assayed is of the order of the estimated concentration of Ca_vs in their most densely known environment, that of the SR junction (Serysheva, 2004). One of the interactions that forms the crystallographic dimer, the Ca²⁺/N-lobe–A-region interaction, has very low intrinsic affinity (*K_d* > 10 μM), and thus, seems unlikely to be able to provide augmentation to the dimerization as suggested (Fallon *et al*, 2009). Alanine mutation of hydrophobic amino acids interactions of which form the heart of the dimer interface in the asymmetric unit, do not affect any of the three well-studied functional properties of the channel

that are known to be regulated by the C-terminal domain: CDI, VDI or CDF. Finally, experiments aimed at determining the association state of full-length channels in cell membranes unequivocally show that the channels are monomers. These results are similar to earlier observations with Ca_v2.3 channels (Ulbrich and Isacoff, 2007), which coiled-coil prediction algorithms suggest should be unable to form a coiled-coil similar to that seen in the crystal (Fallon *et al*, 2009). Thus, our data indicate that, unlike what has been proposed by Fallon *et al* (2009), the Ca_v1.2 C-terminal tail crystallographic dimer is neither robust nor relevant for channel function. This conclusion is also in accord with the observation that Ca_v1.2 channels bearing the PreIQ–IQ domain, but lacking the remainder of the channel C-terminus show no evidence of coupled activity (Navedo *et al*, 2010).

Our biochemical studies identify that the Ca_v1.2 PreIQ–IQ domain can bind two Ca²⁺/CaMs simultaneously and that these two Ca²⁺/CaMs have different properties. These results are corroborated by the recent report by Kameyama and colleagues (Asmara *et al*, 2010). Two half-CaM-binding sites are observed in the crystal, one involving Ca²⁺/N-lobe_{AC} and the other involving Ca²⁺/C-lobe_{AC}. Could either of the two half-CaM interactions represent functionally relevant associations? Biochemical studies (Figure 5) show that interaction of the individual lobes can be measured and that these interactions are affected by mutation of crystallographically observed key residues. However, there is a major difference in the affinities of the half-sites, with the Ca²⁺/C-lobe_{AC} being ~20-fold stronger than the Ca²⁺/N-lobe_{AC} interaction. Importantly, this difference correlates with the findings from our functional studies. Mutations designed to affect either interaction only have clear functional consequences in the case of Ca²⁺/C-lobe_{AC}.

Previous studies of triple-alanine mutations have already implicated the A-region in CDI (Kim *et al*, 2004b). Examination of the impact of single-alanine substitutions at positions that form the Ca²⁺/N-lobe_{AC}-binding site reveals functional changes only at Thr1561 and Leu1565; two residues that make minimal Ca²⁺/N-lobe_{AC} interactions and that do not affect Ca²⁺/N-lobe binding (Figures 5 and 6; Table III). This disconnection of structure and binding from function is unlike what we have observed in previous structural studies of Ca²⁺/CaM modulation (Van Petegem *et al*, 2005; Kim *et al*, 2008). What could explain the breakdown in the connection between structure and biochemistry with function? Insight can be found from recent structural studies of the EF-hand region from the C-terminal tails of Na_v1.2

(Miloushev *et al*, 2009) and Nav1.5 (Chagot *et al*, 2009) voltage-gated sodium channels. The Nav EF-hand region shares a similar location, proximal to the cytoplasmic end of transmembrane segment IVS6, and a high degree of sequence similarity (48.2%) with the homologous Cav1.2 region (Supplementary Figure 6). The Nav1.2 and Nav1.5 structures show that this region forms a paired EF-hand domain containing four helices. Modelling the equivalent Cav1.2 residues onto the Nav1.2 structure indicates that the Cav1.2 A-region corresponds with EF-hand helix IV. Leu1562, Phe1563, Leu1565, and Val1566, which make the main Ca^{2+} /N-lobe_{AC} contacts, are all observed in the core of the four-helix bundle. Thus, given the low affinity of the Ca^{2+} /N-lobe_{AC} for the A-region, and the possibility that the Cav1.2 A-region may be involved in a structure in which the A-region hydrophobic residues would be inaccessible, we conclude that the Ca^{2+} /N-lobe_{AC}–A-region interaction most probably represents an opportunistic non-native interaction driven by the well-known promiscuous target-recognition properties of Ca^{2+} /CaM (Hoeflich and Ikura, 2002). It seems likely that the effects on CDI reported for mutations in this region (Peterson *et al*, 2000; Kim *et al*, 2004b) and A-region mutation induced changes in CDI (Figure 6B; Table III) and CDF (Table IV; Supplementary Figure 5), which are not correlated with our structure or biochemical studies, result from disruption the EF-hand structure.

Our biochemical studies show that the Cav1.2 C-terminal tail forms a stable 2:1 complex with Ca^{2+} /CaM with one Ca^{2+} /CaM bound to the C-region and one bound to the IQ domain. Could the second half-site, which is the site of the labile Ca^{2+} /CaM (Figure 4), represent a functionally important site? Previous gel shift assays of CaM and Cav1.2 constructs containing the A-, C-, and IQ regions reported an 1:1 stoichiometry (Xiong *et al*, 2005). Given the labile nature of the Ca^{2+} /CaM–C-region interaction, this interaction could easily be missed in assays less precise than sedimentation equilibrium. Studies of Cav1.2–CaM fusions suggested that a single CaM is sufficient for Cav1.2 CDI (Mori *et al*, 2004). The presence of the second CaM is not in conflict with the results of Mori *et al* (2004) as disruption of the Ca^{2+} /CaM–C-region interaction does not affect CDI. Our functional experiments indicate that this site has a role in CDF. It is notable that the main anchor, Trp1593, is conserved throughout metazoan evolution and is found in *Ciona* Cav1 (Okamura *et al*, 2003) and in both Cav1 and Cav2 subclasses (Figure 8A). The ability of the Cav1.2 tail to bind multiple Ca^{2+} /CaMs along a largely helical domain is reminiscent of how CaM and CaM-like proteins are organized on myosin (Houdusse *et al*, 2006) and raises the intriguing possibility that there are previously unrecognized connections between these seemingly disparate systems.

Our data suggest that the PreIQ region forms a long α -helix that contains Ca^{2+} /C-lobe-anchoring site and that

Figure 8 (A) Sequence alignments of the PreIQ and IQ domains of human Cav1.2 1561–1647 (Cav α_1 c77) CAA84346; Cav1.1 1466–1552, AA133672; Cav1.3 1534–1620, NP_001077085; Cav1.4, 1526–1612 NP_005174, *Ciona* Cav1 1466–1552, XP_002123864; Cav2.1 1905–1994 NP_001095163; Cav2.2 1794–1883, NP_000709.1; and Cav2.3 1760–1849, Q07652. PreIQ, IQ Domain, A-region, and C-regions are indicated. Conservation of the C-region tryptophan is highlighted blue. Yellow circles indicate residues L1585 and I1588. (B) Cartoon model depicting the possible relative positions of Cav intracellular elements. '?' signifies the potential for a Ca^{2+} /CaM anchored at the C-region site to make bridging interactions with other components of the Cav complex. Yellow indicates the positions of residues L1585 and I1588 that augment CDF effects. Relative orientation of the C-terminal tail is chosen to display the key elements. Its orientation relative to the other intracellular components is not known.

Ca^{2+} /C-lobe is anchored to the C-region in a manner that would permit Ca^{2+} /CaM to form bridging interactions between the PreIQ domain and other channel elements through Ca^{2+} /N-lobe (Figure 8B). Mutations at two positions, L1585 and I1588, which are in the C-region helix but that do not directly participate in the Ca^{2+} /C-lobe-binding site, augment CDF reduction, and reinforce the idea that this portion of the channel interacts with other, yet to be identified, elements. The cytoplasmic $\text{Ca}_v\beta$ subunit is thought to be linked to the pore through a long helix formed by part of the I-II loop (Vitko *et al*, 2008; Zhang *et al*, 2008; Findeisen and Minor, 2009) and is essential for CDF (Grueter *et al*, 2006; Findeisen and Minor, 2009). There are functional interactions between the $\text{Ca}_v1.2$ C-terminal tail and the $\text{Ca}_v\beta$ -I-II loop complex that indicate that both have roles in VDI, CDI, and CDF (Kim *et al*, 2004b; Findeisen and Minor, 2009). It is striking that the long PreIQ helix would position the two elements of the C-terminal tail implicated in CDF, the C-region and the IQ domain (Zühlke *et al*, 1999, 2000; Van Petegem *et al*, 2005), near $\text{Ca}_v\beta$ (Figure 8B). This potential confluence of key regulatory elements provides a framework for dissection of the interactions and structural transitions that drive Ca_v function.

Materials and methods

Protein expression and purification

Constructs encoding the A-region (residues 1561–1580), C-IQ domain (residues 1579–1644), and PreIQ-IQ domain (residues 1563–1637) of human $\text{Ca}_v1.2$ ($\text{Ca}_v\alpha_{1C77}$ isoform; accession number CAA84346) were cloned into a pET28b-derived vector denoted HMT (Van Petegem *et al*, 2004), which contains in sequence a His₆-tag, maltose-binding protein, and a cleavage site for TEV protease. For crystallization, the cloning artefact sequence (His-Met) preceding the PreIQ-IQ domain was reverted to the native sequence (Thr-Leu; residues 1561–1562) by site-directed mutagenesis using QuikChange[®] (Stratagene). Alanine substitutions were also generated using the same mutagenesis protocol. Constructs for N- and C-lobe of CaM (HMT tagged) and full-length CaM (without any tag) were identical to those used previously (Kim *et al*, 2008).

Proteins were expressed and purified using strategies described previously (Kim *et al*, 2008). HMT fusions of the PreIQ-IQ domain or C-IQ domain were co-transformed with CaM in BL21(DE3)-Rosetta cells and grown at 37°C for 3–4 h after IPTG induction at $\text{OD}_{600} = 0.7\text{--}0.8$. After cell lysis by sonication in Buffer A (250 mM KCl, 1 mM CaCl_2 , and 10 mM HEPES (pH 7.4)) containing 1 mM PMSF, the Ca^{2+} /CaM- $\text{Ca}_v1.2$ peptide complexes were applied to a Poros20MC column (Perseptive Biosystems) and eluted with a step elution using 100% Buffer B (Buffer A plus 500 mM Imidazole, (pH 7.4)). Protein eluate was concentrated, diluted (1:10) in Buffer C (10 mM KCl, 1 mM CaCl_2 , 10 mM Tris-HCl (pH 8.8)) and subjected to overnight cleavage with His-tagged TEV protease (Kapust *et al*, 2001). The majority of MBP, uncleaved fusion protein, and TEV protease were removed by purification on a HiLoad[®] Q-Sepharose column (GE Healthcare Life Sciences) using a linear gradient from 15 to 40% of Buffer D (Buffer C plus 1 M KCl). Residual contaminants were removed by passage through tandem Poros20MC/Amylose affinity columns in Buffer A and target proteins were collected from the flow-through. Complexes were further purified by size exclusion chromatography (Superdex 200, GE Healthcare Life Sciences) in Buffer A and concentrated using Amicon[®] Ultra centrifugal filters. For crystallization, protein buffer was exchanged to a low-salt buffer (5 mM KCl, 1 mM CaCl_2 , and 5 mM HEPES (pH 7.4)).

For ITC, the 1:1 Ca^{2+} /CaM- $\text{Ca}_v1.2$ C-IQ domain complex was purified as above but with an additional hydrophobic interaction chromatography step (HiLoad 16/10 Phenylsepharose HP, GE Healthcare Life Sciences) before size-exclusion chromatography. Purified 2:1 Ca^{2+} /CaM- $\text{Ca}_v1.2$ C-IQ domain complexes were applied to a phenylsepharose column in a buffer containing

150 mM KCl, 2 mM CaCl_2 , 25 mM HEPES (pH 7.4). Flow-through containing 1:1 complex was collected, concentrated, and subjected to the gel filtration chromatography. Individual lobes of CaM were purified as described previously (Kim *et al*, 2008).

Wild-type and mutants of HMT-tagged A-region for pull-down assays were initially co-expressed with CaM and passed through a Poros20MC column in the presence of Ca^{2+} . HMT-tagged A-region was further purified by amylose-affinity chromatography. Buffers used at this step contained 10 mM EDTA, so that Ca^{2+} /CaM was removed from the complex. For the last step, gel filtration chromatography was used.

Crystallization and structure determination

Crystals of the Ca^{2+} /CaM- $\text{Ca}_v1.2$ PreIQ-IQ domain complex were obtained against a reservoir solution containing 0.2 M Li_2SO_4 , 24.5% PEG 3350, and 0.1 M bis-Tris (pH 5.5). All the initial crystallization trials were set up by Mosquito nanolitre drop setter (TTP LABTECH) and further optimized by hanging-drop vapour diffusion with a 2:1 volume ratio of protein to mother liquor. Protein concentration used for crystallization is 8–10 mg ml⁻¹. Crystals were transferred to their respective mother liquor containing 5–10% glycerol as cryoprotectant and flash-cooled in liquid nitrogen. Diffraction data were collected at Beamline 8.3.1 (Advanced Light Source, Lawrence Berkeley National Laboratory).

All data were indexed, integrated and scaled with HKL2000 (Otwinowski and Minor, 1997). The structure was solved by molecular replacement using PHASER (Storoni *et al*, 2004) using a full-length CaM (A form of Ca^{2+} /CaM- $\text{Ca}_v1.2$ IQ domain structure (Van Petegem *et al*, 2005)) and N-lobe as a search model. On the basis of clearly visible electron density corresponding to Ca^{2+} ions, 12 Ca^{2+} ions (for two CaM and two N-lobes) were assigned manually. After one round of refinement, clear density for the PreIQ helix and IQ helix appeared and the corresponding alanine backbone was built. Peptide register was established during repetitive rounds of refinement. Due to poor electron density, C-lobe bound to the C-region was partially modelled with only a few side chains defined. Solvent molecules were added manually. Model building was done in COOT (Emsley and Cowtan, 2004). Structures were refined to 2.55 Å using REFMAC5 (Murshudov *et al*, 1997). TLS and NCS parameters were included throughout the refinement. The final model consists of four CaM molecules (chain A): residues 3–147; (chain B): residues 3–76, 82–91, 100–110, and 115–146; (chain C): residues 3–147; (chain D): residues 3–76, 82–91, 101–109, and 115–146; and two PreIQ-IQ molecules (chain E): residues 1560–1602 and 1614–1634; (chain F): residues 1560–1602 and 1614–1634.

Analytical ultracentrifugation analysis

For sedimentation equilibrium experiments, 110 µl of Ca^{2+} /CaM- $\text{Ca}_v1.2$ C-terminus complexes at different concentrations ranging from 5 to 205 µM were loaded in a six-chamber analytical ultracentrifuge cuvette. As a blank, 120 µl of protein buffer (10 mM HEPES (pH 7.4), 250 mM KCl, and 1 mM CaCl_2) was also loaded in the adjacent chamber. All experiments were performed at 4°C in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter). The molecular mass was calculated from a single-species exponential fit (Microsoft Excel) to the distribution of concentration over the radius of the chamber: $M = (2RT/[(1-\nu\rho) \cdot \omega^2]) \cdot (d[\ln(c)]/d(r^2))$, where M is the molecular weight in g mol⁻¹, R is the gas constant (8.314 J (mol K)⁻¹), T is the temperature in K, ν is the partial specific volume of the protein in ml g⁻¹, ρ is the density of the solvent in g ml⁻¹, ω is the angular velocity of centrifugation in rad s⁻¹, and r is the distance in cm from the centre of the rotor to a given position in the cell. Partial specific volume was calculated from the sum of the volumes of individual residues in the protein. Solvent density was calculated from the components of the buffer. Residuals were calculated as the difference between the measured absorbance value and the predicted value extrapolated from the calculated molecular mass.

Isothermal titration calorimetry

All ITC measurements were performed on a VP-ITC MicroCalorimeter (MicroCal) in 10 mM HEPES/KOH (pH 7.4), 5 mM KCl, and 1 mM CaCl_2 at 15°C. Purified Ca^{2+} /CaM- $\text{Ca}_v1.2$ C-IQ (1:1) complex, HMT-tagged A-region, and individual CaM lobes were dialysed against 21 buffer overnight at 4°C. After centrifugation at 40 000 r.p.m. for 30 min at 4°C, protein concentrations were

determined by absorbance at 280 nm. For C-region interaction measurements, degassed CaM C-lobe (100 μM) or CaM N-lobe (300 μM) was titrated into 10 or 30 μM of Ca^{2+} /CaM–Ca $\text{v}1.2$ C-IQ (1:1) complex, respectively. For apo-lobe measurements, 100 μM of CaM $_{12}$ N-lobe or CaM $_{34}$ C-lobe was titrated into 10 μM Ca^{2+} /CaM–Ca $\text{v}1.2$ C-IQ (1:1) complex. For A-region measurements, 100 μM of CaM N-lobe, CaM C-lobe, CaM $_{12}$ N-lobe, or CaM $_{34}$ C-lobe was titrated into 10 μM of HMT-fused A-region under the same conditions described above. Heat of dilution from titrations of protein into buffer was subtracted to correct the baseline. Data were fitted in Origin version 7.0 (MicroCal) assuming a single binding mode. Resulting binding constants were average of at least two independent measurements.

Pulldown experiments

Purified wild type or mutants of HMT-tagged A-region were mixed with N-lobe at a final concentration of 20 and 100 μM , respectively, and incubated on ice for 1–2 h. HMT alone was also used for a negative control. Binding was done in Buffer A (250 mM KCl, 1 mM CaCl_2 , and 10 mM HEPES (pH 7.4)) with a total volume of 100 μl . Protein mixtures were incubated with 80 μl (net volume) amylose beads on ice for 30 min. After unbound proteins were washed out three times with 500 μl Buffer A, proteins bound to amylose resin were eluted with Buffer A plus 10 mM maltose and analysed by Tricine–SDS–PAGE (Schagger, 2006).

Electrophysiology

Human Ca $\text{v}1.2$ (Ca $\text{v}\alpha1c77$; accession number CAA84346) in pcDNA3.1(+)/hygro (Invitrogen), Ca $\text{v}\beta2a$ in pGEM-HE (Promega), and Ca $\text{v}\alpha2\delta1$ in pcDNA3 were used for two-electrode voltage-clamp experiments as described previously (Van Petegem *et al*, 2005). Structure-guided alanine mutants of Ca $\text{v}1.2$ were generated using QuikChange (Stratagene). RNAs of Ca $\text{v}1.2$, Ca $\text{v}\beta2a$, and Ca $\text{v}\alpha2\delta1$ were transcribed *in vitro* using a T7 mMessage mMachine kit (Ambion) according to manufacturer's instructions. *Xenopus* oocytes were injected with 50 nl RNA mixture of Ca $\text{v}1.2$ (33–167 nM wild type or mutants), Ca $\text{v}\beta2a$ (33 nM), and Ca $\text{v}\alpha2\delta1$ (33 nM) using Nanoject II auto-nanoliter injector (Drummond Scientific) and kept at 18°C in ND96 medium containing penicillin and streptomycin. Recordings were carried out 2–5 days after RNA injection. To minimize Ca^{2+} -activated Cl^- current, 47 nl of 0.1 M BAPTA was microinjected into oocytes before recording. Two-electrode voltage-clamp experiments were performed with either a Ba^{2+} (40 mM $\text{Ba}(\text{OH})_2$, 1 mM KOH, 50 mM NaOH, and 10 mM HEPES (pH 7.4) with HNO_3) or a Ca^{2+} (40 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM KOH, 50 mM NaOH, and 10 mM HEPES (pH 7.4) with HNO_3) as a charge carrier using a perfusion system with a Valvelink 16 controller (Automate Scientific). CDI recordings were carried out with a 450-ms pulse protocol from –90 to +20 mV. For CDF, Ca^{2+} currents were measured by applying 40 times of 50-ms pulses to +20 mV at a frequency of 3 Hz. Currents were recorded using CLAMPEX 8.2.0.224 program (Axon Instruments) with GeneClamp 500B amplifier (Axon Instruments) controlled by a computer with a 1200 MHz processor (Gateway) and digitized at 1 kHz with a Digidata1332A (Axon Instruments). Resistance of electrodes ranges from 0.3 to 2.0 M Ω and leak currents were subtracted using a P/4 protocol. All data were produced from more than one oocyte batch and analysed with Clampfit 9.2 (Axon Instruments).

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Subunit-counting experiments

Full-length human Ca $\text{v}1.2$ (splice variant $\alpha1c77$) bearing a C-terminal linker, (GGSGGSRGSGSGG) connected to EGFP, and rat Ca $\text{v}\beta2a$ in pGEMHE were used for RNA preparation and expressed in *Xenopus laevis* oocytes. RNAs were transcribed *in vitro* using a T7 mMessage mMachine kit (Ambion). Oocytes were injected with 50 nl of a 1:1 mixture of 500 ng μl^{-1} Ca $\text{v}1.2$ –EGFP and 100 ng μl^{-1} Ca $\text{v}\beta2a$ cRNA. At 20–24 h after cRNA injection, oocytes were enzymatically treated with 1:1 mixture of neuraminidase (1 U ml^{-1} , Sigma) and hyaluronidase (1 mg ml^{-1} , Sigma), devitellinized by hand and put into an imaging chamber with high refractive index glass ($n = 1.78$) to match the microscope objective (Olympus $\times 100$ NA 1.65). EGFP was excited by a 488-nm argon laser and fluorescence (525/50 nm band pass filter, Chroma) was acquired by an EMCCD camera (Andor iXon DV-897 BV). Total internal reflection microscopy was used to image single molecules without background fluorescence from the interior of the cell (Ulbrich and Isacoff, 2007). Immobile diffraction limited spots with discrete bleaching steps were manually counted; spots with irregular emissions and mobile spots were excluded.

Homology modelling of the Ca $\text{v}1.2$ EF-hand motif

EF-hand domain sequences of human Na $\text{v}1.2$ (residues 1777–1882) and Na $\text{v}1.5$ (residues 1773–1865) were aligned with a corresponding sequence of human Ca $\text{v}1.2$ C-terminus using a web-based ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Using this sequence alignment, a homology model of Ca $\text{v}1.2$ EF-hand motif was automatically generated by SWISS-MODEL (<http://swissmodel.expasy.org/>) (Arnold *et al*, 2006) based on the NMR structure of either Na $\text{v}1.2$ (2KAV) or Na $\text{v}1.5$ (2KBI) EF-hand motif.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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