Crystal Structure of the CaV2 IQ Domain in Complex with Ca^{2+}/Calmodulin: High-Resolution Mechanistic Implications for Channel Regulation by Ca^{2+}

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**SUMMARY**

Calmodulin (CaM) regulation of Ca^{2+} channels is central to Ca^{2+} signaling. CaV1 versus CaV2 classes of these channels exhibit divergent forms of regulation, potentially relating to customized CaM/IQ interactions among different channels. Here we report the crystal structures for the Ca^{2+}/CaM IQ domains of both CaV2.1 and CaV2.3 channels. These highly similar structures emphasize that major CaM contacts with the IQ domain extend well upstream of traditional consensus residues. Surprisingly, upstream mutations strongly diminished CaV2.1 regulation, whereas downstream perturbations had limited effects. Furthermore, our CaV2 structures closely resemble published Ca^{2+}/CaM-CaV1.2 IQ structures, arguing against CaV1/2 regulatory differences based solely on contrasting CaM/IQ conformations. Instead, ala nine scanning of the CaV2.1 IQ domain, combined with structure-based molecular simulation of corresponding CaM/IQ binding energy perturbations, suggests that the C lobe of CaM partially dislodges from the IQ element during channel regulation, allowing exposed IQ residues to trigger regulation via isoform-specific interactions with alternative channel regions.

**INTRODUCTION**

Voltage-gated Ca^{2+} channels of the CaV1-2 family constitute dominant sources of Ca^{2+} entry that trigger numerous biological functions (Dolmetsch, 2003; Dunlap et al., 1995). Fitting with their salient functional roles, these channels are subject to extensive positive and negative Ca^{2+} feedback control of their opening, with calmodulin (CaM) serving as the Ca^{2+} sensor (Evans and Zamponi, 2006). In particular, for CaV2 channels which predominate in triggering central nervous system neurotransmitter release (Wheeler et al., 1994), these feedback regulatory systems are believed to influence short-term synaptic plasticity (Borst and Sakmann, 1998; Chaudhuri et al., 2005; Cuttle et al., 1998; Wykes et al., 2007; Xu and Wu, 2005), and thereby the computational repertoire of the brain (Abbott and Regehr, 2004; Tsodyks and Markram, 1997). In all of these contexts, CaM clearly exhibits powerful and unexpected forms of Ca^{2+} decoding (Dunlap, 2007), the principles of which might generalize to numerous Ca^{2+} signaling systems. As such, the CaM regulation of Ca^{2+} channels not only has specific consequences for Ca^{2+} signaling but might also represent a general mechanistic paradigm for Ca^{2+} decoding and ion-channel modulation.

CaM/Ca^{2+} channel regulation includes several provocative functional features. First, in resting channels, Ca^{2+}-free CaM (apoCaM) is already preassociated with the cytoplasmic region of channels, rendering CaM as a “resident” Ca^{2+} sensor (Erickson et al., 2001, 2003b; Pitt et al., 2001). Second, CaM can “bifurcate” the local Ca^{2+} signal in the channel nanodomain: Ca^{2+} binding to the C-terminal lobe of CaM (C lobe) alone can trigger one type of regulatory process on a channel, and Ca^{2+} binding to the N-terminal lobe (N lobe) can induce a separate form of modulation (Chaudhuri et al., 2005; DeMaria et al., 2001; Yang et al., 2006). Intriguingly, despite evidence that a single CaM molecule orchestrates both processes (Mori et al., 2004; Yang et al., 2007), Ca^{2+}-induced regulation persists with millimolar concentrations of intracellular Ca^{2+} chelators such as EGTA and BAPTA, whereas N lobe processes are largely inhibited by such Ca^{2+} buffering (Liang et al., 2003). Not every channel manifests both C and N lobe functions, but the coexistence of both processes has been clearly demonstrated in CaV2.1 and CaV1.3 channels (Chaudhuri et al., 2005; DeMaria et al., 2001; Lee et al., 2003; Yang et al., 2006). Finally, the polarity of channel modulation by a given lobe of CaM can be inverted between closely similar Ca^{2+} channel types. For CaV2.1 channels, the C lobe triggers a fast Ca^{2+}-dependent facilitation of opening (CDF) (Chaudhuri et al., 2005; DeMaria et al., 2001), whereas the N lobe produces a slower Ca^{2+}-dependent inactivation (CDI) (Chaudhuri et al., 2005; DeMaria et al., 2001; Lee et al., 2003). By contrast, for CaV1.2 channels, Ca^{2+} binding to the C lobe triggers a rapid CDI process, whereas the N lobe initiates a more gradual and distinct CDI mechanism (Alseikhan et al., 2002; Dick et al., 2008; Peterson et al., 1999). Remarkably, structural similarities and single-channel data hint that the CaV2.1 and CaV1.2 C lobe processes might be the same, only with opposing polarity (CDF versus CDI) (Chaudhuri et al., 2007). Probing this C lobe directional inversion thereby promises rich mechanistic insights.
By contrast to function, less is known about the underlying structural bases of CaM/channel regulation. Most of the structural determinants of CaM regulation of CaV1-2 channels are localized to their main, pore-forming $\alpha_1$ subunits, specifically in the upstream third of subunit carboxy termini (Figure 1A left, CI region) (Evans and Zamponi, 2006). Fluorescence resonance energy transfer (FRET) and biochemical evidence indicate that apoCaM is preassociated with a consensus IQ domain and an immediately upstream preIQ region (Erickson et al., 2001, 2003a; Pitt et al., 2001). Ca2+ binding to CaM might cause rearrangements of CaM on these same segments (Evans et al., 2004), as Ca2+/CaM binds to IQ domains of many CaV1-2 channels (DeMaria et al., 2001; Peterson et al., 1999; Zuhlke et al., 1999) as well as to preIQ regions of CaV2.1 (Evans et al., 2004) and CaV1.2 (Erickson et al., 2003a; Kim et al., 2004; Pate et al., 2000; Pitt et al., 2001; Romanin et al., 2000; Tang et al., 2003). These rearrangements somehow modulate channel gating, with the potential involvement of an EF hand-like channel module as a transduction element for C lobe-mediated processes (Chaudhuri et al., 2004; de Leon et al., 1995; Kim et al., 2004; Peterson et al., 2000; Zuhlke and Reuter, 1998). Given the multiplicity of structural determinants within a single molecular complex, the precise sequence underlying Ca2+-dependent regulation of channels remains unclear. Nonetheless, Ca2+/CaM interaction with the IQ domain has been viewed as potentially central to initiating a chain of ensuing regulatory events, because mutations within this domain can eliminate both CDF and CDI in CaV2.1 (DeMaria et al., 2001; Lee et al., 2003) and CDI within CaV2.2, CaV2.3, and CaV2.3 channels (Evans and Zamponi, 2006; Yang et al., 2006). Indeed, these complexes between CaM and channel IQ domains represent sophisticated additions to a family of CaM/IQ assemblies, originally defined for CaM interactions with unconventional myosins and now encompassing over 50 different mammalian molecules (Hoeflich and Ikura, 2002). Unlike classical CaM binding targets, IQ domains support both apoCaM and Ca2+/CaM interactions that underlie exquisite functional modulation of affiliated molecules (Black et al., 2006), nowhere more evident than in the setting of Ca2+ channels.

Very recently, the first atomic-resolution structures of Ca2+ regulatory modules were reported (Fallon et al., 2005; Van Petegem et al., 2005). In keeping with the presumed centrality of the IQ domain, these structures have focused upon Ca2+/CaM interaction with the IQ domain of CaV1.2, from sagittal (top) and coronal (bottom) viewpoints. The IQ domain is colored in red; amino-terminal end toward the left. N and C lobes are differentially colored as labeled. The side chain of I[0] residue is shown as a stick diagram. The four EF hands with Ca2+ (balls) are numbered in order. (C) Analogous CaV2.1 structure. (D) Superimposition of structures in (B) and (C), indicating close similarity.
First, CaM, as fully charged with four Ca$^{2+}$ ions, envelopes an involving the IQ moiety of CaV2.1. Both structures were highly similar representatives of a generic Ca$^2+$/CaM class structure, and correlation with functional analysis of an alanine mutational scan suggests that the C lobe of CaM partially dislodges from the IQ element during channel regulation.

RESULTS

Recapitulation of Distinctive Features of the Ca$_{a}$ IQ Domains Complexed with Ca$^{2+}$/CaM

We utilized molecular replacement to solve two crystal structures: one for Ca$^{2+}$/CaM in complex with the IQ moiety of CaV2.1 channels, at a 2.2 Å resolution, and the second for the analogous complex involving the IQ element during channel regulation. By contrast, point contacts contrast with the traditional emphasis on downstream consensus residues. Figure 2C displays specific examples of intimate docking at both upstream and downstream residues, emphasizing the interaction of I[-6] within a hydrophobic pocket of the N lobe (left), as well as the anchoring of Y[+3] and Y[+4] residues into like features of the C lobe. Indeed, the IQ domain demonstrates greater solvent exposure in its downstream half, compared to an almost completely buried upstream portion (Figure 2A, bottom; Figure 2D). These features contrast with the dominance of C lobe and downstream IQ interactions within the apoCaM/IQ structure for myosin (Houdusse et al., 2006).

Crystals for the analogous Ca$_{a}$2.1 structure manifested a C2 symmetry, where the asymmetric unit contained two CaM/IQ complexes (1 and 2), each with 1:1 stoichiometry. Both complexes adopted nearly indistinguishable conformations, although complex 1 yielded a somewhat more defined electron density map. Accordingly, all subsequent analysis refers to structure 1 (Figures 1C and 2B). The near identity of this Ca$_{a}$2.1 structure to its CaV2.3 counterpart is documented by the overlay in Figure 1D. Additionally, the detailed contact map (Figure 2B) confirmed a highly analogous interface between CaM and the IQ domain of CaV2.1, and the atomic positions were closely similar throughout (Table 1). The details of model refinement for both structures are summarized in Table 2.

Thus oriented to CaV2 structure, we searched for any structural differences relative to the analogous complex for Ca$_{a}$1.2 channels, ones that might rationalize the notable contrasts in regulatory phenotype between these two channel classes. In this regard, no simple answers arose: the Ca$_{a}$2.1 structure is highly similar to the most common Ca$_{a}$1 form, but for the lack of an ~10° bend in the mid-IQ segment of the Ca$_{a}$1 structure (see Figure S1 in the Supplemental Data available with this article online). This minor difference, in a largely buried peptide, seemed insufficient to explain appreciable functional differences. Also, the similarity of an extensive contact map for a published Ca$_{a}$1.2 complex (Van Petegem et al., 2005; Figure S1), and statistical comparisons of atom positions (Table 1), underscored the overall structural similarities.

Systematic Alanine Scanning of the Ca$_{a}$2.1 IQ Domain

To gain perspective on the contrast between structural similarity and functional divergence, we undertook alanine-scanning mutagenesis of the Ca$_{a}$2.1 IQ domain, and correlated mutations with CaM regulatory function as assayed by whole-cell electrophysiological recordings. Previous reports have described the effects of cluster mutations within the IQ domain (DeMaria et al., 2001; Lee et al., 2003), but these scans were far from exhaustive, and could well involve perturbations of main-chain conformation that complicate interpretation. By contrast, point
Figure 2. Residue-by-Residue Interaction of CaV2 IQ Domains with Ca\textsuperscript{2+}/CaM

(A) Contact map of Ca\textsuperscript{2+}/CaM with the IQ domain of CaV2.3. IQ domain sequence in light gray rectangle. CaM residues within 4 Å of indicated IQ domain position are shown above (N lobe) and below (C lobe) IQ sequence. Listings for CaM include number of atoms within each residue that are within 4 Å of the indicated IQ position. Water-mediated hydrogen bonds are shown by "o" symbol. Bar graph at the bottom plots solvent-accessible area for each IQ position.

(B) Contact map for Ca\textsuperscript{2+}/CaM with CaV2.1 IQ domain. Format as in (A).

(C) Close hydrophobic interactions between select IQ domain residues and hydrophobic pockets of Ca\textsuperscript{2+}/CaM, taken from CaV2.3 structure (Figure 1B). Left: upstream residues Y\textsuperscript{[-5]} and I\textsuperscript{[-6]} dock closely with N lobe pocket (shown in space-filling representation). Right, those at positions 3 and 4 nestle tightly with C lobe cavities. CaM is color coded for electrostatic potential (gray, hydrophobic; red, electronegative).

(D) Space-filling representation of CaV2.3 structure. Partial exposure of IQ domain (red) at carboxy-terminal end. Y\textsuperscript{[+4]}, stick figure; V\textsuperscript{[+12]}, colored in yellow.
alanine mutations probe side-chain effects without perturbation of the backbone fold (Cunningham and Wells, 1989).

The conventional concept for IQ domains (bottom, filled circles). The small rise of CDF is readily detected as a slow phase of increasing Ca2+ current seen during short (50 ms) test depolarizations (top, gray trace, arrow). When channels are facilitated by Ca2+ entry during a preceding voltage prepulse, ensuing currents activate rapidly to the facilitated level during the test pulse (top, black trace). To quantify the facilitation produced by the prepulse, we integrate the difference between normalized test-pulse currents in the absence and presence of a prepulse (ΔQ, gray shaded area), and this integral is used to determine the relative facilitation (RF) induced by the voltage prepulse. On average, RF demonstrates a bell-shaped dependence upon prepulse voltage (bottom, open circles), as expected for a genuine Ca2+-driven process (Brehm and Eckert, 1978). By contrast, fitting with the strong preference of CaM for Ca2+ over Ba2+ (Chao et al., 1984), there is little evidence of such prepulse facilitation with Ba2+ as charge carrier (bottom, filled circles). The small rise of RF seen with Ba2+ (~0.05, bottom, open circles) likely reflects background G protein modulation (DeMaria et al., 2001). To index pure CDF, we consider the increase of the Ca2+ over Ba2+ relations (isoleucine at position zero mutated to an alanine [I(0)A], and methionine at position 1 changed to alanine [M(1)A]) indicates that the bulk of attenuation is attributable to the I[0] position (Figure 3E). Beyond this derivative result, the results of alanine scanning were unexpected (Figure 3E). Point mutations at any of the other canonical IQ domain residues (gray highlighted residues) had no appreciable effect on CDF. In fact, no more than minor effects were observed for any of the residues downstream of the initial [I0] at the beginning of the canonical motif; the strongest downstream effect was seen with Y[+3]A and Y[+4]A perturbations, which induced no more than 30%–40% attenuation of function. The conventional concept for IQ domains predicts that the region downstream of I[0] would be functionally dominant (Bahler and Rhoads, 2002). By contrast, alanine point mutations at multiple upstream sites induced surprisingly large reductions in CDF, and this upstream trend was upheld for one of two naturally occurring alanines that were changed to threonines (Figure 3E, positions –4 and –3). In particular, CDF was nearly abolished with I[−6]A, [−4]T, and M[−1]A mutations (Figures 3B, 3C, and 3E), all of these being upstream residues. These findings emphasized a surprising structure-function correlation for the Ca2+-CaM/IQ complex of CaV2 channels: IQ domain contacts at multiple residues upstream of I[0] were functionally dominant for C lobe-triggered regulatory function, compared with the canonical IQ motif residues, which include I[0] and selected downstream positions. This feature likely relates to the substantial structural contrasts between Ca2+-CaM/IQ and apoCaM/IQ complexes (Houdusse et al., 2006), for which the latter forms the basis of traditional IQ motif hotspots (Figure 3E, highlighted positions). The set of key upstream residues identified here might be emblematic of a new canonical IQ motif pertinent to Ca2+/CaM interactions. In this regard, the pattern of upstream dominance might well pertain to the CaV1C a2+-CaM/IQ complex in regard to C lobe signaling (Fallon et al., 2005; Van Petegem et al., 2005).

We next turned to a second form of CaM regulation of CaV2.1 channels, as shown in Figure 3D. Ca2+ binding to the N lobe of CaM triggers a CDI that progresses over hundreds of

| Structure of Ca2+/CaM Complexed with CaV2 IQ Domain |

| Table 1. C2-Root-Mean-Square Deviations (Å2) for Structures of Ca2+/CaM Complexed with IQ Domains of CaV2.3, CaV2.1, and CaV1.2 |

<table>
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<tr>
<th>Structure</th>
<th>CaV2.3</th>
<th>CaV2.1a</th>
<th>CaV2.1b</th>
<th>CaV1.2c</th>
<th>CaV1.2d</th>
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<td></td>
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<td>CaV1.2d</td>
<td>4.03</td>
<td></td>
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</table>

* Denotes structure 1 of the CaV2.1 complex.
* Denotes structure 2 of the CaV2.1 complex.
* C form in PDB code 2BE6.
* PDB code 2F3Y.

| Table 2. Data Collection and Refinement Statistics |

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Highest resolution shell is shown in parentheses.
milliseconds (Chaudhuri et al., 2005, 2007; DeMaria et al., 2001; Lee et al., 2003; Soong et al., 2002). This can be seen from the faster decay of Ca^{2+} versus Ba^{2+} currents (Figure 3D, top). Because the Ba^{2+} current decay reflects a separate form of voltage-dependent channel inactivation (Alseikhan et al., 2002; Jones et al., 1999; Patil et al., 1998), the additional speeding of decay seen in Ca^{2+} versus Ba^{2+} currents reflects pure CDI (Figure 3D, bottom). The maximum difference between Ba^{2+} and Ca^{2+} relations, RF_{800} = 0.36, quantifies average CDI. Whereas substitution of glutamate at IQ positions 0 and 1 does eliminate this CDI (DeMaria et al., 2001), more targeted alanine substitutions have left CDI unchanged, at least for the few residues where such alanine substitutions have thus far been made (DeMaria et al., 2001; Lee et al., 2003). Here, despite an exhaustive scan, none of the alanine cluster/point mutations throughout the IQ domain appreciably affected CDI (Figure 3F). This result was unexpected, given the anticipated centrality of the IQ domain for CaM/channel regulation.

Potential Mechanisms of CaM/Channel Regulation Implicated by Ca^{2+}-CaM/IQ Structures
The demonstrated similarity in Ca^{2+}-CaM/IQ structure for CaV2 and CaV1 channels (Table 1; Figure S1), together with the functional outcomes of systematic alanine scanning of the CaV2.1 IQ region (Figure 3), offered new constraints for evaluating mechanisms of CaM/channel regulation. The simplest scenario for rationalizing the opposing regulatory polarities of C lobe regulation in CaV2.1 and CaV1.2 channels would arise from clear distinctions in the Ca^{2+}-CaM/IQ structures of CaV2.1 versus CaV1.2 channels. Our novel structures (Figures 1 and 2) clearly exclude this scenario, at least for the conformation in our crystals. This exclusion allowed us to focus on two other broad classes of mechanisms.

Class 1
This mechanism postulates insignificant structural differences between CaV2.1 and CaV1.2 channels, when comparing either their apoCaM/IQ complexes or their Ca^{2+}-CaM/IQ assemblies. In this manner, the IQ domain itself would play no distinguishing role in specifying C lobe signaling polarity. Instead, nearly identical Ca^{2+}-induced conformational changes in the CaM/IQ structure would be decoded in different ways by other parts of these two channel types. An important corollary of this class is “context-independent IQ function,” wherein substitution of the IQ domain from one channel subtype into another should not alter C lobe signaling.

To test this prediction, we undertook the chimeric channel experiments displayed in Figure 4. CaV1.2 and CaV1.2.1 channels (with pore-forming subunits z1C and z1A, respectively) both manifest Ca^{2+} regulation triggered by Ca^{2+} binding to the C lobe of CaM, but the regulatory polarity is CDI for CaV1.2, and CDF for CaV1.2.1. To examine the effects of targeted switching of IQ domains, we substituted the crucial proximal third of the carboxy terminus (Figure 1A, CI region) from either an z1C or z1A subunit onto a single, expression-permissive CaV2.3 channel backbone (Figure 4A, left, z1E). Direct swapping of CI regions between z1C and z1A subunits yielded nonfunctional channels (not shown), whereas CaV2.3 channels (z1E subunit backbone) do not exhibit intrinsic C lobe CaM regulation (Liang et al., 2003). Hence, the CaV2.3 backbone furnished an appropriate and uniform context in which the regulatory effects of chimeric channel manipulations could be evaluated. Importantly, all recordings were performed in elevated Ca^{2+} buffering (5 mM EGTA) so as to isolate the C lobe component of CaM regulation, and the electrophysiological protocols and analysis were analogous to those in Figures 3A and 3D.

The experimental results clearly contradicted context-independent function of the IQ domain. For construct 1, with the carboxy tail from CaV1.2 channels, C lobe signaling induced CDI (Figures 4A and 4C, top row), as for the parent CaV1.2 channel. Following the same linkage to the parental channel, construct 2 (carboxy tail from CaV2.1 channels) exhibited CDF (Figures 4B and 4C, row 2). Given this baseline, mechanistically informative results were obtained by selective exchange of IQ segments between constructs 1 and 2. Figure 4C (constructs 3 and 4) shows that this exchange did not preserve the C lobe signaling present in the original constructs. Construct 3, which features insertion of the IQ segment of CaV2.1 into construct 1, failed to exhibit appreciable CDF or CDI. Construct 4, wherein the IQ segment from CaV1.2 has been introduced into construct 2, shows significantly weaker CDF than seen in construct 2. Yet stronger violation of context-independent IQ function came from two further constructs. Upon substituting the EF-hand domain from CaV1.2 into construct 2, the resulting construct 5 still exhibited CDF (Figure 4C, row 5), albeit weaker than in construct 2. However, after switching the CaV1.2 IQ domain into construct 2, the
resulting construct 6 showed unmistakable CDI (Figure 4C, row 6). Thus, substitution of an IQ domain alone, as seen in the conversion from construct 5 into 6, switched regulatory polarity. Hence, class 1 cannot be true.

**Class 2**

This leads to a remaining possibility, wherein our CaV2 IQ/CaM structure might only be partially related to the conformation underlying functional CDF. Instead, the physiologically relevant conformation features some displacement of CaM to expose portions of the IQ domain for interaction with other regions, which in turn triggers channel regulation. In this scenario, the IQ domain would contribute in part to C lobe signaling polarity, whereas interactions with other portions of the channel would also matter.

To investigate this hypothesis, we compared our CaV2 IQ/CaM structure (Figure 1) to the functional outcomes from alanine-scanning mutagenesis (Figure 3) using Robetta, a molecular simulation package for ligand/receptor binding energy perturbation via single point mutations (Kortemme and Baker, 2002). These algorithms have successfully predicted these energies for

Figure 4. Context-Dependent IQ Function

(A) C lobe regulation in chimeric channel with carboxy tail from CaV1.2 α1C subunit (construct 1). Left: cartoon of chimeric channel α1 subunit composition, with carboxy-tail landmarks as in Figure 1A. The “backbone” of all chimeric α1 subunits, from the amino terminus through the beginning of the carboxy terminus, is derived from the CaV2.3 α1E subunit, here and throughout the figure. The proximal third of the carboxy tail from the CaV1.2 α1C subunit, spanning the EF hand through the IQ domain, is fused to this backbone to create construct 1. Middle: exemplar Ca2+ traces showing absence of CDF (format as in Figure 3A). Right: exemplar Ca2+ and Ba2+ traces illustrating the presence of CDI (format as in Figure 3D). All experiments utilized high 5 mM EGTA Ca2+ buffering, to enrich for C lobe-mediated regulation throughout the figure.

(B) C lobe regulation in chimeric channels with carboxy tail from CaV2.1 α1A subunit (construct 2). CDF is present, but CDI is absent.

(C) CDF and CDI population data, for constructs 1–6. Left: schematic showing composition of constructs. Middle: average CDF strength (g). Right: average CDI strength (f). Metrics are defined in Figures 3A and 3D. Context-dependent function of IQ domain is shown by constructs 2 versus 3, and constructs 5 versus 6. All error bars for population data indicate SEM.
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multiple sets of experimentally determined structures and binding energies. Here we utilized our Ca\textsubscript{v}2.3 IQ/CaM structure as an input to Robetta, and the energetic effects of various point mutations to binding were then estimated. Importantly, a core assumption of this algorithm, that main-chain conformation undergoes little perturbation by mutations, is consistent with prior structures showing that Ca\textsuperscript{2+}/CaM in complex with a mutant Ca\textsubscript{v}1.2 IQ domain is nearly indistinguishable from the wild-type configuration (Fallon et al., 2005). Figure 3E (bottom) displays the results of the Ca\textsubscript{v}2.3 analysis as $\Delta \Delta G$ values, each denoting the estimated change in IQ/CaM binding energy for the indicated point mutation. All portions of the IQ segment subject to these energy calculations were characterized by well-defined electron densities, as demonstrated in Figure 3G (bottom). If the extent of IQ/CaM binding, as captured in our crystal structure (Figure 1B), were directly related to the strength of functional CDF, then plotting the strength of CDF ($g$) as a function of $\Delta \Delta G$ should define a 1:1 binding isotherm. Alternatively, if such binding were only indirectly related to function, such as in a hypothetical configurational isotherm featuring some Ca\textsuperscript{2+}/CaM displacement toward another surrogate partner, an absence of correlation between these two variables might result. Figure 5A displays the results of this analysis, where each symbol relates to a single point mutation from Figure 3E. Initial examination would suggest no correlation. However, when the data were segregated according N and C lobe interfaces, a coherent pattern emerged. The plot for data relating to the N lobe interface alone (Figure 5B) renders a clearly recognizable 1:1 binding curve, with half-saturating binding energy $\sim$2 kcal/mol. To further corroborate this relation, we introduced mutations other than alanine at a single position ([I[-6]) where intimate CaM/IQ contact is prominent in Ca\textsubscript{v}2 structure (Figure 2C). Reassuringly, these alternative mutations produced data that also resided close to the same binding curve (Figure 5B, open symbols). Hence, IQ domain binding to the N lobe of CaM appears intimately related to functional CDF.

DISCUSSION

We have solved the crystal structure for Ca\textsuperscript{2+}/CaM in complex with the IQ domains from Ca\textsubscript{v}2.3 and Ca\textsubscript{v}2.1 channels. Both of these can be considered as equivalent to a generic Ca\textsubscript{v}2 structure which shares most of the hallmark features for previously resolved structures of Ca\textsuperscript{2+}/CaM bound to the IQ domain of Ca\textsubscript{v}1,2 channels. One surprising aspect of this structure arises by ala-nine-scanning mutagenesis of the entire Ca\textsubscript{v}2.1 IQ domain. For regulation by the C lobe of CaM, IQ domain contacts upstream of the canonical IQ motif (IQxxGGxxxxR) are functionally dominant, whereas the downstream canonical contacts play a lesser role. This set of key upstream residues encompasses hydrophobic contacts at [−6], [−5], and [−1] positions. As these are not predicted by any known Ca\textsuperscript{2+}/CaM binding motif (Rhoads and Friedberg, 1997), this set of contacts might comprise part of a new canonical IQ motif pertinent to Ca\textsuperscript{2+}/CaM interactions (as opposed to the traditional motif for apoCaM; Rhoads and Friedberg, 1997). Fitting with potential generalization, an IQ domain database indicates conservation of such hydrophobic residues (Marchler-Bauer et al., 2007). Another interesting feature concerns signaling triggered by Ca\textsuperscript{2+} binding to the N lobe of CaM (Figure 3D). In this context, none of the IQ domain contacts appeared functionally consequential. A final key result is that only minor structural differences are present between the Ca\textsubscript{v}2 and Ca\textsubscript{v}1 IQ/CaM structures, and these in themselves seem insufficient to account for the substantial differences in C lobe CaM-mediated regulation of these channels. Together, these results unambiguously exclude the simplest of prevailing hypotheses for channel regulation, wherein clear distinctions in the
Figure 5. Comparing CDF Function and Predicted Binding Energy Perturbation

(A) Apparent absence of correlation between strength of CDF (ordinate) and predicted CaM/IQ binding perturbation by point mutations in IQ domain of CaV2 channels (abscissa). Filled symbols correspond to data from Figure 3E.

(B) Strength of CDF correlates well with predicted binding energy perturbations, if analysis is restricted to IQ residues with N lobe contacts. Filled symbols, N lobe subset of data in (A); open symbols, additional mutations at position +6. Solid curve plots 1:1 binding isotherm, with a half-binding energy value of ~2 kcal/mol.
Ca²⁺-CaM/IQ structures of CaV2.1 versus CaV1.2 channels are primarily responsible for divergent regulatory effects. This exclusion now emphasizes more complex mechanisms, wherein multiple binding sites and structural determinants codominate in specifying CaM regulation of channels.

Specifically, by using molecular simulation (Robetta) to correspond our CaV2 structure with the functional outcomes of alanine-scanning mutagenesis across the IQ domain of CaV2.1 (Kortemme and Baker, 2002), we favor a working proposal (class 2) in which the configuration in our CaV2 Ca²⁺-CaM/IQ structure might not fully capture the configuration underlying functional CDF. Instead, the C lobe might partially dislodge from the IQ segment, enabling contacts between exposed IQ surfaces and other channel domains to trigger C lobe-mediated channel regulation. These alternative contacts might be customized to produce opposing C lobe regulatory effects between CaV2.1 and CaV1.2 channels. Likewise, the absence of appropriate contacts might explain the lack of C lobe-mediated regulation in CaV2.2 and CaV2.3 channels (Liang et al., 2003). Importantly, this proposal rationalizes the overall sparing of C lobe-driven CDF by mutations at Y[+3] and Y[+4] positions (Figure 3E), despite intimate anchoring of these residues within a C lobe cavity of the crystal structure (Figure 2C).

A remaining challenge for this class 2 proposal concerns the suggested importance of IQ contacts with the N lobe in producing CDF (Figure 5B); this importance might initially appear to contradict previous results that Ca²⁺ binding only to the C lobe of CaM is required to produce CDF (Chaudhuri et al., 2005; DeMaria et al., 2001). However, although our structure contains the Ca²⁺-bound N lobe in complex with the IQ domain, the contacts and energetics for a Ca²⁺-free N lobe interaction with the IQ element might be similar, and we would propose that either form of N lobe/IQ binding supports CDF. Another form of Ca²⁺/CaM displacement could explain the uniform absence of IQ mutational effects on regulation triggered by Ca²⁺ binding to the N lobe of CaM (Figures 3D and 3F). In particular, displacement of the N lobe from the IQ domain, and subsequent binding to another site, could trigger the slower form of CaM modulation (CDI, Figure 3D), in a manner insensitive to IQ manipulation. Indeed, there is evidence that the Ca²⁺-bound N lobe interacts with an aminoterminal channel locus to produce CDI in CaV1.3 and CaV1.2 channels (Dick et al., 2008), and we would propose that CaV2 channels contain a similar site elsewhere in the channel. Given this view, the slow induction of CDI would terminate CDF, because CDF appears reliant upon N lobe/IQ interactions.

One other class of mechanism deserves consideration. There could be appreciable differences in the structures of apoCaM in complex with the IQ domains of CaV2.1 and CaV1.2 channels, whereas the analogous structures for Ca²⁺/CaM are very similar. This proposal readily explains differences in regulatory polarity, in terms of distinct apoCaM/IQ configurations for different channel types. Moreover, this scheme could partially explain the alanine-scanning data for CaV2.1 (Figure 4C), as some discord between function and Ca²⁺/CaM binding to the IQ domain might reflect actual changes in apoCaM/IQ binding. However, live-cell FRET experiments between apoCaM and holochannels indicate a similar apoCaM/channel configuration in CaV1.2, CaV2.1, and CaV2.3 contexts (Erickson et al., 2001), at odds with this scenario. Although more direct data would be required to conclusively exclude this proposal, we favor class 2 mechanisms as more consistent with a comprehensive view of experimental constraints.

In all, our results emphasize that CaM/channel regulation likely reflects a multiplicity of binding configurations and structural determinants, likely extending beyond the IQ domain (Dunlap, 2007). Combined consideration of structure, alanine scanning, and molecular simulation hint at a scenario wherein Ca²⁺/CaM displaces from the IQ domain to facilitate further interactions triggering channel regulation. This provisional proposal forms an important framework for ongoing work. Future studies must peer beyond Ca²⁺/CaM in complex with the IQ domain, refine identification of additional effector sites, and resolve further related structures at the atomic level. In these structures, it might be necessary to simultaneously incorporate CaM with multiple sites, to fully capture the CaM/channel configurations most closely linked to modulation of channel gating. In this process, simple forms of molecular simulation (Baker, 2006) to correlate function with structure might continue to prove advantageous.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Crystallization** Recombinant rat CaM (as cloned into pET24b, Novagen, EMD Chemicals, San Diego, CA, USA) was expressed in Escherichia coli BL-21 (DE3) and purified to homogeneity by octyl-sephrose column chromatography (GE Healthcare, Piscataway, NJ, USA). The purified CaM was shown to be greater than 95% pure by SDS-PAGE. Synthetic peptides of the IQ domains for CaV2.1 and CaV2.3 channels were generated at the Synthesis and Sequencing Facility at the Johns Hopkins University School of Medicine. To confirm molecular weights, these peptides were analyzed by MALDI-TOF mass spectrophotometry (Applied Biosystems, Foster City, CA, USA). Mixtures of CaM (10 mg/ml) and each IQ domain peptide (3 mg/ml) were incubated in a solution containing 20 mM MOPS-NaOH, 10 mM CaCl₂ (pH 7.4). The mixture was then applied to a gel-filtration column (Superdex 75 10/300, GE Healthcare) equilibrated with same buffer. A purified CaM/IQ complex was thus isolated, and subsequently crystallized using hanging-drop vapor diffusion. Hanging drops were composed of 1 μl of protein solution and 1 μl of a 1:2 dilution of reservoir buffer (described below) with water. Each drop was vapor equilibrated against 500 μl of undiluted reservoir buffer, and incubated at 18°C. Further details of the crystallization conditions and related information for each type of complex are elaborated below.

**CaV2.1 IQ/CaM Complex** The purified complex of CaM and the IQ domain of CaV2.1 was further concentrated to 10 mg/ml using a Centricon spin column (YM-3, Millipore, Bedford, MA, USA). This concentrated complex was mixed into droplets as described (C) Discordance of strength of CDF and predicted binding energy perturbations for C lobe contacts. Filled symbols, C lobe subset of data in (A); open symbols, additional mutations at positions –1, +3, and +4. Locus 1, cases where energy calculations predict major disruption of CaM binding to IQ domain, yet CDF is largely preserved. Locus 2, cases where energy predictions suggest little perturbation of CaM binding to IQ domain, yet there is nearly complete elimination of CDF.

(D) Y[+3]E mutant preserves CDF.

(E) Y[+4]E mutant also spares CDF.

All error bars for population data indicate SEM.
above, with a reservoir buffer containing 2.3 M ammonium sulfate, 0.15 M sodium tartrate, and 0.05 M sodium citrate (pH 5.0). Jagged, trapezoidal-shaped crystals appeared within 2 d, and achieved their full size in approximately 2 wk. Diffraction-quality crystals were grown by microseeding initial jagged crystals into freshly mixed droplets that had been pre-equilibrated for 3 hr. This produced single, rectangular-shaped crystals that grew in space group C2 and contained two CaM/IQ complexes in the asymmetric unit. The crystals used for X-ray diffraction analysis were soaked briefly in a fresh aliquot of reservoir buffer. The diffraction data were collected at room temperature, with crystals held in a wax-sealed glass capillary tube. The X-ray source was CuKα radiation produced by a Rigaku RU-200 generator (Tokyo, Japan).

CaV2.3 IQ/CaM Complex

The purified complex of CaM and the IQ domain of CaV2.3 was concentrated to 10 mg/ml, mixed into droplets as above, and crystallized using a reservoir buffer containing 2.1 M ammonium sulfate and 0.05 M sodium citrate (pH 5.0). Pyramidal crystals appeared within 2 d, and achieved their full size in approximately 1 wk. Crystals exhibited an I4122 space group, with one CaM/IQ complex within the asymmetric unit. The crystals used for diffraction analysis were soaked briefly in a cryoprotectant (20% glycerol in reservoir buffer), and flash-frozen in a gaseous nitrogen stream at ~180°C. Diffraction data were then collected as above.

Analysis of X-Ray Diffraction Data

Data were processed using HKL2000 (Otwinowski and Minor, 1997). The C lobe of CaM, taken from a previously solved structure of CaM/Ca2+ in complex with the IQ domain of CaV2.3 (PDB code: 2BE6; C form), was used as the search model in a molecular replacement approach to solving the structure of CaV2.3 IQ/CaM in complex with the IQ domain of CaV2.3. In this process, we used the program MOLREP (Vagin and Teplyakov, 1997) from the CCP4 program suite (CCP4, 1994). Structure refinement was accomplished with iterative rounds of model building and refinement, using Coot (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997). Data were processed using HKL2000 (Otwinowski and Minor, 1997). The C lobe of CaM, taken from a previously solved structure of CaM/Ca2+ in complex with the IQ domain of CaV2.3 (PDB code: 2BE6; C form), was used as the search model in a molecular replacement approach to solving the structure of CaV2.3 IQ/CaM in complex with the IQ domain of CaV2.3. In this process, we used the program MOLREP (Vagin and Teplyakov, 1997) from the CCP4 program suite (CCP4, 1994). Structure refinement was accomplished with iterative rounds of model building and refinement, using Coot (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997) software packages, respectively. The structure for CaV2.3 IQ/CaM in complex with the IQ domain of CaV2.3 was obtained in a similar manner, except that the search model was the solved structure for CaV2.3 IQ/CaM in complex with the IQ domain of CaV2.3. In addition, the refinement also incorporated TLS algorithms, with TLS groups derived from TLSMD (Painter and Merritt, 2006). All data collection and model refinement statistics for both structures are summarized in Table 2. Molecular graphics were prepared with CCP4 mg software (Pottorff et al., 2004). Total buried surface area was determined by the AREAIMOL program in the CCP4 suite (Lee and Richards, 1971), as applied to our CaV2.3 structure.

Transfection of Human Embryonic Kidney 293 Cells

Human embryonic kidney (HEK293) cells were transiently transfected (calcium phosphate protocol) and cultured in 6 cm plates, as described (Brody et al., 1998). Human embryonic kidney (HEK293) cells were transiently transfected (calcium phosphate protocol) and cultured in 6 cm plates, as described (Brody et al., 1998). For expression of recombinant calcium channels, we applied 4 μg of cDNA encoding the desired α1 subunit (CaVα), along with 4 μg each of rat brain β3 (Perez-Reyes et al., 1992), rat brain δ subunits (Tomlinson et al., 1993), and, finally, 1 μg of the RSV T antigen. The α1xα pore-forming subunit (CaV2.1) (Soong et al., 2002) was cloned from a human source and the CaV1.2,α1C subunit from rabbit (Wei et al., 1991) (GenBank number X15539). All chimeric constructs were made from the carboxy tails of these two parental α1x and α1C channel subunits, as fused in various combinations onto the rat brain CaV2.3 α1E subunit (Soong et al., 1993) (GenBank number NM_019294), which served as a channel backbone (Figure 4).

Electrophysiology

Whole-cell current recordings were performed 1–3 d after transfection, using glass pipettes with resistances of 1.5–3.5 MΩ prior to series resistance compensation. For characterization of regulatory processes initiated by CaVα binding to the C lobe of CaM (all figures except Figures 3D and 3F), which can occur when CaVα elevations are restricted to within tens of nanometers of the channel cytoplasmic pore, the internal solution contained a relatively high concentration of CaVα buffer, with a specific composition containing 135 mM Cs-MesSO4, 5 mM CsCl, 5 mM EGTA, 1 mM MgCl2, 4 mM MgATP, and 10 mM HEPES (pH 7.3); 290 mMOSm, adjusted with glucose. To characterize N lobe-mediated channel regulation, which favors global CaVα elevations, the EGTA was reduced to 1 mM in the internal solution (Figures 3D and 3F, only). The bath solution contained 140 mM TEA-MesSO4, 10 mM HEPES (pH 7.3), and 5 mM CaCl2 or 5 mM BaCl2; 300 mMOSm, adjusted with glucose. Standard patch-clamp techniques were used with an Axopatch 200A amplifier (Axon Instruments/Molecular Devices, Union City, CA, USA). Currents were filtered at 2 kHz and sampled at 10 kHz; series resistance was typically 1–2 MΩ after >70% compensation; and leaks and capacitive transients were subtracted by a P/B protocol. Test pulse depolarizations were delivered every 30 s for local Ca2+ regulation protocols, and 60 s for global Ca2+ regulation protocols. Population data are presented as mean ± SEM, after analysis by custom-written software in MATLAB software (MathWorks, Natick, MA, USA) and Microsoft Excel.

Construction of Mutant and Chimeric Molecules

Single or cluster mutations in the IQ domain were generated using the Quik-Change procedure (Stratagene, La Jolla, CA, USA), with the template being a small portion of the CaV2.1 α1E subunit, which spanned the IQ domain. The mutated IQ domain was then cloned into a plasmid vector, which was transfected into CaV2.3-expressing cells. For the chimeric channels in Figure 4, the carboxy tail of the CaV2.3 α1E subunit was replaced by the N-terminal third of the carboxy tail (containing EF hand and IQ regions) derived from various combinations of CaV1.2 α1C and CaV2.3 α1E subunits. Thus, amino acids 1–1712 of the CaV2.3 α1E subunit served as a “backbone” for all chimeric channels. For construction 1 (Figure 4C), PCR was used to amplify CDNA encoding amino acids 1513–1670 of the CaV1.2 α1C subunit, followed by a stop codon. This product was cloned into unique upstream Xhol and downstream XbaI sites of the CaV2.3 α1E subunit. The upstream Xhol site in the PCR product was introduced as a silent mutation at the upstream end of the PCR product. For construction 2 (Figure 4C), amino acids 1818–1982 of the CaV2.1 α1E subunit were introduced into the CaV2.3 α1E subunit by the same strategy and restriction sites. For constructs 3–6 (Figure 4C), overlap extension PCR (Ho et al., 1989) was used to generate the various carboxy tail inserts, which were introduced into the CaV2.3 α1E subunit by the same strategy and restriction sites. The detailed amino acid composition of constructs 1–6 are presented in Figure 5. Throughout, all portions of constructs subject to PCR or QuickChange were confirmed in their entirety by sequencing.

Energy Calculations for Point Mutations

The web-based computational algorithm Robetta (http://www.roberta.org/ alascano/submit.jsp) was used to predict the change in ligand-receptor (i.e., IQ domain and CaVα/CaM) binding energy produced by point mutation of interface residues (Kortemme et al., 2004). These algorithms accommodate changes not only to alanine but also to other amino acids (e.g., Figure 5; M[-1]T and Y[+4]E). For mutations to alanine, the default operational mode of the algorithm was used. For mutations from alanine to nonalanine residues (A→X), we modified the target residue in the submitted structure file, so as to rename the target residue as X and delete all side-chain atoms. This change triggered the computational algorithm to build in the missing atoms for the desired mutant residue (X), and then optimize rotomer conformation. The calculated ΔΔG (X→A) then corresponded to an A→X mutation. The energy for the desired A→X mutation is then determined as ΔΔG (A→X) = ΔΔG (X→A). For Y→X mutations, where neither X nor Y is alanine, a concatenation of the former manipulations was used. Specifically, ΔΔG (Y→X) = ΔΔG (Y→A) + ΔΔG (X→A). Calculations were generated based on our structure of CaVα/CaM in complex with the IQ domain of CaV2.3, rather than for the CaV2.1 analog, owing to the advantages of the higher resolution in the former structure (2.1 Å versus 2.6 Å).

ACCESSION NUMBERS

Coordinates and structure factors have been deposited in the Protein Data Bank under codes 3BXK and 3BXL for CaV2.1 and CaV2.3, respectively.

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.structure.org/cgi/content/full/16/4/607/DC1/.
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Structure of Ca^{2+}/CaM Complexed with Ca_{v}2 IQ Domain


