Progress in the structural understanding of voltage-gated calcium channel (Ca_v) function and modulation

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Voltage-gated calcium channels (CaVs) are large, transmembrane multiprotein complexes that couple membrane depolarization to cellular calcium entry. These channels are central to cardiac action potential propagation, neurotransmitter and hormone release, muscle contraction and calcium-dependent gene transcription. Over the past six years, the advent of high-resolution structural studies of CaVs components from different isoforms and CaV modulators has begun to reveal the architecture that underlies the exceptionally rich feedback modulation that controls CaV action. These descriptions of CaV molecular anatomy have provided new, structure-based insights into the mechanisms by which particular channel elements affect voltage-dependent inactivation (VDI), calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF). The initial successes have been achieved through structural studies of soluble channel domains and modulator proteins and have proven most powerful when paired with biochemical and functional studies that validate ideas inspired by the structures. Here, we review the progress in this growing area and highlight some key open challenges for future efforts.

Electrical activity drives each heartbeat and thought. This activity arises from the action of ion channels, membrane proteins that control ion passage across cell membranes and that constitute the molecular foundations of cardiac contraction and nervous system function.1 Voltage-gated calcium channels (CaVs) comprise a family of multisubunit transmembrane proteins that control cellular calcium entry in response to membrane depolarization.2,3 Calcium ions have a special signaling role as they act as agents of membrane potential change and as potent effectors of signaling pathways.4 Consequently, CaVs form a nidus for signals that couple calcium entry in response to membrane depolarization to many physiological processes such as neurotransmitter and hormone release, cardiac action potential propagation, excitation-contraction coupling and calcium-dependent gene regulation.1,2 Because of these roles, CaV regulation plays a vital role in both the normal function and pathological states of the cardiovascular, endocrine, sensory and nervous systems.5,7 Understanding how these molecular switches work, are regulated, and are integrated into macromolecular complexes and signaling pathways, and how they become deranged in cardiovascular and neuronal diseases, remain important questions at the frontier of modern biology.

There are a number of CaV subtypes that have diverse physiological and pharmacological properties that depend largely on the identity of the pore-forming CaVα subunit. High-voltage activated channels, CaV1s (CaV1.x, L-type3,8) and CaV2s (CaV2.x; 2.1, P/Q-type; 2.2, N-type; and 2.3, R-type)3,7,9 are activated by strong depolarizations (+30 to +50 mV), whereas low-voltage activated CaV3s (CaV3.x, T-type10,11) are activated by weaker depolarizations (~0.5 MDa). Presently, the best data available on complete channels are low-resolution (~30 Å) electron microscopy reconstructions.18-24 Although progress is being made in identifying the possible orientation of the different components,22-24 this level of resolution reveals only the gross shape of the channel and is not sufficient for developing detailed mechanistic hypotheses.

CaVα pore-forming subunits are polypeptide chains of ~1,600–2,400 amino acids in which the ion-conducting pore is formed from four homologous repeats that each bear six transmembrane segments (Fig. 1B). Interdomain intracellular loops of various sizes bridge the four repeats and serve as docking sites for auxiliary subunits and regulatory molecules that control channel
SH3 domain has a number of unusual features. Its structure is incompatible with the canonical mode of polyproline binding for which SH3 domains are well known as it lacks key residues required for ligand engagement and the surface that could be used for polyproline interactions is blocked by an α-helix that leads to the V2/HOOK loop. More strikingly, one of the β-strands that forms the central SH3 core occurs in the primary sequence after the V2 domain (also called the HOOK domain, after a similar domain in MAGUKs) and is adjacent to the NK domain. This arrangement, also found in MAGUKs, results in an interdependence of the SH3 and NK domains that is crucial for the ability of CaVβ to modulate CaVα function.

The CaVα1 I-II loop bears the high affinity CaVβ binding site, known as the ‘α-interaction domain, AID’. CaM is shown bound to the C-terminal cytoplasmic tail at the site of the IQ domain. The membrane associated CaVαβ subunit is shown in orange and green. (B) Topology of the pore-forming CaVα subunit. Positions of the CaVβ/AID complex, Caβ/CaM-PrelQ complex and Caβ/CaM-IQ domain complex are shown. CaVα intracellular loops are drawn to scale according to the human CaV1.2 sequence.

**CaVβ Subunits**

The cytoplasmic CaVβ subunit is an essential component of CaV1 and CaV2 channel complexes. Four isoforms are found in mammals and have a wide range of splice variants. The first high-resolution structural studies came from a set of studies that reported the X-ray crystallographic structures of the core, conserved domains of CaVβ2, CaVβ3, and CaVβ4. The structures showed, in line with sequence prediction, that CaVβs have two folded, conserved domains: an SH3 domain fold and a nucleotide kinase (NK) fold (Fig. 2A). The CaVβ domain structure is related to that seen in a class of scaffolding proteins known as membrane-associated guanylate kinases (MAGUKs), but has number of key differences including the relative orientation of the SH3 and NK domains. The SH3 domain has a number of unusual features. Its structure is incompatible with the canonical mode of polyproline binding for which SH3 domains are well known as it lacks key residues required for ligand engagement and the surface that could be used for polyproline interactions is blocked by an α-helix that leads to the V2/HOOK loop. More strikingly, one of the β-strands that forms the central SH3 core occurs in the primary sequence after the V2 domain (also called the HOOK domain, after a similar domain in MAGUKs) and is adjacent to the NK domain. This arrangement, also found in MAGUKs, results in an interdependence of the SH3 and NK domains that is crucial for the ability of CaVβ to modulate CaVα function.

The CaVα I-II loop bears the high affinity CaVβ binding site, known as the ‘AID’ (α-interaction domain). AID-CaVβ co-crystal structures revealed the molecular details of this interaction and showed that the region from CaVβ previously thought to be the main AID-interaction site, the ‘BID’, was buried in the center of the NK domain and made no direct contacts with the AID. Instead, the AID binds to a deep groove on the NK domain, termed the α-binding pocket (ABP) (Fig. 2A and B), that encompasses ~730 Å² and that involves sidechain contacts from twenty-four residues, a large number of which are highly-conserved. Isothermal titration calorimetry studies show that the AID-ABP interaction is strong, regardless of the CaV1 or CaV2 AID isoform (Kd ~3.5–53.5 nM). The good correspondence between these values and those measured using larger portions of the I-II loop provides solid support for the idea that the AID is the only high-affinity CaVβ binding site on the I-II loop. Alanine scanning mutagenesis reveals that the binding energy...
Figure 2. Ca_β-AID interactions. (A) Structure of the Ca_β-AID complex from reference 30. SH3 and NK domains are shown in green and blue, respectively. The AID is shown in red with the sidechains depicted as sticks. Variable domains V1, V2 and V3 are indicated. (B) Energetics of the Ca_β-AID interaction. Residues are colored according to their impact on Ca_β-AID binding as follows: <0.5 kcal mol^{-1}, dark blue; 0.5–1.5 kcal mol^{-1}, light blue; 1.5–3.0 kcal mol^{-1}, orange; >3 kcal mol^{-1}, red. Select AID (L434, L438, Y437, W440 and I441) and Ca_β (M245 and L352) residues are labeled. (C) View of the complementary Ca_β-AID hotspots from the perspective of Ca_β. Colors and labels are as in (B). (D) Disruption of the AID-Ca_β hotspot interaction by the AID mutant (HotA) abolishes Ca_β-dependent modulation. Data in (B–D) are from ref. 40.
of the AID-ABP interface is focused into two complementary energetic hotspots. Nearly all of the binding energy comes from the interaction of just four amino acids, three from the AID (Y437, W440 and I441) and one from Caß (Met245) (Fig. 2B and C). Targeted disruption of this interaction is sufficient to prevent association and channel modulation (Fig. 2D).46,44 The restricted area of the interaction hotspots fits a general profile45 that makes it an attractive target for the development of small molecule or peptidomimetic strategies to control Caß function by blocking the AID-ABP protein-protein interaction.

The presence of the AID helix, the relatively short distance from the cytoplasmic end of the IS6 segment from the pore forming subunit, and the predicted high helical propensity of the IS6-AID sequence prompted the structure-based hypothesis that Caß exerts control on Caα function through a continuous α-helix that would connect the IS6 and AID helices (Fig. 3A).29,30 Subsequent tests of this idea have provided good support for this model.36-44 Polyglycine replacement of residues in the middle of the IS6-AID segment uncouples the effects of Caß on voltage-dependent inactivation (VDI) and voltage-dependent activation in exemplars from both types of high-voltage activated channels: CaV1.2 (Fig. 3B and C),47 CaV2.1,47,48 and CaV2.2.46 While direct high-resolution structural data for IS6-AID region are still lacking, circular dichroism studies of the IS6-AID segment from CaV1.2 and CaV2.2 support the ability of this conserved region to form an α-helix and also validate the detrimental impact of the polyglycine substitutions on helix formation.47,49 Strikingly, disruption of the Caαα-Caß functional coupling, not only affects VDI, but also affects calcium-dependent feedback modulation processes that traditionally have been associated with calmodulin (CaM) interactions with the Caαα C-terminal tail (Fig. 3D and E).47 This unexpected functional link suggests that both Caß-dependent and CaM-dependent channel modulation couple to the pore by a common mechanism that requires Caß and an intact IS6-AID linker.49 Another type of channel modulation, voltage-dependent inhibition of CaV2.1 by the G-protein, Gßγ, also requires the presence of Caß and helical structure in the IS6-AID segment.48 Together, these studies point to a situation in which Caß relays a diverse set of modulatory signals to the pore via its action on the IS6-AID helix.

The IS6-AID is not the only element through which Caßs can affect Caα function. The various Caß isoforms have different effects on channel properties including stereotyped effects on the rank order of VDI50-53 that arise from the Caß variable domains54,55 and that persist even when the IS6-AID segment structure is disrupted.47 Structural information for the variable V1, V2 and V3 segments is presently limited. In the crystallographic Caß studies, the variable regions were either removed to enable crystallization, or if present, lacked sufficient order to produce clear electron density. Presently, the only direct structural data for a Caß variable region comes from nuclear magnetic resonance (NMR) structure determination of the Caßßß V1 domain. These studies included the α1-helix present in the Caß core structures and show that the preceding region can adopt a compact folded structure consisting of a short α-helix and a β-hairpin.56 Whether the other variable regions have structures in isolation or only fold upon interaction with Caαα remains unknown. The persistence of the Caßß rank order effects in the Caßß, IS6-AID polyglycine mutants and clear functional cross-talk between the different intracellular domains supports the likelihood of physical interdomain interactions between Caßß and other channel elements. Definition of the structures of the Caß variable domains and their sites of interaction on Caαα remains a challenging and open question.

**Interaction of Caßs with Ca²⁺/CaM**

Calcium influx is a potent activator of intracellular signaling pathways but is toxic in excess. Therefore, its cellular entry is tightly regulated. Because Caßs are major sources of activity-dependent calcium influx, their activity is strongly controlled by self-regulatory and extrinsic mechanisms that tune Caß action in response to electrical activity, neurotransmitter stimulation and hormone initiated cues. This intense regulation employs multiple mechanisms and intracellular signaling molecules. Calmodulin (CaM) has a preeminent role in the intrinsic mechanisms of Caß1s and Caß2 calcium-dependent regulation and in its calcium free form (apo-CaM) is thought to be tethered constitutively to the Caαα C-terminal cytoplasmic tail, making an arrangement that places CaM in a privileged position as a detector of Caß-generated calcium signals.57,58 CaM is a bilobed, 16.7 kDa, calcium binding protein from a large family of calcium sensor proteins that has a central role in intracellular Ca²⁺ signaling and can interact with more than 300 different target proteins in a variety of binding modes.59-61 CaM has two independently folded lobes,62,63 N-lobe and C-lobe, that each contain two Ca²⁺-binding motifs known as EF-hands.64-66 The CaM lobes undergo calcium-dependent conformational changes that expose a phenylalanine and methionine-rich hydrophobic pocket that is the principal site of target engagement.66,67 CaM is responsible for driving two opposing Caß feedback modulation processes: calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF). The main site of Ca²⁺/CaM binding is an isoleucine-glutamine 'IQ' domain67-80 located in the Caαα subunit C-terminal cytoplasmic tail ~150 amino acids after the last transmembrane segment, IVS6 (Figs. 1B and 4A).

Elegant experiments using CaM mutants impaired in calcium binding at specific lobes have demonstrated that the individual lobes control CDI in a manner that is exchanged between Caß1 and Caß2 isoforms. In Caß1,2, the C-lobe governs CDI,67 whereas in Caß2.1,68,69 Caß2.2.70 and Caß2.3,70 the N-lobe governs CDI. Further, the C-lobe has been shown to control Caß2.1 CDF.68,69 The high level of sequence similarity among the Caß1 and Caß2 IQ domains (Fig. 4B) and apparent inversion of lobe specific functions, pushed the desire to understand how the same molecule, Ca²⁺/CaM, could affect separate calcium-driven processes using different lobes and yet interact with a highly conserved portion of the pore-forming subunit. This question has motivated a number of recent structural studies of Ca²⁺/CaM complexes of IQ domains from both Caß1 and Caß2s71-75 (Table 1). To simplify comparisons in the following discussion, and because the Caß IQ sequences have different
Figure 3. Caβ controls channel function via the IS6-AID helix. (A) Model showing the proposed continuous helix between the IS6 transmembrane segment and the AID. Model is based on the likely gross similarity between the transmembrane portions of Ca and K_v channels. A surface representation (white) of the K_v transmembrane portion (PDB 2A79) is used to depict the Ca transmembrane domains. The helical IS6-AID segment (red) was modeled manually by building an α-helix corresponding to the length between the K_v1.2 S6 C-terminus and the helix from the Caβ2a-AID complex (PDB 1T0J). The Caβ2a SH3 and NK domains are colored green and blue, respectively. Arrow indicates the site of the depicted polyglycine and polyalanine substitutions studied in ref. 47. Effects of IS6-AID substitutions on (B) CaV1.2 VDL, (C) CaV1.2 voltage-dependent activation, (D) CaV1.2 netCDI and (E) CaV1.2 CDF. Experimental details can be found in ref. 47. (B–E) Reprinted from Findeisen et al. 47
numbering schemes depending on the organism of origin and particular splice variant used, the IQ domain positions are designated by their relative position in the sequence with respect to the central isoleucine, Ile(0). Negative and positive numbers signify the position of each residue N-terminal or C-terminal to Ile(0), respectively.

**Cao 1 IQ Complexes**

The initial crystallographic studies of Ca²⁺/CaM-Ca₁ IQ domain complexes investigated the IQ domains of the L-type channel Ca₁.2. These structures were not only the first descriptions of Ca²⁺/CaM-Ca₁ IQ domain complexes, but were also the first description of a Ca²⁺/CaM-IQ domain complex from any source. The structures uncovered two key, unanticipated features. Ca²⁺/CaM wraps around an α-helix formed by the IQ domain in an unusual parallel binding orientation in which Ca²⁺/N-lobe and Ca²⁺/C-lobe bind to sites on the N- and C-terminal ends of the IQ α-helix, respectively (Fig. 4C). Previously, all other examples of Ca²⁺/CaM-peptide complexes, except that of the Ca²⁺/CaM dependent kinase kinase, had been found to adopt an antiparallel arrangement with respect to Ca²⁺/CaM and the bound helical peptide. The structures also revealed that each Ca²⁺/CaM lobe interacts with three aromatic residues, termed ‘aromatic anchors’ arranged on opposite faces of the IQ helix. Ca²⁺/N-lobe interacts with Phe₁₆₁₈(-6), Tyr₁₆₁₉(-5) and Phe₁₆₂₂(-2), Ca²⁺/C-lobe interacts with Tyr₁₆₂₇(+3), Phe₁₆₂₈(+4) and Phe₁₆₃₁(+7). Ile₁₆₂₄(0) is completely buried by interactions with Ca²⁺/C-lobe whereas Gln₁₆₂₅(+1) interacts with both lobes. These principal contacts are similar in both X-ray structures (PDB: 2BE6 and 2F3Y). Additionally, Fallon et al. determined the structure of an IQ→AA mutant at positions 0 and +1 (PDB: 2F3Z). This Ca₁.2 double mutant eliminates CDI and causes strong CDF, but does not alter the ability of Ca²⁺/CaM to bind the IQ domain. The structure of the IQ→AA mutant complex is essentially identical to the wild-type complex and leaves the reason for the strong functional effect of the IQ→AA mutation unresolved.

The interactions between Ca²⁺/N-lobe and the Ca₁.2 aromatic anchors were unanticipated. Tests of the importance of this interaction by a triple alanine mutation (TripleA) of Phe₁₆₁₈(-6), Tyr₁₆₁₉(-5) and Phe₁₆₂₂(-2), abolished Ca₁.2 CDF(32,33). This result, together with the unexpected parallel binding mode raised the hypothesis that the apparent inversion of lobe-specific function between Ca₁.2 and Ca₂.1 originates from an exchange of binding orientations between Ca₁.1 and Ca₂.2,12,57. The Ca₁.1 IQ domain is the most divergent from the Ca₁.1 family and has an H→Y change in the (+3) Ca²⁺/C-lobe anchor position (Fig. 4B). Ca₁.1 acts in skeletal muscle as the voltage-sensing subunit for the intracellular ryanodine receptor. This function requires a physical link between Ca₁.1 and the ryanodine receptor but does not require calcium permeation through Ca₁.1. Nevertheless, Ca₁.1 subunits have been shown to have CDI. The structure of a Ca²⁺/CaM-Ca₁.1 IQ domain complex (PDB: 2VAY) reveals a parallel binding conformation that is very similar to that observed in the Ca₁.2 complexes. The structure shows that His₁₅₃₂(+3) occupies the same Ca²⁺/C-lobe binding pocket as Ca₁.2 Tyr₁₆₂₇(+3) and requires only a minor rearrangement of the sidechains that line the Ca²⁺/C-lobe pocket. Introduction of Y→H(+3) into the Ca₁.2 IQ domain is sufficient to reduce Ca²⁺/CaM binding and simultaneous introduction of Y(+3)→H(+3) and K(+8)→M(+8) into Ca₁.2 eliminates CDI. Although there are no reported structures for Ca²⁺/CaM complexes with Ca₁.3 or Ca₁.4 IQ domains, the extremely high sequence conservation among Ca₁.1 IQ domains (Fig. 4B) that includes the aromatic anchors, strongly suggests that the parallel binding mode will be found throughout the Ca₁ family.

**Cao 2 IQ Complexes**

Structure determination of Ca²⁺/CaM-Ca₂ IQ domain complexes from Ca₂.1, Ca₂.2 and Ca₂.3 (PDB: 3DVM, 3DVE and 3DVK, respectively), revealed that unlike the Ca₁.2 and Ca₁.1 Ca²⁺/CaM-IQ domain complexes, all three Ca₂ IQ domain complexes are antiparallel (Fig. 4E). Rather than just a simple exchange of lobe binding positions, Ca²⁺/CaM is also situated further towards the N-terminal end of the IQ-helix. Consequently, the two lobes are anchored by different constellations of residues than are used in the Ca₁.1 and Ca₁.2 complexes. Ca²⁺/N-lobe interacts with four main residues: methionine (-1), isoleucine (0) and the aromatics at positions (+3) and (+5). This positional shift is consistent with the loss of the aromatic anchor at the (+7) position, which is a small hydrophilic residue in all of the Ca₂ IQ domains (Fig. 4B). Ca²⁺/C-lobe, which is bound to the N-terminal end of the IQ helix, is anchored by two major positions in the Ca₂.1 and Ca₂.3 complexes, positions (-6) and (-2) and has additional contacts with position (+9). The Ca₂.1 and Ca₂.3 complexes are essentially identical. The Ca₂.2 complex has some structural differences that involve a slightly altered IQ helix pose and a relative displacement of the Ca²⁺/C-lobe towards the center of the IQ helix.
Figure 4. For figure legend, see previous page.
of these sites is exchanged between CaV1 and CaV2 isoforms (Fig. 5B). The observed inversion of structural polarity provides an attractive explanation for the inversion of lobe-specific roles (however, consult ref. 74 for alternative scenarios). The observation that the occupant of the N-terminal site in both CaV1 and CaV2 IQ domains makes fewer contacts and binds more weakly73,75 suggests that these physical properties are also important for the CDF mechanism. Nevertheless, the IQ domains are distant from the pore-forming subunit and it remains unclear as to how binding events in the IQ domain are coupled to changes in the behavior of the pore. Structural determination of larger portions of the C-terminal tail and clarification of the structural basis of apo-CaM binding, which is a key state in the reaction cycle that drives calcium-dependent channel modulation, will be required to gain further insight into this question.

Multiple Ca2+/CaMs Can Bind the C-terminal Tail But Do Not Mediate Channel Dimerization

Two recent studies have succeeded at obtaining structural information about larger portions of the CaV1.2 C-terminal tail that contain the PreIQ region and the IQ domain (Figs. 1B and 4A). The PreIQ region contains two Ca2+/CaM binding sites known as the A-region and C-region.83,84 Surprisingly, both studies found structures that contained crystallographic dimers made from interactions between the Ca2+/CaM complexes. In one,82 the components were well enough resolved to show two C-terminal tails and four bound Ca2+/CaMs (Fig. 6A). The Ca2+/CaM-IQ domain structures are essentially identical to those solved using the individual CaV1.2 IQ domains and indicate that the parallel orientation is maintained in the context of a substantially longer portion of the CaV1.2 C-terminal tail. The PreIQ region forms a long helix (42 residues) that connects to the Ca2+/CaM-IQ complex by a short turn.

In both the parallel CaV1 and antiparallel CaV2 Ca2+/CaM IQ domain complexes, each Ca2+/CaM lobe interacts with an extensive network of IQ domain residues. Although there are differences in the details of the interactions, one common element is that the occupant of the N-terminal binding site, Ca2+/N-lobe for CaV1s and Ca2+/C-lobe for CaV2s, buries much less surface area than the occupant of the C-terminal binding site (Ca2+/N-lobe vs. Ca2+/C-lobe: CaV1.1 1,209 Å2, 1,630 Å2; CaV1.2 1,450 Å2, 1,819 Å2; CaV2.1 1,652 Å2, 921 Å2; CaV2.2 2026 Å2, 1,224 Å2; CaV2.3 1,673 Å2, 950 Å2; respectively).

In addition to the above structures, complexes of Ca2+/CaM with CaV2.1 and CaV2.3 IQ peptides shorter than those described above, (21 vs. 25 residues, Table 1) have also been determined (PDB: 3BXK and 3BXL, respectively). Strikingly, these complexes are not antiparallel, but are parallel structures very similar to the CaV1.2 complex determined using the short IQ peptide reported by Fallon et al.72 The ability of Ca2+/CaM to bind similar peptide sequences in opposite orientations is remarkable. It should be noted, however, that the peptides in these parallel structures lack four residues that contribute to interactions with Ca2+/C-lobe in the antiparallel complexes, including residue (-9). Further, adoption of the antiparallel pose on the short peptide would place the positively charged IQ peptide N-terminus near the Ca2+/C-lobe hydrophobic pocket.

Structure-based alanine mutations designed to test the importance of the Ca2+/C-lobe contacts from the antiparallel orientation, show that loss of these interactions eliminates binding of Ca2+/C-lobe to the IQ domain in titration calorimetry experiments and greatly reduces CDF.73 (Fig. 4F). This direct connection between structure, biochemistry and function suggests a clear structural basis for the swap of lobe-specific roles (Fig. 5). These data indicate that the CaV1 and CaV2 IQ domains contain dedicated sites that control CDF and that the occupant of these sites is exchanged between CaV1 and CaV2 isoforms (Fig. 5B). The observed inversion of structural polarity provides an attractive explanation for the inversion of lobe-specific roles (however, consult ref. 74 for alternative scenarios). The observation that the occupant of the N-terminal site in both CaV1 and CaV2 IQ domains makes fewer contacts and binds more weakly73,75 suggests that these physical properties are also important for the CDF mechanism. Nevertheless, the IQ domains are distant from the pore-forming subunit and it remains unclear as to how binding events in the IQ domain are coupled to changes in the behavior of the pore. Structural determination of larger portions of the C-terminal tail and clarification of the structural basis of apo-CaM binding, which is a key state in the reaction cycle that drives calcium-dependent channel modulation, will be required to gain further insight into this question.

### Table 1. Ca2+/CaM IQ domain complexes

| Native channel | Pore forming CaV subunit | CaV IQ domain complexes | Ca2+/CaM IQ domain complexes |
|----------------|--------------------------|--------------------------|-----------------------------|
|                | Classification           | Resolution               | Peptide length (amino acids) | Residues | IQ numbers | Source | PDB code | Ref. |
|                | Alphabetical Numerical   |                          |                            |          |            |        |          |     |
| L-type         |                          |                          |                            |          |            |        |          |     |
| α1s            | CaV1.1                   | 1.94 Å                    | 21                         | 1522–1542 | I1529/Q1530 | Human | 2VAY     | 82  |
| α1c            | CaV1.2                   | 2.00 Å                    | 34                         | 1611–1644 | I1624/Q1625 | Human | 2BE6     | 76  |
| α1m            | CaV1.3                   | 1.45 Å                    | 21                         | 1655–1685 | I1672/Q1673 | Human | 2F3Y     | 69  |
| α1p            | CaV1.4                   | 1.60 Å                    | 21                         | 1655–1685 | I1672A/Q1673A | Human | 2F3Z     | 69  |
| Non-L-type     |                          |                          |                            |          |            |        |          |     |
| α1a            | CaV2.1                   | 2.60 Å                    | 25                         | 1963–1984 | I1971/M1972 | Rabbit | 3DVM     | 79  |
| α1b            | CaV2.2                   | 2.60 Å                    | 21                         | 1960–1980 | I1967/M1968 | Human | 3BXK     | 80  |
| α1c            | CaV2.2                   | 2.35 Å                    | 25                         | 1855–1876 | I1863/F1864 | Rabbit | 3DVE     | 79  |
| α1d            | CaV2.3                   | 2.80 Å                    | 25                         | 1853–1876 | I1863/F1864 | Rabbit | 3DVJ     | 79  |
| R-type         |                          |                          |                            |          |            |        |          |     |
| α1a            | CaV2.1                   | 2.30 Å                    | 25                         | 1818–1839 | I1826/M1827 | Rat    | 3DKV     | 79  |
| α1b            | CaV2.2                   | 2.20 Å                    | 21                         | 1819–1839 | I1826/M1827 | Human | 3BXL     | 80  |
flexible linker. In the asymmetric unit of the crystal, the two PreIQ helices interact via crossbridges formed by the binding of Ca\textsuperscript{2+}/C-lobe to one PreIQ C-region and Ca\textsuperscript{2+}/N-lobe to the A-region of the adjacent PreIQ segment as well as through helix-helix interactions that resemble five turns of an anti-parallel coiled coil.

Although recent studies have suggested coupled activity among Ca\textsubscript{1.2}s,\textsuperscript{85} Ca\textsubscript{1.2}s are generally not thought to function as dimers. Based on the crystallographic dimer and a modest effect on CDI resulting from an E→P change in the PreIQ region, Fallon et al. proposed that Ca\textsubscript{1.2}s dimerize through a functionally relevant anti-parallel coiled-coil interaction augmented by bridging interactions between Ca\textsuperscript{2+}/CaM and A-region- and C-region-binding sites.\textsuperscript{81} Discernment of oligomerization state from crystallographic contacts alone is difficult\textsuperscript{86-88} and requires some alternative method for validating whether the observed oligomerization is robust or only a consequence of the ordered array of the crystallographic lattice. Experiments performed using a Superdex 75 size exclusion column were presented as evidence that the crystallized complex could dimerize in solution.\textsuperscript{81} However, it is worth noting that the size of the dimeric complex (85.6 kD) that forms the asymmetric unit composed of a 4:2 Ca\textsuperscript{2+}/CaM:PreIQ-IQ complex is beyond the resolution limit of the column (3–70 kDa). Size exclusion experiments using a Superdex 200 column (resolution range 10–600 kDa), on which, in principle, 2:1 and 4:2 complexes could be resolved (42.8 kDa vs. 85.6 kD) yielded an ambiguous assessment of molecular weight for the complex (~66 kDa).\textsuperscript{82} This ambiguity persisted regardless of whether the construct contained the full PreIQ-IQ segment, or a version that could not dimerize because it lacked one of the binding sites required to form the PreIQ crossbridge.\textsuperscript{82} These results suggested that elongated nature of the complex was a likely confounding factor for size determination by this methodology, which relies on comparison to globular proteins standards.\textsuperscript{27} As analytical ultracentrifugation studies are insensitive to molecular shape,\textsuperscript{27,80} this technique was used to measure the molecular mass of the complex in solution unambiguously. The results demonstrate that the Ca\textsuperscript{2+}/CaM:PreIQ-IQ complex has a 2:1 stoichiometry (Fig. 6B and C).\textsuperscript{82} This complex shows no evidence of dimerization, even at concentrations that are on the order of that experienced by Ca\textsubscript{1.2}s in the most densely packed environment known (~100 μM), that of the SR junction.\textsuperscript{90}

Biochemical studies of the full-length channel in cardiac microsomes were interpreted as evidence for channel dimers;\textsuperscript{91} however, these studies do not account for the contribution of the detergent to the apparent molecular weight of the protein-detergent complex and were done in the apparent absence of reducing agents. In contrast, single molecule subunit counting experiments of Ca\textsubscript{1.2} channels in live cell membranes unequivocally demonstrate that the full-length channels are monomers (Fig. 6D).\textsuperscript{82}

Two types of mutational studies were used to test the importance of the PreIQ helix. Simultaneous alanine mutations of two residues that form the core of the asymmetric unit dimer interface, and that would be expected to preserve the helical structure while being very detrimental to the PreIQ helix interaction,\textsuperscript{91,92} had no functional effects on VDI, CDI or CDF.\textsuperscript{82} The PreIQ E→P change\textsuperscript{81} had modest effects on both VDI and CDI but is at a residue that is on the opposite side of the PreIQ helix crystallographic interface. Although the proline substitution could affect the PreIQ helix structure, this change also removes a charge and thus, could produce the modest functional changes by perturbing interactions of the PreIQ helix with other channel components. Further, Ca\textsubscript{1.2} channels bearing the PreIQ-IQ domain but lacking the remainder of the Ca\textsubscript{1.2} C-terminal tail show no evidence of coupled activity.\textsuperscript{85} Thus, the weight of evidence indicates that the crossbridged, dimeric structure is neither robust nor relevant for function, and is simply a consequence of crystallization.

The demonstration that the Ca\textsuperscript{2+}/CaM:PreIQ-IQ complex has a 2:1 stoichiometry\textsuperscript{82} shows that multiple Ca\textsuperscript{2+}/CaMs can bind the Ca\textsubscript{1.2} C-terminal tail simultaneously (Fig. 6C), a result that is corroborated by recent independent biochemical studies.\textsuperscript{82} This property is likely to be conserved among Ca\textsubscript{1.1}s and Ca\textsubscript{2.3}s as the residue central to the Ca\textsuperscript{2+}/CaM:PreIQ C-region interaction, Trp1593, is present from mammals through the sea squirt, Ciona intestinalis. The binding of multiple Ca\textsuperscript{2+}/CaMs along a largely helical segment is reminiscent of how CaM and CaM-like proteins are organized on myosin\textsuperscript{94} and may indicate a previously unrecognized connection between these classes of CaM-modulated proteins.

Investigation of the biochemical properties of the 2:1 Ca\textsuperscript{2+}/CaM:PreIQ-IQ complex showed that the two bound Ca\textsuperscript{2+}/CaMs have different properties. The one bound to the PreIQ C-region is labile, whereas the one bound to the IQ domain is not.\textsuperscript{82} Further, the C-region has a strong preference for binding Ca\textsuperscript{2+}/lobes over apo-lobes. Electrophysiological experiments show that the PreIQ C-region has a previously unrecognized role in CDF.\textsuperscript{82} Together, the data suggest that the PreIQ region forms a long α-helix that contains a Ca\textsuperscript{2+}/C-lobe binding site that could be used as an anchor for making bridging interactions to other channel elements via the Ca\textsuperscript{2+}/N-lobe (Fig. 6E).\textsuperscript{82}

The idea that Ca\textsuperscript{2+}/CaM could form bridges between channel elements, such as the N-terminal and C-terminal...
Figure 6. For figure legend, see opposite page.
cytoplasmic domains,\textsuperscript{35} is attractive; however, biochemical support for such interactions remains elusive.\textsuperscript{36} The presence of two long $\alpha$-helices in the Ca$_{\alpha}$, cytoplasmic domains, the IS6-AID helix\textsuperscript{46-48} and the PreIQ helix could position the two elements that have functional crosstalk, the Ca$_{\beta}$/I-II loop and the Ca$^{2+}$/CaM:PreIQ-IQ complexes, near each other (Fig. 6E). The intriguing possibilities raised by this possible convergence of key modular protein-protein complexes highlights the pressing need for further biochemical and structural work to identify how the various intracellular elements interact in both apo-CaM and Ca$^{2+}$/CaM bound states and how the structural changes that underlie VDI, CDI and CDF are communicated with the pore.

Other elements from the intracellular regions of the channel are likely to have some role in both CDI and CDF. Functional data point towards potential roles for interaction of the Ca$^{2+}$/CaM-IQ domain with the putative EF hand proximal to transmembrane segment IV\textsuperscript{6,74,97} elements in the N-terminal 95,98,99 structure. These channel elements and their interactions is an important near-term goal. Additionally, the large dodecameric protein kinase, CaMKII, has been shown to be a key component for both Ca$_{\alpha}$,1\textsuperscript{107-111} and Ca$_{\alpha}$,2\textsuperscript{112} CDF. Exactly how this large, multiprotein complex engages the channel remains unknown.

**Progress on Ca$_{\alpha}$ Modulator Structures**

Besides the work on conventional channel elements, the past six years have produced advances in structural studies of two channel modulators that have dramatic effects on Ca$_{\alpha}$ function. These studies form the starting point for understanding how Ca$_{\alpha}$ activity can be customized to particular cellular contexts.

**CaBP1, Inhibitor of Ca$_{\alpha}$1 CDI**

In cerebellar and hippocampal neurons,\textsuperscript{113,114} photoreceptor synapses,\textsuperscript{115} and auditory hair cells,\textsuperscript{116,117} the composition of Ca$_{\alpha}$,1 complexes is changed in a way profoundly effects channel activity. Members from a family of calcium binding proteins homologous to CaM, known as CaBPs,\textsuperscript{118} and the C-terminal Ca$_{\alpha}$, cytoplasmic domains,\textsuperscript{100-104} and the Ca$_{\beta}$ subunit,\textsuperscript{47,105,106} Structural definition of these channel components and their interactions is an important near-term goal. Additionally, the large dodecameric protein kinase, CaMKII, has been shown to be a key component for both Ca$_{\alpha}$,1\textsuperscript{107-111} and Ca$_{\alpha}$,2\textsuperscript{112} CDF. Exactly how this large, multiprotein complex engages the channel remains unknown.

**Figure 6** (See previous page). Structure and characterization of the Ca$^{2+}$/CaM-PreIQ-IQ domain complex. (A) Two Ca$_{\alpha}$,1,2 PreIQ helices (red and blue) form a crystallographic dimer cross-bridged by Ca$^{2+}$/CaMs. Ca$^{2+}$/CaM N-lobe and C-lobe are colored green and blue, respectively. N- and C-termini of PreIQ-IQ domain are indicated. 48 (B) Sedimentation equilibrium analysis of 100 $\mu$M Ca$^{2+}$/CaM-Ca$_{\alpha}$,1,2 PreIQ-IQ complex at 11,000 rpm and 4°C and measured at 293 nm. Raw data (black open circles) and single species fit (black line) are compared to predicted curves for complexes having Ca$^{2+}$/CaM:Ca$_{\alpha}$,1,2 tail ratios of 1:1 (red), 2:1 (yellow), 2:2 (green) and 4:2 (blue). Inset shows the distribution of residuals as a function of radial distance. Reprinted from Kim, EY, et al. 48 (C) Cartoon diagram of the 2:1 Ca$^{2+}$/CaM-Ca$_{\alpha}$,1,2 PreIQ-IQ complex. C-lobe$_{\alpha}$ indicates the Ca$^{2+}$/CaM lobe bound to the C-region site. (D) TIRF image of Ca$_{\alpha}$,1-2-GFP in a live cell membrane of a X. laevis oocyte. Blue circles mark the selected spots used for subunit counting. Inset shows distribution of fluorescent spots that bleach in one or more steps. Each bleaching step indicates one Ca$_{\alpha}$,1 subunit. Reprinted from Kim, EY, et al. 48 (E) Model depicting the possible relative positions of Ca$_{\alpha}$ intracellular elements based on Figure 3A. ‘??’ signifies the potential for a Ca$^{2+}$/CaM anchored at the C-region site to make bridging interactions with other components of the Ca$_{\alpha}$,1 complex. 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. The N-terminal cytoplasmic domain, II-III loop and III-IV loop are not depicted.
RGK proteins have a core G-domain, that binds GDP and GTP\(^{130}\) and that is flanked by N- and C-terminal domains of 44–88 and 29–37 residues, respectively. Structural data for the RGK family of channel modulators is limited to Mg\(^{2+}\)•GDP complexes of the G-domains of Rad\(^{131}\) and Gem,\(^{132}\) and an Mg\(^{2+}\)•GDP complex of a Gem construct bearing the G-domain and part of the C-terminal domain.\(^{130}\) Compared to canonical Ras-GTPases, the RGK family has unusual sequences in two elements, Switch I and Switch II, which are important for G-protein activation and inactivation. These regions show substantial structural differences from Ras. Switch I is either disordered\(^{130,131}\) or in a widely different orientation compared to Ras\(^{32}\) (Fig. 8B) and leads to a very high solvent exposure of the bound GDP. Structure-based alanine scanning identified three residues on one face of Gem that reduce binding to Ca\(\beta\) (Fig. 8B).\(^{133,134}\) Surprisingly, a triple alanine mutant at these sites did not block Gem modulatory activity.\(^{134}\) Thus, the importance of the Gem-Ca\(\beta\) interaction for channel modulation remains unclear. Acquisition of additional structural and biochemical data regarding the nature of RGK-Ca\(\beta\) interactions and nucleotide-hydrolysis driven changes should help clarify the basic mechanisms of action. Further, as engineered RGK-based Ca\(\alpha\) modulators offer a novel way to affect Ca\(\alpha\) function,\(^{135}\) structural characterization of the RGK-Ca\(\beta\) interaction should aid protein design based efforts to make new generations of Ca\(\alpha\) modulators.\(^{136}\)

**Prospectus**

The protein dissection approach has proved illuminating for the initial studies of Ca\(\alpha\) structure. Given the power of this approach, particularly when integrated with biochemical and functional studies, such studies are likely to continue to drive the next set of advances.

Although there is good evidence supporting the model that Ca\(\beta\) influences the pore forming domain via a long helix, it is less clear how structural transitions of CaM and the C-terminal tail are coupled to the pore. There is a growing body of functional evidence that multiple regions of the channel interact to bring about the two processes that dominate voltage-gated...
calcium channel (Ca\(_V\)) inactivation, VDI\(^{105,136}\) and CDI\(^{57,58,105}\). The simplest hypothesis is that, at least in one functional state, there is a direct physical interaction between the major VDI and CDI components, the Ca\(\beta\)/Ca\(\alpha\)-I-II loop and CaM-Ca\(\alpha\)_I C-terminal tail complexes.\(^{57}\) Interactions of these components with the N-terminal cytoplasmic domain may also be important for both CaM\(^{58,99}\) and CaBP1 modulation.\(^{119}\) Nevertheless, robust evidence for such direct physical interactions remains elusive. Given the substantial amount of Ca\(_V\) mass in the cytoplasm, \(-150\) kDa, it seems likely that interactions between the Ca\(\beta\)/Ca\(\alpha\)-I-II loop and CaM-Ca\(\alpha\)_I-C-terminal tail complexes may involve other yet to be determined points of contact. It will be important to determine structures of Ca\(^{2+}\)/CaM bound to larger portions of the Ca\(_{1}\) and Ca\(_{2}\) tails to place the IQ domain conformations in a larger structural context. Further, the calcium-free state of CaM, apo-CaM, binds the Ca\(\alpha\)_C-terminal tails of both L-type and non-L-type Ca\(_{1}\) and is thought to be essential for the ability of Ca\(_{1}\)s to decode different types of calcium signals;\(^{99}\) however, the exact apo-CaM binding site and interaction details remain controversial. Definition of the structural basis for apo-CaM interactions with Ca\(_{1}\)s remains a major missing piece that will be required for understanding the structural transitions that underlie CDI and CDF.

Among the subunits, Ca\(_{2}\delta\) remains the most mysterious. It is a target for drugs such as gabapentin (Neurontin) and pregabalin (Lyrica) that are used to treat epileptic seizures, pain from diabetic neuropathy, post-herpetic neuralgia, fibromyalgia and generalized anxiety disorder.\(^{139,140}\) Defining the structural basis for the interaction of these drugs with Ca\(_{2}\delta\) should provide new ideas regarding their mechanisms of action and also the general role of Ca\(_{2}\delta\) in Ca\(_V\) function. Because it is a large, membrane-anchored glycoprotein, Ca\(_{2}\delta\) poses a number of extra challenges for structure determination efforts.

There is no doubt that some questions about Ca\(_V\) function can only be answered by obtaining structural data on holo-channels in different states of the functional cycle (open, closed, inactivated, etc.,) and bound to compounds from the rich and the well-characterized Ca\(_V\)-directed pharmacology toolkit.\(^{141,142}\) As the association of multiple channel subunits is necessary to make a properly functioning high-voltage activated Ca\(_V\), effort on the structure of Ca\(_V\) should benefit from the ongoing development of technologies aimed at producing full-length, multiprotein membrane protein complexes.\(^{143,144}\) In this regard, ability of Ca\(_3\)s to function alone, may make them better candidates for initial work in full-length channels. The ability to produce large quantities of Ca\(_V\) holo-channels of defined composition or bearing well-studied mutations would not only provide a great advance for structural work, but would also allow the pursuit of biochemical and reconstitution experiments that could directly address questions about interdomain interactions and structural transitions. In the meantime, further studies that exploit the ‘divide-and-conquer’ approach are likely to be productive at yielding new insights and posing new questions. Such studies will be most revealing when combined with biochemical validation and functional studies of structure-based hypotheses in the full-length channels.

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Figure 8. RGK functional effects and Gem G-domain structure. (A) Suppression of Ca\(_{1}\) function by co-expression of Kir/Gem. Redrawn from Fig. 2 from Beguin et al.\(^{126}\) (B) Cartoon depiction of the Gem G-domain structure.\(^{132}\) Switch regions and residues implicated in Ca\(\beta\) interaction\(^{133,134}\) are indicated. Bound GDP (cyan and orange) and Mg\(^{2+}\) (yellow sphere) are also shown.
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