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Ca^{2+} channel β-subunits: structural insights AID our understanding

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It has taken 17 years from the first identification of a voltage-gated Ca^{2+} channel (Ca_V) β-subunit as a band on a gel following purification of skeletal muscle dihydropyridine (DHP) receptors in 1987 to the publication of key information on the structures of Ca^{2+} channel β-subunits. Three recent X-ray crystallographic studies have now solved the structures of the core domains of three Ca^{2+} channel β-subunits. In this article, the properties of these cytoplasmic auxiliary subunits will first be summarized. Then the Ca_Vβ structures and the information they provide regarding how these proteins interact with the Ca_Vα1 subunit will be discussed and the possible implications of these new data for G-protein modulation of Ca^{2+} channels will be examined.

Following the identification, in 1987, of a β-subunit as an auxiliary subunit of voltage-gated Ca^{2+} channels [1], the gene encoding the skeletal muscle isoform of this subunit, subsequently termed β1a, was cloned soon after [2]. This led rapidly to the identification, by homology, of three further genes encoding Ca_Vβ subunits, termed Ca_Vβ2, β3 and β4, in addition to a neuronal splice variant of β1, termed β1b [3]. Now, within the space of a few weeks, three research groups have provided a wealth of fascinating structural information and insights into the function of Ca_Vβ2, β3 and β4 [4–6].

Modulation of Ca^{2+} channel function by β-subunits

All Ca_Vβ subunits are cytoplasmic proteins that bind to an intracellular linker region of the main pore-forming Ca_Vα1 subunits of voltage-gated Ca^{2+} channels [3] (Figure 1). Ca_Vβ subunits affect Ca^{2+} channel function in several ways. Among other effects, they increase current amplitude and hyperpolarize the voltage-dependence of activation, so that the channels open at less depolarized membrane potentials [3].

The basis for the effect of Ca_Vβ subunits on current amplitude is still controversial, with several research groups suggesting that a major component of this effect involves an increased number of channels at the plasma membrane [7,8]. This increase in the number of channels is thought to be due to the Ca_Vβ subunit masking an unknown endoplasmic reticulum retention signal within the cytoplasmic I–II linker between transmembrane domains I and II of the Ca_Vα1 subunit. Furthermore, it has been shown that the I–II linker attached to an unrelated transmembrane domain is trafficked to the plasma membrane by a Ca_Vβ subunit [7]. However, it remains controversial whether Ca_Vβ subunits influence gating charge movement, which is a good measure of functional channels integrated into the plasma membrane because it results from the net outward movement of the charge associated with the channel voltage sensors. Some groups have shown an increase in gating charge movement associated with voltage-gated Ca^{2+} channels as a result of coexpression of β-subunits with Ca_Vα1 subunits in HEK293 cells [9]. However, another group has shown no increase in charge movement following β-subunit coexpression in *Xenopus* oocytes [10,11], and has suggested that the effect of Ca_Vβ subunits is to increase the ratio of ionic current to gating charge moved. Recently, the issue was addressed biochemically using a Ca_V1.2 channel construct with an extracellular epitope tag; in this study Ca_Vβ subunit coexpression clearly increased the amount of Ca_V1.2 channel protein in the plasma membrane [12].

One difficulty associated with this type of research is that some expression systems contain endogenous Ca_Vβ subunits. In particular, *Xenopus* oocytes have been shown to contain mRNA for a β3-like β-subunit [13] and subsequently to contain β3 protein at an estimated concentration of ~17 nM [14]. In both these studies [13,14], an antisense oligonucleotide directed against β3 mRNA reduced the current amplitude resulting from a heterologously expressed Ca_Vα1 subunit, and in one study [14] this antisense approach was also shown to reduce the amount of endogenous β3. Both studies indicate that oocytes normally contain sufficient Ca_Vβ to have at least a partial trafficking effect to enable some channels to reach the plasma membrane. Nevertheless, heterologously expressed Ca_Vβ subunits do hyperpolarize the voltage-dependence of the open probability of voltage-gated Ca^{2+} channels, which also serves to further enhance the current amplitude [10,14].

The concept of the AID region

The primary binding region for all Ca_Vβ subunits on Ca_Vα1 subunits, referred to as the α1-interaction domain (AID), is an 18 amino acid region in the I–II linker of Ca_Vα1 subunits, beginning ~23 amino acids from the end of the sixth transmembrane segment of

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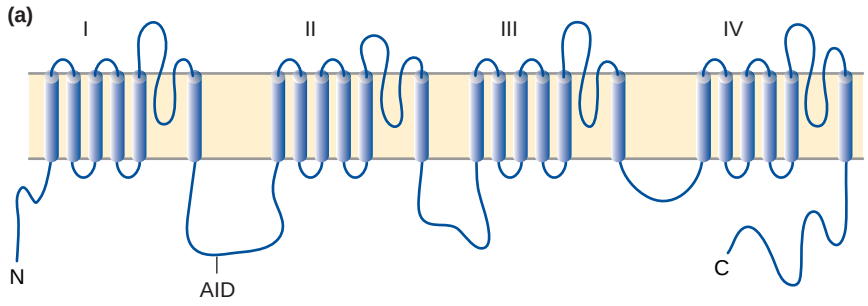

(b)

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L-type:
Human        CaV1.1   335 GEFTKEREKAKSRGTFQKLREKQQLDEDLRGYMSWITQGEVMDVE
Mouse        CaV1.2   406 GEFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQAEDIDPE
Rat          CaV1.3   407 GEFSKEREKAKARGDFQKLREKQQLEEDLKGYLDWITQAEDIDPE
Human        CaV1.4   373 GEFSKEREKAKARGDFQKQREKQQMEEDLRGYLDWITQAEELDME
Jellyfish    CaV1     382 GEFAKEKARQTKSGEFHKIREKHMLDDAVKGYLDWINQASDIENV
vertebrate L-type consensus                    QQ--EDL-GY--WITQ-E

non-L-type:
Rat          CaV2.1   363 GEFAKERERVENRRAFLKLRRQQQIERELNGYMEWISKAEEVILA
Rat          CaV2.2   357 GEFAKERERVENRRAFLKLRRQQQIERELNGYLEWIFKAEEVMLA
Rat          CaV2.3   303 GEFAKERERVENRRAFMKLRRQQQIERELNGYRAWIDKAEEVMLA
Flatworm     CaV2A    361 GEFAKERERVEKRRAFLKLRRQQQSDRELNGYLDWIQRAEEVILA
Vertebrate non-L-type consensus                QQIERELNGY--WI-KAE

vertebrate HVA consensus                       QQ----L-GY--WI---E
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TRENDS in Pharmacological Sciences

Figure 1. (a) The α1-subunit of the voltage-gated Ca^{2+} channel ($Ca_V\alpha1$). This subunit contains four transmembrane domains, each comprising six transmembrane segments. $Ca_V\beta$ subunits bind to the $Ca_V\alpha1$ subunit at an α1-interaction domain (AID) within the I–II linker of the $Ca_V\alpha1$ subunit. **(b)** Sequence alignment of the AID within $Ca_V\alpha1$ subunits. $Ca_V\beta$ subunits associate with the $Ca_V\alpha1$ subunit through the AID, an 18-residue region that begins ~23 amino acids from the end of the sixth transmembrane segment of domain I. The AID can be defined by the consensus motifs shown below the alignments. The eight residues in the consensus sequence underneath the lower panel are conserved in all known vertebrate high-voltage-activated (HVA) $Ca_V\alpha1$ subunits (both L-type and non-L-type). Several of the intervening residues of the AID are well conserved separately within the L-type (Ca_V1) and non-L-type (Ca_V2) families (blue), but there are important distinctions between the characteristic motifs of the two families.

domain I (IS6) [15], which has a characteristic consensus motif (Figure 1).

The recent crystal structures [4–6] show that the AID in complex with $Ca_V\beta$ subunits forms an amphipathic helix, and that the spacing of the best conserved AID residues through its length locates them along one side of this helix where they can pack into a complimentary hydrophobic groove on the $Ca_V\beta$ subunit (Figure 2).

Following identification of the AID sequence, the affinity of the binding of $Ca_V\beta$ subunits to either the AID peptide or the full length I–II linker was determined for a variety of combinations of subunits and shown, using several methods, to be in the low nanomolar range [14,16–20] (Table 1). In agreement with this, the concentration of the $Ca_V\beta$ subunit required to produce a half-maximal increase of the maximum Ca^{2+} channel conductance was ~17 nM [14], and similarly the half-maximal concentration of β4 subunit protein required for enhancement of $Ca_V2.1$ channel current was 7 nM [20].

Specific amino acids, including tryptophan and tyrosine, within the AID motif have been reported to be crucial for binding $Ca_V\beta$ subunits [21]; these amino acid residues have now been shown to have the most intimate

Table 1. Measured affinities of $Ca_V\beta$ subunits and Gβγ for binding to $Ca_V\alpha1$ I–II linkers[a,b]

α1 species	I–II linker construct	β species	Affinity (nM)	Refs	Gβγ affinity (nM)	Refs	Method
$Ca_V2.2$	GST fusion of I–II linker	β1b	22	[18]	62	[18]	SPR
		β3	26	[14]			
Ca_V 1.3	GST fusion of I–II linker	β1b	10	[18]	0	[18]	SPR
$Ca_V2.1$	AID–GST fusion	β1b	6	[17]	63	[16]	Pull-down
		β2	4, 36				
		β3	55				
		β4	3, 76				
$Ca_V2.1$	AID–GST fusion	β4	9	[20]	ND	–	SPR
$Ca_V1.2$	AID peptide	β2	16	[19]	ND	–	Fluorescence polarization

[a]The measured affinities were determined using three different methods: a surface plasmon resonance (SPR) assay, involving binding of purified $Ca_V\beta$ to a sensor chip to which the I–II linker or the α1-interaction domains (AIDs) are attached; binding of radiolabelled *in vitro* translated $Ca_V\beta$ to beads (pull-down); or a fluorescence polarization solution assay. In the pull-down assay both a low and a high affinity were observed in some cases.
[b]Abbreviations: $Ca_V\beta$ subunits, β-subunits of the voltage-gated Ca^{2+} channel; GST, glutathione S-transferase; ND, not determined.

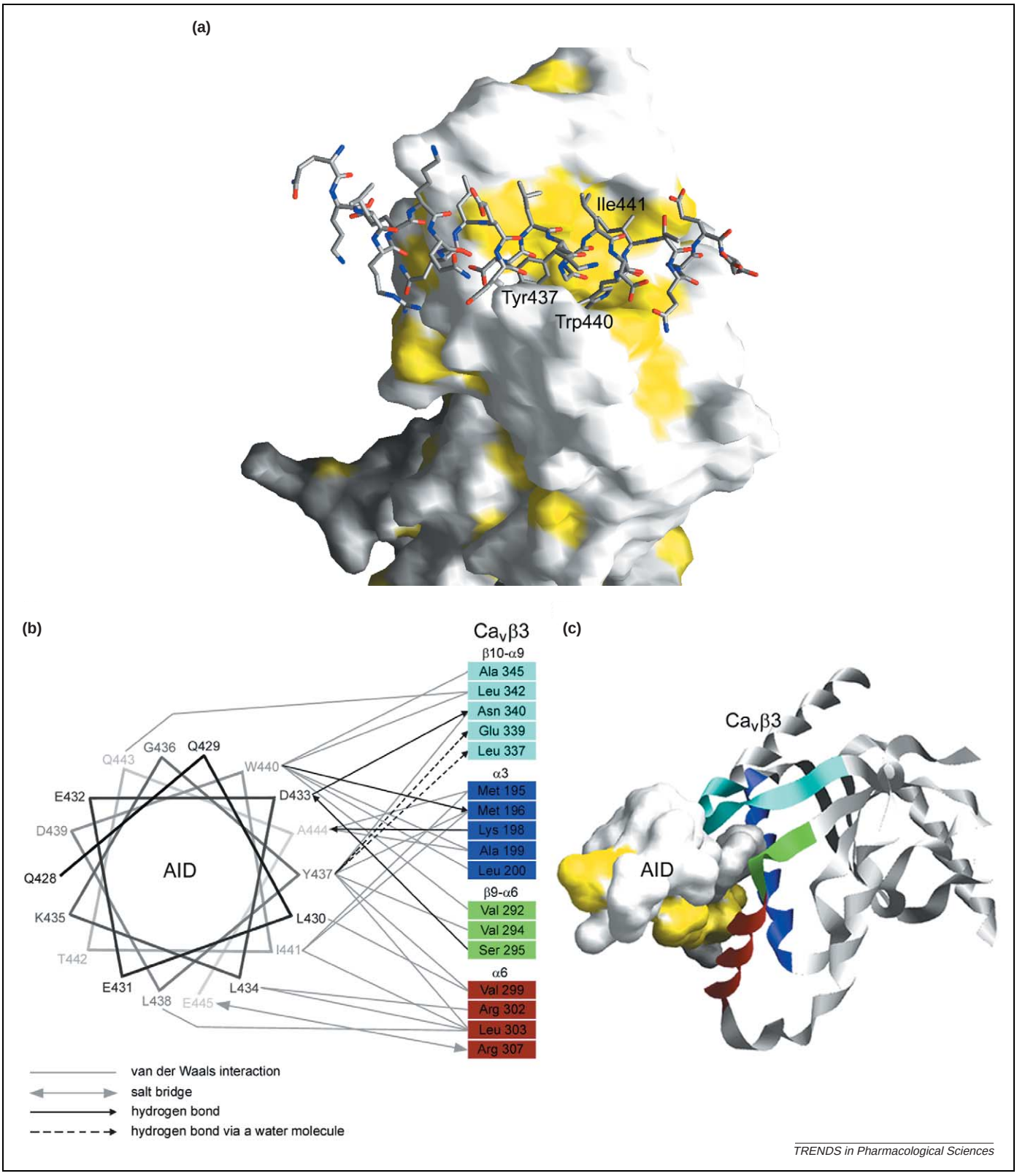

Figure 2. Binding of the α1-interaction domain (AID) of the $Ca_V1.2$ channel as an amphipathic α helix to the AID-binding pocket (ABP) on the $Ca_V\beta3$ subunit [6]. **(a)** Surface representation of the $Ca_V\beta3$ subunit with bound AID peptide. The ABP forms a deep hydrophobic groove in the surface of the $Ca_V\beta$ subunit to which the AID is complimentary. The surface of $Ca_V\beta3$ is shown rendered by hydrophobicity (yellow). The AID is shown as a stick representation. The three most crucial AID residues for binding are labelled (Ile441, Tyr437 and Trp440). **(b)** The large number of interactions between the AID and the ABP (extracted from [6]). The AID is shown on the left as a helical wheel. **(c)** Surface representation of the AID binding to the ABP of the $Ca_V\beta3$ subunit. The surface of the AID is shown rendered by hydrophobicity (yellow) to demonstrate its amphipathic character. The orientation of the complex and the colouring of structural elements comprising the ABP relate to (b). (a) and (c) were generated and rendered using GRASP [42].

interactions with the $Ca_V\beta$ subunit (of all amino acids within the AID) [6] (Figure 2).

The minimal region of the $Ca_V\beta$ subunit required to stimulate $Ca_V\alpha1$ expression was determined to be a 35 amino acid stretch at the beginning of the second conserved domain of β1b (residues 211–245) [22] and a 41 amino acid sequence was later termed the β-interaction domain (BID) [23]. However, as we now see from the structural studies discussed later [4–6], although several amino acids of the BID are involved in important interactions with the AID, the concept of the BID peptide binding directly to the AID is unfounded because the BID actually forms a structural part of the hydrophobic core of the $Ca_V\beta$ subunit. Therefore, it is unclear how the small BID sequence alone could produce stimulation.

$Ca_V\beta$ is a member of the MAGUK family

In a modelling study, we showed that the first conserved domain of $Ca_V\beta$ subunits was likely to be a Src homology 3 (SH3) domain linked by a flexible loop to the second conserved domain, which is a guanylate kinase (GK)-like domain [24]. In this regard, the $Ca_V\beta$ subunit is similar to other SH3–GK domain-containing proteins such as the 95-kDa postsynaptic density protein (PSD-95), which are thought to be scaffold proteins because they contain several protein–protein interaction domains. This family of proteins has been dubbed membrane-associated GK (MAGUK) proteins, although the GK domains within them do not usually have GK activity because of mutation of the specific enzymatic signature sequence. Crystal structures of the SH3–GK region of PSD-95 have been solved, demonstrating that these two domains fold together intimately as a module [25,26]. SH3 domains in other proteins bind to PxxP proline-rich motifs [27], and GK domains also have binding partners, such as GK-associated protein (GKAP) for PSD-95 [28].

Within proteins of the MAGUK family an intramolecular interaction is known to exist between their SH3 and GK domains [29]. This provided the impetus for examining the similarity between $Ca_V\beta$ and other MAGUK proteins. It was found that, analogous to the GK and SH3 domains of PSD-95, the isolated SH3 and GK domains of $Ca_V\beta$ subunits interact with each other and form a stable, functional core [30]. $Ca_V\beta$ subunits also contain N-terminal, C-terminal and centrally located non-conserved regions that are each highly variable in sequence and length among different isotypes and splice variants. In a limited proteolysis study each of these regions was found to be exposed in a protease-sensitive manner [19]. The two remaining regions, approximately corresponding to the SH3 and GK domains, interacted and formed a stable core that was ultimately amenable to crystallization. In one of the three structural studies, the two isolated domains of the β2-subunit were coexpressed and the complex crystallized [4]. A second study used the SH3–GK core construct of $Ca_V\beta2$, which had the central variable region removed [5]. A third study used SH3–GK core domains of β3- and β4-subunits, which have short linker regions [6].

The revelations of the structures

The SH3 domain

Appended to the N-terminus of the $Ca_V\beta$ subunit SH3 domain is a prominent α-helix, at least 20 amino acids in length, extending away from the protein. This addition might be unique to the SH3 domains of $Ca_V\beta$ subunits because no such helix was observed in the structure of PSD-95, and true MAGUK proteins have PDZ domains N-terminal to their SH3 domains that are absent from $Ca_V\beta$ subunits. The arrangement of the first four β-strands of the $Ca_V\beta$ subunit SH3 domain as an orthogonally packed sandwich is similar to canonical SH3 domains. However, as in PSD-95, the fourth β-strand is followed by a second long α-helix that extends away from the protein. The distal ends of both these α-helices (α1 and α2 in Figure 3) give way to non-conserved and probably flexible regions. The fifth β-strand is formed by residues C-terminal to the central variable region, and therefore falls within the second conserved region, giving the SH3 domain of the $Ca_V\beta$ subunit a 'split architecture' like that of PSD-95 (Figure 3). The two conserved domains of the $Ca_V\beta$ subunit are intimately associated, as was observed in the structure of PSD-95. However, the orientations of the SH3 domain with respect to the GK domain observed in the crystal structures of the $Ca_V\beta$ subunit and PSD-95 differ by ~90°. Furthermore, in PSD-95 a β-strand from the C-terminus of the GK domain forms a β-sheet with part of the fifth β-strand of the SH3 domain, whereas in the $Ca_V\beta$ subunit the last β-strand of the GK domain is absent and the fifth β-strand of the SH3 domain is much shorter.

The function of the SH3 domain in $Ca_V\beta$ subunits is uncertain because it binds to the GK domain but has no direct role in interacting with the AID. Despite this, the GK domain alone cannot function exactly like intact $Ca_V\beta$ subunits, although some enhancement of Ca^{2+} currents has been observed with this isolated domain [6,22]. Many of the residues that are crucial for interactions with PxxP motifs are conserved in the SH3 domains of $Ca_V\beta$ subunits but the crystal structures have shown that the putative

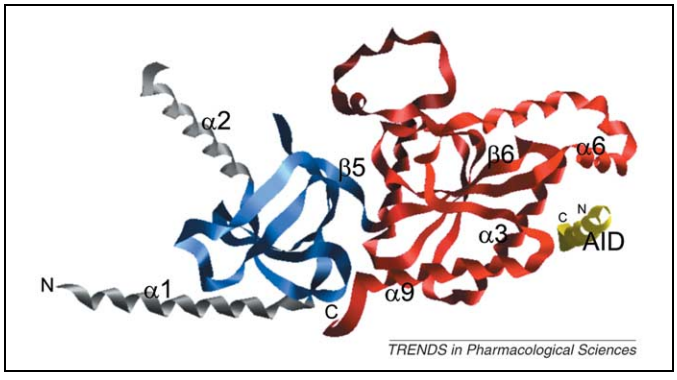

Figure 3. Structure of a complex between the β-subunit of a voltage-gated Ca^{2+} channel ($Ca_V\beta$) and the α1-interaction domain (AID) of the $Ca_V\alpha1$ channel subunit. A $Ca_V\beta2a$–$Ca_V1.1$ AID complex is shown as a ribbon representation [5]. $Ca_V\beta$ subunits consist of an Src homology 3 (SH3) domain (blue) and a guanylate kinase (GK) domain (red). Two long helices (α1 and α2; grey) that are not present in canonical SH3 domains are appended onto the SH3 domains of $Ca_V\beta$ subunits. The AID (yellow) binds into its pocket on the opposite side of the GK domain from the SH3. The central variable region would fall in the gap that is visible between α2 and β5. For consistency, structure elements are numbered according to [6]. The ribbon representation was prepared using GRASP [42].

polyproline binding site is occluded, unless a major rearrangement were to be induced. It is possible that, rather than interacting with proline-rich regions in the classical manner, the SH3 domains of $Ca_V\beta$ subunits support the pair of prominent α-helices that protrude from them, the function of which remains unknown. It will be of great interest to determine with what structures these α-helices interact.

The GK domain and the AID-binding pocket

All three structural studies investigated the binding of the AID peptide to the β-subunit protein and provided similar evidence that the interaction site of AID is in a deep groove on the GK domain (Figure 2). This has now been dubbed the AID-binding pocket (ABP) [4]. The $Ca_V\beta$ structure is only slightly altered by binding the AID peptide [6]. It is clear from the structure that the amino acids defined previously as essential in the BID region [22] are actually involved in maintaining the integrity of the GK domain and also, by contributing the fifth β-strand of the SH3 domain, maintaining the central interdomain interaction.

The GK domain has no predicted enzymatic activity because key residues in the glycine-rich ATP-binding motif that are present in true GKs (GxxGxGK) [31], in addition to other residues that are crucial for coordinating ATP, are not conserved in $Ca_V\beta$ subunits. Nevertheless, the conserved tryptophan residue of the AID is positioned in the binding groove in an equivalent place to where the adenosine moiety of ATP would fit in a catalytically active GK [5]. As these authors discuss, the GK domain has evolved from an enzyme into a protein interaction module, but has retained the skeleton of the enzymatic nucleotide-binding site for this purpose. The GK domain of the $Ca_V\beta$ subunit has also lost the GMP-binding subdomain that is present in PSD-95.

There are a pair of putative protein kinase C (PKC) phosphorylation sites within the previously defined BID that are highly conserved among vertebrate $Ca_V\beta$ subunits [21,32]. From the structures, these appear to be buried within the core along with the rest of the BID, and therefore are unlikely to be phosphorylated in the absence of structural rearrangement.

The AID α-helix

One structural study made the salient point that when the isolated AID peptide is in solution it is an unstructured random coil but, when bound to the $Ca_V\beta$ subunit, it becomes structured as an α-helix, within the ideal folding environment of the hydrophobic binding groove [5]. In the intact I–II linker, this α-helix is predicted to continue back from the AID to the end of IS6 [5]. Thus, $Ca_V\beta$ could impose α-helical structure not only onto the AID but also onto regions proximal to it, acting to induce folding. A rigid α-helical rod could be the means by which the effects of $Ca_V\beta$ subunits on the voltage-dependence of channel gating are elicited [4,5]. Alternatively, $Ca_V\beta$ subunits, associated with the AID region, could directly interact with other parts of the channel that are involved in gating, such as the base of transmembrane segments forming the pore [4].

Implication for G-protein modulation of Ca^{2+} channels

The high affinity of $Ca_V\beta$ subunit binding to the AID (Table 1) and the suggestion that there is an induced fit [5] have implications for G-protein modulation of Ca_V2 Ca^{2+} channels, which is mediated by G-protein βγ-subunits. It has been shown that Gβγ can bind to the isolated I–II linker in Ca_V2 channels but this does not occur in Ca_V1 channels, which are not modulated by the same pathway. The main binding site is on the proximal half of the AID, incorporating QQIER [16,33], although a second binding site has also been identified on the distal part of the I–II linker [16]. It was initially hypothesized that activated G proteins might achieve their modulation by displacing the $Ca_V\beta$ subunit from its binding site on the I–II linker because many of their effects oppose that of $Ca_V\beta$ subunits [34]. However, other data suggested that even if the entire I–II linker from a Ca_V2 channel was replaced by that from a Ca_V1 channel, substantial voltage-dependent G-protein modulation was still preserved [35]. Furthermore, in an expression system that contains no $Ca_V\beta$ subunits (COS-7 cells), in the absence of coexpressed $Ca_V\beta$ subunit, G-protein modulation is still present. However, in this case G-protein modulation is not voltage dependent, as shown by the fact that a depolarizing prepulse cannot remove such modulation [36]. This suggests that Gβγ produces a direct effect on channel gating in either the presence or the absence of $Ca_V\beta$ subunits. We hypothesized that there was, however, a role for $Ca_V\beta$ subunits and suggested that there is a depolarization-induced alteration in the interaction between the $Ca_V\beta$ subunit and the α1-subunit of the Ca^{2+} channel that destabilizes Gβγ binding at depolarized potentials and induces their removal, resulting in prepulse potentiation [14,36]. However, a recent article using fluorescent resonance energy transfer (FRET) suggests that displacement of the $Ca_V\beta$ subunit by Gβγ is a key feature of G-protein modulation [37]. There is still much to investigate in this modulatory pathway, not least, the nature of its voltage dependence.

In the structural study from Minor's group [4], it was speculated that, because the AID binds so tightly into the ABP of the $Ca_V\beta2a$ subunit, if this were a Ca_V2 AID sequence, it would be unlikely to be displaced by a Gβγ subunit because of the large difference in their affinities; the difference is between 3- and 20-fold, depending on which pair of estimates are taken (Table 1). Simply put, if two ligands compete for the same site, the proportion of each ligand occupying the site at equilibrium will depend on both their relative affinity and their concentration. The concentration to which Gβγ rises in intact cells at the peak of an agonist response is difficult to estimate because it is essentially in two dimensions, but it will parallel that of activated Gα. The concentration of activated $G\alpha_{transducin}$ has been estimated to rise to ~250 nM in retinal photoreceptors [38], which probably puts an upper limit on the peak level of Gβγ in other cell types. There is no published evidence concerning endogenous cytoplasmic $Ca_V\beta$ subunit levels, but it can be assumed, from a comparison of prepulse potentiation rates [39], that most plasma membrane Ca^{2+} channels are associated with a $Ca_V\beta$ subunit in neurons, and that there is therefore sufficient excess in the cytoplasm to ensure saturation (100–200 nM).

Van Petegem *et al.* [4] also hypothesized that it was unlikely that Gβγ would bind to the AID in addition to the $Ca_V\beta$ subunit, and thus it must bind elsewhere. So far the structure of the $Ca_V\beta$ subunit has only been solved with the AID regions from $Ca_V1.1$ and $Ca_V1.2$ channels, leaving open the possibility that there could be more flexible binding of the proximal part of $Ca_V2.x$ channel AID sequences to $Ca_V\beta$ subunits. An early mutational study suggested that the amino acids proximal to the tyrosine residue in the AID contributed little to the affinity of binding of AID to the $Ca_V\beta$ subunit [21]. Therefore, it remains possible that even if Gβγ does not displace $Ca_V\beta$ binding, they might both bind at the same time [36], although the AID tyrosine residue that is deeply embedded in the GK binding groove of the $Ca_V\beta$ subunit was also found to be essential for Gβγ binding to the AID [16], which argues against this hypothesis. However, it also remains the case that the positions within the AID corresponding to those residues of $Ca_V2.x$ channels that are most closely identified with Gβγ binding are located within a patch on the outside of the AID α-helix, and remain exposed when the AID is bound into its cleft on the $Ca_V\beta$ subunit (Figure 2). Because it was shown initially that Gβγ binds to two sites on the I–II linker, a site associated with the AID motif, and a more distal site [16], the interplay between these two sites could be altered in the presence or absence of $Ca_V\beta$ subunits.

Implications for drug discovery

The knowledge of any protein structure brings with it the possibility for rational drug design. The interaction between $Ca_V\alpha1.2$ and $Ca_V\beta$ subunits has already been used to screen drugs for inhibition of this association in the context of a yeast two-hybrid assay [40]. Another example of a drug possibly interfering with the $Ca_V\alpha1$–β interaction is the anti-schistosome drug praziquantel. The effects of praziquantel include the disruption of Ca^{2+} homeostasis in the parasite [41] and it has been suggested that it inhibits the interaction between schistosomal $Ca_V\alpha1$ subunits and a structurally unusual $Ca_V\beta$ subunit [32], although it remains to be determined whether this is indeed its mechanism of action, particularly in the light of the new structural data.

Future directions

In the studies reviewed in this article, the structures of the conserved core domains of $Ca_V\beta$ subunits have been solved. The N- and C-terminal regions have not been included but are predicted to be extended [19], and could interact with other regions on $Ca_V\alpha1$ subunits or elsewhere. It will be fascinating to determine these interactions, in addition to a structure of Gβγ interacting with the I–II linker of a relevant channel. Furthermore, it should now be feasible to crystallize together all the major intracellular loops of a $Ca_V\alpha1$ subunit, as has been performed for various other channels, and, if this were done, it is possible that a full-length $Ca_V\beta$ subunit could be crystallized together with this structure.

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