The Role of Auxiliary Subunits for the Functional Diversity of Voltage-Gated Calcium Channels

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Voltage-gated calcium channels (VGCCs) represent the sole mechanism to convert membrane depolarization into cellular functions like secretion, contraction, or gene regulation. VGCCs consist of a pore-forming α1 subunit and several auxiliary channel subunits. These subunits come in multiple isoforms and splice-variants giving rise to a stunning molecular diversity of possible subunit combinations. It is generally believed that specific auxiliary subunits differentially regulate the channels and thereby contribute to the great functional diversity of VGCCs. If auxiliary subunits can associate and dissociate from pre-existing channel complexes, this would allow dynamic regulation of channel properties. However, most auxiliary subunits modulate current properties very similarly, and proof that any cellular calcium channel function is indeed modulated by the physiological exchange of auxiliary subunits is still lacking. In this review we summarize available evidence in support of alternative functions of the auxiliary subunits. At the heart of the discussion is the concept that, in their native environment, VGCCs function in the context of macromolecular signaling complexes and that the auxiliary subunits help to orchestrate the diverse protein–protein interactions found in these calcium channel signalosomes. Thus, in addition to a putative differential modulation of current properties, differential subcellular targeting properties and differential protein–protein interactions of the auxiliary subunits may explain the need for their vast molecular diversity.

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Auxiliary Calcium Channel Subunits

The molecular diversity of VGCCs is greatly enhanced by the auxiliary calcium channel subunits, which promote membrane expression and modulate the current properties. The original purification of VGCCs from skeletal muscle revealed that calcium channels are protein complexes consisting of the aforementioned four non-covalently associated subunits (Fig. 1 A and C): α1, β, α2δ, and γ (Curtis and Catterall, 1984; Takahashi et al., 1987).

The α2δ subunits are highly glycosylated extracellular proteins encoded by four genes in mammals (Dolphin, 2013; Geisler et al., 2014). The gene product is post-translationally cleaved into the αδ and the δ polypeptides which remain associated with each other by disulfide bonds (Ellis et al., 1988; Calderon-Rivera et al., 2012). Initially the δ subunit was proposed to form a transmembrane helix with the short C-terminus exposed to the cytoplasmic compartment (Ellis et al., 1988). More recent evidence suggests that α2δ is anchored to the membrane by a GPI-anchor at the C-terminus of the δ polypeptide (Davies et al., 2010). Due to their large and highly glycosylated extracellular structure α2δ subunits are ideally situated to interact with components of the extracellular matrix (Geisler et al., 2014). Furthermore, α2δ-1 and -2 contain a binding site for gabapentin and pregabalin, two drugs used to treat epilepsy and neuropathic pain (Dolphin, 2013). The nature of α2δ binding and interaction with the α1 subunit is poorly understood, but co-expression of an α2δδ subunit with an α1 subunit in heterologous cells consistently augmented the expression of functional channels (Dolphin, 2013). Knockdown or knockout of α2δ in muscle cells results in a loss of the specific activation kinetics of the skeletal and cardiac muscle Cav1 isoforms (Sipos et al., 2000; Obermaier et al., 2005; Tuluc et al., 2007; Obermaier et al., 2008; Fuller-Bicer et al., 2009). Therefore, α2δδ appears to function in both targeting and/or stabilization of the channel in the membrane and in shaping the specific gating properties of different channel isoforms.

The calcium channel β subunit is a cytoplasmic protein that binds to Cav1 and Cav2 channels at a high-affinity binding site (AID, for α interaction domain) in the calcium channel in cholesterol and essential for the channel in cholesterol and essential for the channel to mediate calcium influx. The γ subunit is a cytoplasmic protein that binds to Cav1 channels and is important for the channel to mediate calcium influx.

The original purifications of VGCCs from skeletal muscle revealed yet another auxiliary subunit, the transmembrane γ subunit, which contains an N-terminal palmitoylation or a positively charged sequence, respectively, that target them to the plasma membrane and upon co-expression with α1 subunits dramatically slow down the rate of current inactivation (Olcese et al., 1994; Chien et al., 1998; Miranda-Laferte et al., 2014). In native cells β subunits may play additional roles in subcellular targeting and in the specific incorporation of the channel in macromolecular signaling complexes (see below).

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not necessarily required for functional expression of CaV channels in heterologous cells. In contrast, γ1 belongs to a family of eight genes predominantly expressed in the brain where they are known as transmembrane AMPA receptor regulatory proteins (TARPs) (Catterall, 2011). They associate with the glutamate receptor and play an important role in targeting and anchoring these ionotropic receptors in the postsynaptic membrane of neurons as well as in glia cells (Chen et al., 2007). A recent study demonstrated that different γ subunits differentially modulate the inactivation properties of the cardiac CaV1.2 channel in HEK293 cells (Yang et al., 2011). However, apart from the CaV1.1/γ1 interaction in skeletal muscle, it is not known whether γ and α1 subunit isoforms functionally interact with each other in native tissues.

What defines an auxiliary calcium channel subunit?

The complement of canonical VGCC subunits was originally defined by their co-purification from muscle tissue. This definition has served us well in that it described a minimum set of proteins that are part of many CaV channels and more or less important for their normal functions. However, since then many other calcium channel interacting and modulating proteins have been identified, and conversely the known roles of the classical channel subunits are no longer limited to CaV channel functions. These developments raise the question as to which properties actually define a protein as a calcium channel subunit. Is it its co-purification with an α1 subunit or its functional interaction with the channel, the exclusiveness of its
association with CaV channels or the absolute requirement for its function, or a combination of any or all of these properties? No matter which of these properties one would consider, no definition would include all classical auxiliary subunits or necessarily exclude other VGCC-interacting proteins.

For example today we know that T-type (CaV3) channels do not contain any of the classical auxiliary subunits and that inclusion of the γ subunit may be specific to CaV1.1 channels in skeletal muscle (Zhang et al., 2013). Thus, the classical VGCC subunits are not universally associated with all members of the CaV family. The classical VGCC subunits also fall short of being exclusive to CaV channels. Based on the number of γ genes expressed in the brain versus muscle and on their importance as AMPAR regulatory proteins, γ proteins primarily have to be considered as being AMPAR subunits, with one notable exception, γ1, which associates with a calcium channel (Chen et al., 2007). But also for α2δ and β subunits CaV channel-independent functions have been reported, indicating that their functional importance and molecular interactions may not be limited to CaV channels (Eroglu et al., 2009; Subramanyam et al., 2009).

Also the importance of the classical auxiliary subunits for specific CaV channel-dependent functions differs greatly. Although β subunits are important for normal surface expression and current properties of CaV channels in most expression systems, α2δ subunits seem to be less important and γ subunits even dispensable for normal muscle function (Obermair et al., 2008; Catterall, 2011). On the other hand the ubiquitous calcium sensing protein calmodulin is associated with CaV1 and CaV2 channels and plays an important role in feedback regulation (i.e., calcium-dependent inactivation and facilitation) as well as in the subcellular localization of CaV channels (Wang et al., 2007; Minor and Findeisen, 2010). Yet, it has no rank among the canonical auxiliary calcium channel subunits. Calmodulin belongs to a group of VGCC associated proteins, which are constitutively associated with most, if not all, CaV1 and CaV2 channels and modulate channel expression as well as their current properties. Therefore we will refer to these proteins as non-canonical auxiliary calcium channel subunits (see Section non-canonical auxiliary calcium channel subunits).

Apparently the importance of an associated protein for CaV channel function is neither necessary nor sufficient to define it as auxiliary calcium channel subunit. Rather the current definition of α, β, and γ as auxiliary calcium channel subunits is mostly based on historical reasons. However, 25 years of calcium channel research have led to a much more complex picture of auxiliary calcium channel proteins. Therefore it is necessary to re-think the physiological role of auxiliary calcium channel subunits and perhaps question some of the cherished traditional concepts.

**Auxiliary Subunits Increase the Molecular Diversity of Calcium Channels**

Mammals have four genes each encoding for α2δ and β subunits (Fig. 1C), both with several known splice variants and probably many more to be identified (Buraei and Yang, 2010; Dolphin, 2013). (Because of the limited importance of the γ1 as calcium channel subunit we will from here on focus our discussion on α2δ and β subunits.) Counting only the number of α2δ and β genes together with the seven CaV1 and CaV2 α1 subunit genes, there are 112 possible subunit combinations. Further considering the multiple splice variants of each gene we would likely arrive at a number of over thousand possible molecularly distinct calcium channel combinations. Do we need such a great molecular diversity and if so, for what purpose?

The calculation above is based on the assumption that every subunit variant can associate with every α1 subunit. Therefore, we first need to question whether such unlimited subunit combinations are at all possible. A host of co-expression studies in heterologous cells support the concept of a great promiscuity in calcium channel subunit interactions (Obermair and Flucher, 2013). In fact, the authors of this review are not aware of a single study demonstrating that a given α2δ or β subunit cannot functionally interact with any specific CaV1 or CaV2 α1 subunit, when heterologously co-expressed. There is also evidence that a similar heterogeneity in channel interactions occurs in differentiated and native cell systems (Obermair et al., 2010; Campiglio et al., 2013). In those cases where isolated channel combinations serve specific cellular functions this typically is linked to the co-expression of a unique set of channel isoforms (Obermair and Flucher, 2013; Geisler et al., 2014).

The overwhelming functional evidence for highly promiscuous subunit interactions is supported by the available studies measuring binding affinities between α1 and β subunits, or β subunits and AID peptides (Van Petegem et al., 2008). AID binds different β isoforms with affinities in the low nM range and any isoform-specific differences in the strength of binding are rather small (Buraei and Yang, 2010). Together these studies do not suggest that isoform-specific subunit combinations form spontaneously in vitro or when expressed in heterologous cells. Thus, we need to consider the possibility of an extensive molecular diversity of VGCC subunit combinations.

However, cell- and tissue-specific expression patterns or differential developmental expression of the calcium channel subunit isoforms can limit the extent of possible subunit combinations in specific cell types (Obermair and Flucher, 2013; Geisler et al., 2014). For example skeletal muscle exclusively expresses CaV1.1, α2δ-1 and β1a, whereas heart muscle expresses CaV1.2, α2δ-1 and β2a. The hair cells of the inner ear express CaV1.3, α2δ-3 and β2, whereas retinal photoreceptor cells express CaV1.4 and α2δ-4 (two isoforms that seem to be unique to this tissue) and β3 (Lee et al., 2015). Since these cells express only a single isoform of each subunit, null-mutants of the respective gene result in the corresponding disease phenotypes (Gregg et al., 1996; Neef et al., 2009; Stockner and Koschak, 2013). Some neuron types show a predominance of specific channel subunits. For example cerebellar granule cells express high levels of CaV2.1 together with β3 and α2δ-2 (Schlick et al., 2010). Although in such neurons other isoforms may be expressed at lower levels, these cannot fully compensate the loss of any one of the dominant isoforms. Accordingly, null-mutants of any of these dominant calcium channel subunit genes—in tottering (CaV2.1), ducky (α2δ-2), and lethargic (β4) mice—result in very similar neurological defects characterized by ataxia and cerebellar epilepsy (Felix, 2002). On the other hand, the majority of neurons in the central nervous system expresses multiple isoforms at comparable levels, allowing a great molecular diversity of calcium channel combinations and loss of a single isoform readily can be compensated by the other isoforms (Schlick et al., 2010; Obermair and Flucher, 2013; Geisler et al., 2014).

Similarly the expression of some calcium channel subunit isoforms is limited to specific stages of development. Developing and differentiated cells have different requirements for calcium signaling that are reflected in the expression of distinct CaV channel isoforms. Recently we identified a splice variant of CaV1.1 which is the dominant isoform in embryonic muscle but is almost exclusively replaced by expression of a functionally very distinct CaV1.1 splice variant in mature skeletal muscle (Tuluc et al., 2009). Another example is provided by the expression of β2c, β2d, and β2e in embryonic cardiac muscle, whereas in adult heart expression of these
isoforms declines and β2b becomes the predominant β subunit (Buraei and Yang, 2010).

Together these examples demonstrate that differential expression of calcium channel isoforms and splice variants gives rise to both spatially and temporally distinct calcium channel complexes. These mechanisms dramatically decrease the number of possible subunit combinations within a given cell. Nevertheless, in many cell types the simultaneous expression of multiple α and β isoforms and their promiscuous association with several expressed α subunits still allows for a considerable combinatorial complexity and molecular diversity of voltage-gated calcium channels in many excitable cells.

What is the functional importance of the molecular diversity of auxiliary subunits?

Usually molecular diversity is equated with functional diversity. With regard to calcium channels the studies of subunit interactions are strongly grounded on the belief that association of a given α1 subunit with alternative β or α2δ subunits endows the channel with different current properties. This concept is supported by the distinct voltage sensitivity of activation and the slow inactivation kinetics found in channels co-expressed with the membrane-associated β2a or β2b isoforms compared to those of other isoforms (Olicese et al., 1994; Miranda-Laferte et al., 2014). In contrast to β2a and β2b all other examined β isoforms, or mutated β2a, lacking the N-terminal palmitoylation site, show very similar effects on voltage-dependence and current density (Buraei and Yang, 2010). Likewise, also different α2δ subunit isoforms appear to exert very similar effects upon co-expression with specific α1 subunits. Apparently, the distinct effects on CαV current properties of the membrane-associated β2a and β2b are the exception among the auxiliary subunits, not the rule. Therefore, the concept that the large molecular diversity among auxiliary subunits exists in order to tune current properties to the specific requirement may need to be abandoned in light of the paucity of evidence obtained in 25 years of co-expression studies.

If two molecularly distinct subunit isoforms or splice variants endow a channel with similar functional properties, they will create functional redundancy rather than functional diversity. Functional redundancy among different β and α2δ isoforms in vivo is supported by evidence from several mutant animal models. Whereas null-mutants of some β and α2δ subunit genes do show disease phenotypes, these are for the most part associated with calcium channel functions in cells or tissues that express only a single isoform (Obermair and Flucher, 2013; Geisler et al., 2014). However, in the brain, where the majority of cells express multiple α2δ and β isoforms these knockout animal models cause little or no defects (Buraei and Yang, 2010; Dolphin, 2013). Apparently, other isoforms are functional redundant and thus can compensate the loss of the mutated subunit isoform. As the studies in the knockout mouse models show, functional redundancy increases the safety factor for cellular function and thus may by itself represent a benefit of molecular diversity. Whereas this may explain why some cells express more than one auxiliary subunit gene, it appears to be a poor explanation for the large molecular diversity among α2δ and β genes and certainly does not explain the need for multiple splice variants, which often would be equally affected by genetic defects.

One alternative explanation might be that distinct auxiliary subunits can differentially interact with proteins involved in targeting of VGCCs or in the formation of signaling complexes. In polarized epithelial MDCK cells recombinant Cα2.1 is targeted to the apical membrane when co-expressed with β1b or β4, while it is trafficked to the basolateral membrane when co-expressed with β2a (Brice and Dolphin, 1999). This study suggested for the first time that different β isoforms could target calcium channels to structurally and functionally distinct membrane domains of polarized cells. A study from our laboratory reported differential localization of various β isoforms in cultured hippocampal neurons (Obermair et al., 2010). In fact, while all examined β isoforms were incorporated in the presynaptic terminals, β1 isoforms were transported poorly to the distal axons compared to the other β isoforms. This indicates that specific axonal targeting mechanisms may in part specify the subunit combination of presynaptic calcium channels. This notion is further substantiated by a recent study demonstrating differential somato-dendritic and axonal targeting properties of three β splice variants in differentiated neurons (Etemad et al., 2014b). Differential axonal targeting properties may also enrich specific α2δ subunits in the presynaptic compartment. Unpublished data from the laboratory of G. Obermair also indicate differential axonal targeting properties in α2δ subunit isoforms (personal communication). Together these studies provide evidence that individual channel subunits are specifically targeted to the presynaptic compartment, where they may assemble into specific channel complexes based on their relative availability.

Theoretically, specific β subunit targeting mechanisms might function to target entire channel complexes into specific subcellular compartments. However, axonal targeting of preassembled channel complexes again would require their specific assembly in the soma, where a multitude of channel subunits are present. Given the promiscuous association this is unlikely to create the highly specific composition of presynaptic channels. Moreover, to date there is no compelling evidence for the existence of an auxiliary subunit guided targeting mechanism. In contrast, β subunits require the α1 subunits for their own subcellular targeting, not vice versa. For example, without an α1 subunit β subunits fail to incorporate in the triad junctions of skeletal myotubes (Neuhuber et al., 1998; Campiglio et al., 2013). When an α1 subunit is expressed and targeted into the junctions, any co-expressed β subunit associates with it (Subramanyam et al., 2009; Campiglio et al., 2013; Etemad et al., 2014a). Similarly the subcellular localization of β subunit in neurons chiefly depends on the interaction with specific α1 subunits (Obermair et al., 2010). Thus, existing evidence suggests that specific targeting of auxiliary subunits to a subcellular compartment may contribute to the generation of specific subunit composition of the channel complexes in the presynaptic membrane. Obviously, such a mechanism would be limited to neurons or a few other cell types where a similar spatial segregation of channel subunits by specific targeting mechanisms is possible.

An alternative but plausible explanation for a high subunit complexity is that different auxiliary subunits might specifically interact with distinct up- or down-stream signaling proteins. Thereby channel complexes containing distinct auxiliary subunits could be differentially modulated or initiate differential cellular responses. Below, we describe several such VGCC signaling complexes and review the experimental evidence in support of the notion that auxiliary calcium channel subunits function as important coordinators of these signaling complexes, in part in an isoform-specific manner.

**VGCCs Function in the Context of Calcium Channel Signaling Complexes**

Calcium-mediated signaling processes achieve their remarkable speed and specificity by a tight spatio-temporal regulation of the calcium signal. For less than a millisecond calcium concentration near the cytoplasmic mouth of VGCC can rise by three orders of magnitude (from 100 μM to 100 mM) before it dissipates due to diffusion, buffering, and active removal processes. Consequently, to become activated,
low affinity calcium sensing proteins need to be located within the close vicinity of the channel. Therefore many VGCCs are colocalized with calcium sensors, regulatory proteins, and effector proteins in macromolecular signaling complexes encompassing calcium micro- and nanodomains. In this context VGCCs not only function as voltage sensors and the calcium source, but also serve as signaling platforms for up- and down-stream pathways (De Waard et al., 1996; Tsien and Curtis, 2005). The large cytoplasmic domains of the \( \alpha \) subunits with multiple protein–protein interaction domains provide many binding sites for modulatory and effector proteins (Dai et al., 2009). Furthermore, auxiliary subunits greatly increase the potential of VGCCs to form transmembrane signaling complexes. The intracellular \( \beta \) subunit belongs to the MAGUK family of scaffold proteins containing an SH3 and a catalytically inactive GK domain, which has evolved into a highly specific protein interaction domain (Buraei and Yang, 2010). In contrast, the relatively short extracellular domains of the pore-forming subunit provide few potential interaction sites. However, the potential for extracellular interactions is substantially increased by the \( \alpha_{2}\delta \) subunit, which is completely extracellular. \( \alpha_{2}\delta \) is highly glycosylated and contains a von Willebrand factor type A (VWA) cell–cell adhesion domain that is typically found in extracellular matrix proteins and integrin receptors (Geisler et al., 2014). Consequently, the \( \alpha_{2}\delta-\alpha_{1}\beta \) channel complex comprises a platform for transmembrane signaling in which the \( \alpha_{1} \) subunit primarily determines the specific gating and current properties and the different \( \alpha_{2}\delta \) while \( \beta \) subunit isoforms determine the subcellular localization and the molecular context for the calcium signal.

In the following sections we will describe macromolecular signaling complexes formed by calcium channels, and present evidence indicating that the auxiliary subunits serve as scaffold proteins in these VGCC complexes. In some cases specific auxiliary subunit isoforms determine whether particular proteins associate with the signaling complex or whether a channel complex is sensitive to a particular mode of modulation or not (summarized in Fig. 2 and Table I). These findings suggest the importance of the molecular subunit diversity in the formation of various functional calcium channel signaling complexes.

The excitation-contraction (EC) coupling apparatus

The best described VGCC-mediated mechanism is EC coupling, the process in which an electric signal, the action potential, is converted into muscle contraction. This fundamental process in muscle physiology is mediated by Ca\( \text{V} \)1.1 and Ca\( \text{V} \)1.2 VGCCs, also called dihydropyridine receptors (DHPR), which in response to membrane depolarization activate channel opening and the opening of calcium release channel (ryanodine receptors or RyR) in the sarcoplasmic reticulum (SR). EC coupling takes place in junctions between the SR and the plasma membrane or its invaginations the T-tubules, where Ca\( \text{V} \)1 channels and the RyR are located in direct apposition. Different subunit compositions of Ca\( \text{V} \)1 and the RyR in skeletal and cardiac muscle determine distinct molecular signaling mechanisms (Franzini-Armstrong et al., 1998).

In skeletal muscle the DHPR is composed of Ca\( \text{V} \)1.1, \( \alpha_{2}\delta-1 \), and \( \beta_{1d} \), which physically interact with the type 1 RyR. Upon depolarization the Ca\( \text{V} \)1.1 undergoes a conformational change which leads to the opening of the RyR1. The physical interaction of Ca\( \text{V} \)1.1 and RyR1 is also reflected by the organization of Ca\( \text{V} \)1.1 in ordered tetrad arrays (groups of four) opposite to one RyR, which can be visualized in freeze–fracture electron microscopy. Influx of calcium through the Ca\( \text{V} \)1.1 channel is not necessary for functional EC coupling and in many fish (e.g., zebrafish) Ca\( \text{V} \)1.1 is non-conducting and functions merely as voltage sensor (Schredelseker et al., 2010). The essential features for skeletal muscle EC coupling and for tetrad formation (i.e., the correct incorporation into the functional signaling complex) are the II-III intracellular loop of Ca\( \text{V} \)1.1 and the SH3 domain and C-terminus of \( \beta_{1a} \). In fact, EC coupling fails in null mutants of either Ca\( \text{V} \)1.1 or \( \beta_{1a} \). A chimera of Ca\( \text{V} \)1.2 containing the II-III loop of Ca\( \text{V} \)1.1 can reconstitute EC coupling and tetrad formation in Ca\( \text{V} \)1.1-null myotubes (Grabner et al., 1999; Wilkens et al., 2001; Kugler et al., 2004). Similarly, a chimera of \( \beta_{3} \) containing the SH3 domain and the C-terminal region of \( \beta_{1a} \) can fully restore EC coupling in relaxed \( \beta_{1a} \)-null myotubes (Dayal et al., 2013). Importantly, the specific \( \beta \) subunit isoform is critical for tetrad formation and thus for the direct Ca\( \text{V} \)1.1-RyR1 coupling mechanism in skeletal muscle (Schredelseker et al., 2005). In contrast, the \( \alpha_{2}\delta-1 \) subunit seems to be dispensable for EC coupling (Obermair et al., 2005).

Recent reports revealed a hitherto unnoticed essential component of the EC coupling machine. STAC3 is a cytoplasmic Ca\( \text{V} \)1.1 binding protein containing a cysteine rich region and two SH3 domains. It is necessary for membrane expression of Ca\( \text{V} \)1.1 in heterologous cells and its deletion in skeletal muscle impairs EC coupling in mouse and zebrafish (Horstick et al., 2013; Nelson et al., 2013; Polster et al., 2015). Both, the \( \beta_{1a} \) subunit and STAC3 associate with the Ca\( \text{V} \)1.1 \( \alpha_{15} \) subunit, whereas their possible interactions with the RyR1 depend on the presence of Ca\( \text{V} \)1.1 (Campiglio et al., 2013; Polster et al., 2015). Thus, it appears that \( \beta_{1a} \) and STAC3 are important for the correct assembly of the skeletal muscle EC coupling apparatus. Whether they also participate in the signal transduction process remains controversial.

| Protein | \( \beta \) isoform in the VGCC | Interaction partner | Function | Reference |
|---------|-------------------------------|--------------------|----------|----------|
| RIM1    | any \( \beta \) isoform | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| RGK     | any \( \beta \) isoform | Ca\( \text{V} \)1,2, Ca\( \text{V} \)2.2 | Current inhibition | Yang and Colecraft (2013) |
| PKC     | any \( \beta \) isoform | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| RyR1    | \( \beta_{1a} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| PKA     | \( \beta_{1a}, \beta_{3} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| Ahnak1  | \( \beta_{3} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| Synaptotagmin I | \( \beta_{3}, \beta_{4d}, \text{not } \beta_{4b} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| G\( \alpha_{1} \) | \( \beta_{1a}, \beta_{2a} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| CAMKII  | \( \beta_{1b}, \beta_{2a} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| Akin     | \( \beta_{1a}, \beta_{2a}, \text{not } \beta_{4b} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
| AA      | \( \beta_{1a}, \beta_{2a} \) | Ca\( \text{V} \)1,2 | Targeting and docking of vesicles in proximity to VGCCs | Coppola et al. (2001) |
Whereas skeletal muscle EC coupling is independent of calcium influx, the β-adrenergic receptor mediated increase in the force of the contraction depends on extracellular calcium. At rest the C-terminus of CaV1.1 is proteolytically cleaved but remains associated with the channel, forming an autoinhibitory complex with reduced calcium currents. Upon activation of the β-adrenergic/PKA signaling pathway both the C-terminus of CaV1.1 and β1d are phosphorylated (Catterall, 2010). PKA dependent inositol trisphosphate (PtdIns(4,5)P3) turnover of the plasma membrane induces its autoinhibitory function and the calcium current and EC coupling are augmented. This signaling mechanism critically depends on the anchoring of PKA to the C-terminus of CaV1.1 by the adaptor protein AKAP15 (Catterall, 2010).

In cardiac muscle EC coupling is carried out by a channel complex consisting of CaV1.2, α2-δ-1, mainly β2b (but also β1d), β2a-d, and β3) and the RyR2. CaV1.2 and RyR2 are also localized in close proximity in the junctions between the sarcoplasmic reticulum and the plasma membrane, but unlike in skeletal muscle, they are not directly coupled. As a consequence the DHPRs do not form tetrads and cardiac EC coupling requires calcium influx through CaV1.2 (Franzini-Armstrong et al., 1998) Thus, CaV1.2 calcium triggers the opening of RyR2 in a process called calcium-induced calcium release (CICR). Importantly, CICR in cardiac muscle also necessitates the colocalization of the CaV1.2 channel in the close proximity of the RyR2 (Leach et al., 2007). Heterologously expressed CaV2.1 channels, which conduct even larger currents but are not targeted into triad junctions, fail to induce CICR in dysgenic myotubes (Tanabe et al., 1990). Therefore, even without a direct physical link between CaV1.2 and RyR2, these channels form a functional signaling complex.

Like in skeletal muscle this calcium channel signaling complex is modulated by β-adrenergic stimulation in the fight or flight response. Again the mechanism involves the relief of an autoinhibitory protein–protein interaction within the C-terminus of CaV1.2, as well as the association of PKA by AKAP15. In vitro PKA also phosphorylates three sites of β2a (Gerhardtstein et al., 1999). However, these phosphorylation sites were shown to be not essential in the PKA upregulation of L-type current in cardiac myocytes (Miriyala et al., 2008). Nevertheless, the β subunits play an important role in the β-adrenergic facilitation of CaV1.2 currents. The extent of PKA modulation is highest with β1b, followed by β3 and β4, while β2a shows the weakest modulation. As PKA modulation of CaV1.2 signaling also can occur also in brain the differential effects of different β subunits may be involved in the tissue-specific tuning of this modulatory process (Miriyala et al., 2008).

In the heart the large membrane scaffold protein Ahnak1 has been suggested to play a role in the β-adrenergic modulation of CaV1.2. Ahnak1 contains docking sites for several cytoskeleton proteins and for auxiliary β subunits. It has been suggested that Ahnak1 binds to the GK domain of β2, acting as a repressor of the β2 functionality during basal conditions (Pankonien et al., 2012). Upon PKA activation β2δ is phosphorylated at Ser296, which reduces its binding to Ahnak1 allowing full augmentation of calcium influx (Pankonien et al., 2012). Together these examples indicate that in cardiac muscle, in which calcium influx through CaV1.2 controls the force of contraction, auxiliary β subunits are critically involved in the current modulation by functionally and structurally interacting with various signaling and scaffolding proteins.

The presynaptic calcium channel signaling complex

One of the most studied mechanisms controlled by VGCCs is regulation secretion and specifically presynaptic neurotransmitter release. Presynaptic CaV2 channels conduct P/Q- and N-type calcium currents, which trigger the fusion of synaptic vesicles with the plasma membrane. The efficiency of synaptic release critically depends on the amount of calcium entering the nerve terminal and on the proximity of the calcium channels to the fusion apparatus. The synaptic vesicle membrane contains the v-SNARE protein VAMP/ syntobrevin, which interacts with two t-SNARE proteins of the plasma membrane, SNAP-25 and syntaxin. The calcium sensor protein synaptotagmin I, a transmembrane protein that binds syntaxin and the SNARE complex, triggers vesicle fusion upon calcium binding (Catterall, 2011). The colocalization of the VGCCs and the fusion apparatus within a calcium nanodomain allows transmitter release within 200 μs of the action potential (Sudhof, 2013).

Presynaptic VGCCs directly interact with the synaptic vesicle fusion apparatus through a specific interaction site called synprint, located in the II-III loop of the α1 subunit. Synprint has been suggested to interact with syntaxin, SNAP25 and synaptotagmin I in a calcium- and phosphorylation-dependent manner (Sheng et al., 1998), and it has also been shown to be important for synaptic targeting of calcium channels (Szabo et al., 2006). Synaptotagmin I also binds to specific β isoforms (β2 and β4, but not β3a) (Vendel et al., 2006). Furthermore the Rab-interacting molecule 1 (RIM1), a presynaptic scaffold protein which binds the synaptic vesicle protein Rab3, also binds to auxiliary β subunits, and thus may be involved in the targeting and docking of vesicles in proximity of the VGCCs. RIM1 binding to the auxiliary β subunits not only has a scaffolding function, but also modulates the current properties of the channel. It slows down voltage-dependent inactivation and shifts its voltage-dependence to hyperpolarizing potentials (Coppola et al., 2001). These findings indicate a dual function of auxiliary β subunits in anchoring synaptic vesicles to presynaptic VGCCs and in the modulation of the calcium current that triggers vesicle fusion.

It has also been suggested that the auxiliary subunit α2δ plays a role in targeting VGCC to the presynaptic boutons (G. Obermair, personal communication), presumably through interactions with extracellular active-zone-specific proteins (Geisler et al., 2014). In the nerve terminal α2δ may be involved in coupling presynaptic CaV to the synaptic release machinery (Hoppa et al., 2012). Furthermore, α2δ subunits have been suggested to play a role in synaptogenesis. The α2δ-1 subunit has been identified as a receptor of thrombospondin, an astrocyte-secreted protein that promotes synaptogenesis (Eroglu et al., 2009). In that study overexpression or knockdown of α2δ-1, respectively, promoted or inhibited excitatory synapse formation in cultured retinal ganglion cells.

Auxiliary β subunits may play a crucial role not only in the targeting of presynaptic VGCCs in the proximity of the synaptic release machinery, but also in the tuning of the VGCC properties. Because of their crucial role in synaptic release, presynaptic calcium channels are subject to regulation by several modulatory pathways. For example CaV2 channels undergo negative-feedback inhibition through G-protein coupled receptor pathways, which represent mechanisms of presynaptic inhibition and short-term synaptic plasticity (Buraei and Yang, 2010). Nerve terminals contain G-protein coupled receptors that are activated by spillover of neurotransmitters and provide negative feedback by inhibiting the presynaptic calcium channels; thus, reducing neurotransmitter release. Specifically the Gβδ2 dimer has been demonstrated to bind directly to CaV2 channels at multiple sites, including two in the I-III loop, one up-stream and one down-stream of the I-III loop. Binding of Gβδ2 to CaV2 channels results in a shift in the voltage-dependence of activation to more depolarized potentials and a slowing of activation kinetics. The auxiliary β subunit appears to be essential for this inhibition (Buraei and Yang, 2010). When the β subunit binds to the AID, the I-III loop adopts a rigid α-helical structure and this structural property is crucial for the Gβδ2 inhibition.
Furthermore it was demonstrated that distinct β isoforms have different effects on the extent of the Gβγ inhibition, with β3 being the most effective (Feng et al., 2001). The presence of an auxiliary β subunit is also essential also for PKC modulation of specific CaV subunits (Stea et al., 1995). PKC activity can relieve Gβγ inhibition, by increasing the activity of R-type and N-type channels, which potentiate synaptic release. Upon activation PKC phosphorylates CaV2 channels on the I-II loop, which releases the Gβγ dimer and abolishes its inhibition on the channel activity in a β-dependent manner (Dai et al., 2009).

Together these examples highlight the importance of the auxiliary calcium channel subunit in presynaptic function. They are involved in the molecular interactions regulating the tightness of coupling between excitation and synaptic release, as well as in orchestrating up-stream signaling pathways, which dynamically regulate the release probability by modulating the gating properties of the presynaptic channels. Emerging evidence indicates isoform-specificity of these functions. Interestingly, a proteomics approach identified around 200 proteins as potential interaction partners of neuronal CaV2 channel complexes, but only less than 10% of these were known presynaptic signaling proteins (Muller et al., 2010). Apparently the complexity of the presynaptic calcium channel signaling machinery vastly exceeds our current models.

The postsynaptic calcium signaling complex

The postsynaptic CaV channels (predominantly CaV1.2 and CaV1.3) are localized in the soma of neurons as well as in dendritic shafts and spines (Obermaier et al., 2004). There they contribute to the neuronal excitability and they activate the excitation-transcription coupling pathway. The postsynaptic channels form macromolecular signaling complexes and require interactions with specific proteins for their own proper function (Calin-Jageman and Lee, 2008; Striessnig et al., 2014).

The C-terminus of CaV1.2 subunits is a major modulatory domain that contains multiple binding sites for regulatory proteins and for scaffold proteins. As mentioned in section 3.1, the distal C-terminus of CaV1.2 as well as that of CaV1.3 can be cleaved, but remains non-covalently associated to the proximal C-terminus. This intramolecular interaction exerts an autoinhibitory effect and reduces the channel activity by more than 10-fold (Hulme et al., 2006; Striessnig et al., 2014). PKA is anchored to the C-terminus of the channels through AKAP15, and upon activation phosphorylates the proximal C-terminus and disinhibits CaV1.2 and CaV1.3 currents (Dai et al., 2009; Catterall, 2010). PDZ-binding motifs in the very C-terminus of CaV1 channels seem to be important for coordinating calcium channel signaling complexes in the postsynaptic compartment of neurons (Stanika et al., 2015). For example, the PDZ domain-containing protein erbin mediates voltage-dependent facilitation (VDF) of CaV1.3 channels, by relieving the autoinhibitory action of the C-terminus (Calin-Jageman et al., 2007). Importantly, erbin’s effect on the CaV1.3 current profoundly depends on the auxiliary β subunit isoform in the complex. It increases VDF of CaV1.3 channels containing β1b, but not of those containing β4 (Calin-Jageman et al., 2007).

Densin, another PDZ domain containing protein, has been reported to anchor the calcium/calmodulin protein kinase II (CaMKII) to CaV1.3 and thereby promote calcium-dependent facilitation (CDF) during high-frequency stimulation (Jenkins et al., 2010). CaMKII activity also regulates CaV1.2 and CaV2.1 channels, slowing down inactivation and positively shifting their voltage-dependence (Dzhura et al., 2000; Jiang et al., 2008). Interestingly, CaMKII binds in vitro to β1, but not to β3 and β4 (Grueter et al., 2008), and it co-immunoprecipitates with forebrain CaV1 channel complexes containing β1b or β2b, but not with those containing β4 (Abiria and Colb, 2010).

A different signaling mechanism regulating VGCC activity are the membrane lipids and their metabolic products, like arachidonic acid (AA). AA inhibits the calcium current of all VGCC, including the CaV3 channels which do not contain auxiliary subunits. Therefore one could speculate that the auxiliary subunits play no role in the AA inhibition of calcium currents. However, when a CaV1.3 channel complex contains β2a, AA exerts little inhibition. Interestingly this dampening of the AA action critically depends on the palmitoylation of β2a (Roberts-Crowley and Rittenhouse, 2009). This suggests that even in regulatory pathways that act directly on the α1 subunit, auxiliary subunits can modify the extent of the modulation of the channel properties. These are striking examples demonstrating how different β isoforms are involved in mediating specific up-stream signaling pathways that modulate neuronal calcium channel function; but auxiliary subunits are similarly involved in down-stream signaling pathways.

The excitation-transcription coupling pathway

VGCCs are important regulators of activity-dependent regulation of gene expression in excitable cells. Since CaV1 channels are preferentially localized in the soma and proximal dendrites of neurons, they have a privileged role in activity-dependent signaling to the nucleus, as opposed to the predominantly presynaptic CaV2 channels and the NMDA receptor (Bading et al., 1993; Dolmetsch et al., 2001; Di Biase et al., 2008). VGCC regulate transcription via activation of classical signal transduction cascades and by nuclear targeting of channel fragments or subunits.

Calmodulin, the ubiquitous and yet neglected calcium channel subunit, plays a central role in gene regulation in neurons (Bito et al., 1996; Dolmetsch et al., 2001). Calcium-calmodulin activation of CREB has been implicated in a wide array of neuronal functions, such as survival, neuronal morphology and synaptic plasticity (Lonze and Ginty, 2002). Calcium entering the cell through VGCC binds calmodulin and activates CaMKI, CaMKII and CaMKIV. Considering the differential effects of β isoforms on CaMKII function (see previous paragraph), it will be interesting to investigate β specificity in CaMKII mediated transcriptional signaling as well. CaV1-dependent calcium influx also activates the calcium/calmodulin dependent protein phosphatase calcineurin (CN). CN is anchored to the C-terminus of CaV1 channels by the scaffolds protein AKA79/150. In cultured hippocampal neurons, desphosphorylation of the transcription factor NFATγ4 by CN results in its migration of the nucleus, where it regulates transcription (Oliveria et al., 2007).

In addition to these classical calcium-dependent signaling pathways, VGCC can directly regulate transcription by the nuclear targeting of channel fragments or β subunits. The C-terminus of CaV1.2 was detected in the nuclei of neurons where it appears to regulate transcription. In the nucleus the CaV1.2 C-terminus binds to the transcriptional regulator p54 (nrb/NonO) and to the enhancer region of the connexin 3.1 gene (Gomez-Ospina et al., 2006). In cardiac myocytes, the distal C-terminus of CaV1.2 acts as a repressor of the CaV1.2 promoter, suggesting that CaV1.2 auto-regulates its own expression (Schroder et al., 2009). Furthermore, a C-terminal fragment of CaV2.1 has been found to translocate to the nuclei of cerebellar nuclei (Kordasiewicz et al., 2006). This nuclear-targeted C-terminus has been implicated in the cytotoxic effect of spinocerebellar ataxia type 6 CaV2.1 mutations which carry an expanded polyglutamine stretch in their C-terminus.

A similar channel-independent function in transcriptional regulation has been reported for auxiliary β subunits. The first β isoform found to translocate to the nucleus and regulate gene transcription was β4c, a truncated β4 variant isolated from chicken cochlea. In the nucleus, β4c interacts with the chromobox protein 2/heterochromatin protein 1 (CHC2/
HP1γ), decreasing its gene silencing activity and thus promoting gene expression (Hibino et al., 2003). The corresponding β4 isoform was subsequently isolated from human brain, where it is prominently expressed in vestibular neurons (Xu et al., 2011). Immunohistochemistry analysis of cerebellar granule and Purkinje cells of mice revealed β3 in the nucleus (Subramanyam et al., 2009). Of the β4 splice variants particularly β4b showed nuclear targeting when expressed in neurons, skeletal muscle or in HEK cells (Subramanyam et al., 2009; Etemad et al., 2014b). A chimeric approach revealed that an N-terminal double arginine motif confers the nuclear targeting property to β4b (Subramanyam et al., 2009). However, the mechanism of nuclear localization of β4b remains controversial: while our group demonstrated in multiple studies that the nuclear localization of β4b is negatively regulated by spontaneous electrical activity and calcium influx (Subramanyam et al., 2009; Etemad et al., 2014a; Etemad et al., 2014b), another group suggested that the accumulation of β4b in the nucleus is activated by membrane depolarization (Tadmouri et al., 2012). These authors also proposed a mechanism by which in response to VGCC activation β4b recruits the B56δ/PP2A complex and translocates to the nucleus. There it promotes histon modification and interacts with the transcription factor TRα, inhibiting the transcription of the tyrosine hydroxylase (TH) gene transcription. An Affymetrix GeneChip analysis on mRNA extracts from β4-null cerebellar granule cells reconstituted with three different β4 splice isoforms revealed that their differential ability for nuclear targeting correlates with their potential to regulate genes. Interestingly, CaV2.1, the partner of β3 in cerebellar synapses, was among the genes regulated by nuclear β4 splice variants (Etemad et al., 2014b). These findings suggested a feedback mechanism by which accumulation of β4b in the nuclei of inactive neurons represses expression of the CaV2.1 channel. Upon activation nuclear export of β4b relieves repression of CaV2.1 and is available for incorporation of new CaV2.1 channel complexes into synapses of cerebellar neurons (Etemad et al., 2014b).

A channel-independent role of β subunits in transcriptional regulation is not limited to β4 splice variants. Also the β3 subunit has also been identified as an interaction partner of a transcription factor. Upon co-expression with Pax6(S), β3 translocates into the nucleus of HEK cells and blocks the transcriptional activity of Pax6(S) (Zhang et al., 2010). Together these diverse functions of β subunits suggest that the high molecular diversity among the auxiliary subunits is not only important for organizing a multitude of up- and down-stream signaling pathways around the calcium channels, but also for calcium channel independent functions of auxiliary subunits.

Non-Canonical Auxiliary Calcium Channel Subunits

Calcium Sensor Proteins

In addition to the canonical CaV auxiliary subunits (β, α2δ, and γ), a number of proteins are part of calcium channel complexes and participate in functions like the regulation of channel properties or channel trafficking. Several of these non-canonical channel subunits are members of the superfamily of calcium sensor proteins (CaS). The prototypical calcium sensor protein calmodulin (CaM) governs calcium-dependent inactivation (CDI) and facilitation (CDF) of CaV1 and CaV2 channels. CaM is constitutively tethered to the IQ domain in the C-terminus of CaV1 and CaV2 channels (Halling et al., 2006). It contains two high affinity calcium binding domains (EF hands) in its N- and C-terminal lobes, and calcium binding to either one of the lobes causes conformational changes which influence CDI and CDF (Peterson et al., 1999; Van Petegem et al., 2005). Using CaM mutants impaired in calcium binding in either the N- or the C-lobe, it was demonstrated that the individual lobes control CDI and CDF in a CaV isoform-specific manner. In CaV1.2 the C-lobe governs CDI, while the N-lobe controls CDF. In CaV2 it is the opposite, with the N-lobe controlling CDI and the C-lobe CDF (DeMaria et al., 2001; Lee et al., 2003). In addition to controlling CDI and CDF, CaM has also been implicated in the trafficking of CaV1.2 channels in neurons (Wang et al., 2007). Therefore CaM has all the characteristics of an auxiliary subunit: it is constitutively associated to the channel, it modulates its gating properties, and it is involved in the membrane expression of CaV1 and CaV2 channels (Lee et al., 2002; Tippens and Lee, 2007). In CaV1 channels co-expression of another calcium-binding protein, CaBP1, causes suppression of CDI (Yang et al., 2006) by displacing CaM from the IQ domain and interacting with the cytoplasmic N-terminal domain of the channel (Zhou et al., 2004). In CaV2.1, CaBP1 binds to a site down-stream of the IQ domain and has opposite effects. It enhances the rate of inactivation and shifts the voltage-dependence of activation to more positive potentials, with a simultaneous loss of CDF (Lee et al., 2002). Visinin-like protein 2 (VILIP2), a neuronal calcium binding protein related to CaBP1, has the opposite effects. Upon co-expression with CaV2.1, VILIP2 slows down the rate of inactivation and enhances CDF (Lauter밀, 2005). Therefore differential expression of CaBP1 and VILIP2 at synapses yield opposite modulation of the CaV2 calcium currents in response to trains of action potentials, with CaBP1 decreasing the vesicle release and VILIP2 enhancing it.

Monomeric G-proteins

The most potent endogenous regulators of VGCCs are the members of the RGK family of Ras-related monomeric G-proteins. The RGK family was named after its four members (Rad, Rem1, Rem2 and Gem/Kir), each of which can powerfully inhibit CaV1 and CaV2 currents (Yang and Colecraft, 2013). Three distinct mechanisms for channel inhibition by RGK proteins have been described: (1) enhancement of dynamin-mediated endocytosis of channels, (2) reduction of the open probability (Po) without changes in voltage sensor movement, and (3) voltage-sensor immobilization (Yang and Colecraft, 2013). All RGK proteins contain a guanine nucleotide binding domain (G-domain), two regions that alter their conformation upon GTP/GDP exchange, and a C-terminus with basic and hydrophobic residues that targets RGK proteins to the plasma membrane. Notable, membrane targeting of the RGK proteins is necessary for VGCC current inhibition (Correll et al., 2008; Fan et al., 2010). All RGKs bind to all β isoforms at a region distinct from the α binding pocket (ABP), which binds to the AID in the α1 subunit. The affinity of the RGK/β association is an order of magnitude lower than the interaction between β and the AID (Fan et al., 2010; Xu et al., 2015). In addition to the binding sites on the β subunit, RGK-binding sites also have been suggested on the α1 subunits, particularly on the N-terminus (Yang and Colecraft, 2013). Using a mutated β subunit, which interacts with the α1 subunit but not with the RGK proteins, it was demonstrated that two of the three identified mechanisms of current inhibition by RGK, namely enhanced endocytosis and decreased Po, rely on β-binding of RGK proteins. In contrast, immobilization of the voltage-sensor by RGKs is independent of β-binding (Yang and Colecraft, 2013). However β subunits are absolutely required for RGK inhibition of CaV1 currents. Using a β subunit with a weakened affinity for the AID, which could be easily washed off from inside-out patches, it was demonstrated that in the absence of the β subunit Gem can still bind the channel, but not inhibit its current (Fan et al., 2010). The necessity of a β subunit in the VGCC complex for RGK inhibition is consistent with the notion that RGK have no effect on CaV3 channels that do not contain β. The requirement of a β subunit in the VGCC complex for RGK inhibition differs from that of CaM, inhibition
(Fan et al., 2010). Unlike G_{B_{2yb}} inhibition, RGK inhibition does not require a rigid α-helical structure of the I-II loop. The physiological relevance of RGK proteins remains elusive, although RGK modulation of CaV channels has been implicated in diverse processes such as cell morphogenesis, migration and apoptosis. Furthermore it has been suggested that Rad may shorten QT intervals and prevent arrhythmias in heart (Yada et al., 2007). In dorsal root ganglia CaV2.1 inhibition by RGK proteins may contribute to the homeostatic mechanisms, which promote plasticity and neuro-regeneration after injury (Scamps et al., 2015).

Dynamic Subunit Composition of VGCC Complexes

So far we have described how the great molecular diversity of calcium channels is important to determine which up-stream signaling proteins can associate with and modulate the calcium current and which down-stream signaling pathways will be initiated by the calcium influx through CaV channels. If the protein–protein interactions between the channel and its various subunits and regulatory proteins are stable, changes of these modulatory mechanisms or their down-stream signaling processes could only occur at the rate of protein turnover. Altering the composition of the channel complex would require endocytosis of the existing complex and synthesis and incorporation of a new complex with different subunit composition. On the other hand, if interactions within the channel complex are dynamic, the regulation of the channel properties and the down-stream effects could be quickly changed by dynamic exchange of channel subunits and/or regulatory proteins.

In native signaling complexes of cultured neurons and skeletal myotubes the pore-forming α1 subunits of VGCC are stably incorporated and turn over on a minute/hour-timescale (Di Biase et al., 2011; Campiglio et al., 2013). An earlier report of depolarization-induced turnover of CaV1.2 channels on a millisecond timescale could not be confirmed (Green et al., 2007). However, FRAP analysis and single particle tracking provided experimental evidence for the existence of a minor fraction of mobile CaV1.2 channels in neurons (in addition to the major fraction of stable channels), providing a limited capacity for short-term adaptation (Di Biase et al., 2011). This rapid modulatory capacity could be increased if the auxiliary subunits could dynamically interact with the pore-forming α1 subunit. This seems to be the case for specific β and α2δ subunits.

The β subunit interacts with the AID, a conserved 18 amino acid peptide on the I-II loop of α1 (Pragnell et al., 1994). All currently available studies measured an affinity of the AID/β interaction in the low nanomolar range, suggestive of stable AID/β association. This high affinity interaction was independent of the AID/β pairs and of the method employed (De Waard et al., 1995; Geib et al., 2002; Opatowsky et al., 2003; Van Petegem et al., 2008). However, in intact cells, several studies demonstrated competition for α1 or β binding by peptides or proteins, indicative of a reversible α1/β interaction. For example application of AID peptide on inside-out patches of HEK cells expressing CaV1.2/β2 subunit complexes resulted in a rapid decrease in the open probability, consistent with the dissociation of β2a from CaV1.2 and β2b binding to the AID peptide (Hohaus et al., 2000). In Xenopus oocytes expressing CaV2.3/β1δ channel complexes, injection of β2a caused a decrease in the inactivation rate of the calcium current, consistent with β1δ in the channel complex being replaced by β2a (Hidalgo et al., 2006). Finally, single channel recordings of HEK cells co-expressing CaV1.2 with different ratios of β1δ and β2a showed changes in the open probability, suggesting that distinct β subunits can sequentially associate with the same CaV1.2 channel on a minute time scale (Jangsangthong et al., 2011). In a recent study we analyzed the mobility and interactions of calcium channel subunits in the context of a functional signaling complex in differentiated myotubes (Campiglio et al., 2013). Using a FRAP approach we demonstrated that the skeletal muscle channel isoforms α1_{54} and β_{1b} form a stable complex in the triads. In contrast, non-skeletal muscle β subunits (β_{2a} and β_{2b}) dynamically interacted with L-type calcium channels. The observed dissociation properties of these β isoforms would enable rapid modulation of the channel by dynamic exchange of different β subunits. As of today experimental proof that channel modulation by dynamic exchange of β subunit occurs under physiological conditions is still missing. Also unresolved is the question as to how the necessary rapid change in the concentration of the β isoforms could be accomplished. However, many neurons simultaneously express multiple isoforms of auxiliary subunits, and in cerebellar neurons the activity-dependent export of nuclear β_{4} isoforms could rapidly change the relative availability of different β_{4} splice variants. Alternatively, a specific β isoform may become available after rapid post-translational modification, e.g. phosphorylation/ dephosphorylation, or after dissociation from a binding partner. A recent study suggests that BARP (β-anchoring and -regulatory protein), a transmembrane protein expressed in the brain and in pancreas, could act as such a donor of β subunits (Beguin et al., 2014).

The molecular determinants of the interaction between the α3δ and the α1 pore-forming subunit remain elusive. However it has been reported that the MIDAS motif in the VWA domain of α3δ-1 and α3δ-2 is essential for the increase in expression and the decrease of the turnover of CaV1 and CaV2 calcium channels in the plasma membrane (Canti et al., 2005; Hoppa et al., 2012). Although α3δ-1 was purified from the calcium channel complexes of skeletal muscle (Curtis and Catterall, 1984), mass spectrometry failed to detect α3δ as stable parts of CaV2 channel complexes in brain (Muller et al., 2010). According to this study CaV2 channels are part of macro-molecular signaling complexes in which they interact, directly or indirectly, with a selection of about 200 up- and down-stream signaling and scaffold proteins. Biochemically some of these interactions may be stronger than that between CaV2 and its α2δ subunit (Muller et al., 2010). Also, upon co-expression in muscle or nerve cells, colocalization of α3δ subunits and VGCCs is weaker than that of β subunits (Schredelseker et al., 2005; Obermair and Flucher, 2013).

Together these data suggest that α3δ subunits with VGCCs interact with a lower affinity than β subunits and that the interaction of α3δ with the channel complex may be reversible. The dynamic interaction of α3δ-1 with the DHPR in skeletal muscle might result in quick change of the current activation properties of the VGCC currents. siRNA-mediated depletion of α3δ-1 in skeletal myotubes resulted in an acceleration of CaV1.1 current activation (Obermair et al., 2005). A component of fast-activating currents was also observed in normal myotubes (Obermair et al., 2005; Obermair et al., 2008). Therefore dissociation and association of α3δ-β from the DHPR complex may occur in muscle cells under physiological conditions.

A dynamic interaction of auxiliary subunits with the calcium channels can result in differential modulation of the channel properties. In the case of β subunits, the β2 subunit confers to the channel slower inactivation kinetics compared to the other β isoforms (Olcese et al., 1994; Buraei and Yang, 2010). However in the case of calcium binding proteins the channel properties are modulated in opposite directions. For example in CaV1 channels displacement of CaM by CaBP1 causes a suppression of CDI. In CaV2.1 channels CaBP1 enhances the rate of inactivation and shifts the activation to more positive potentials, while VILIP2 slows down the inactivation and enhances CDF. Therefore CaV2.1 channels that exchange CaBP1 for VILIP2 will go from a low to a high conductance
regime and, thus, will increase the release probability of synaptic vesicles (Catterall, 2010).

Because the auxiliary subunits play an important role in organizing molecular machines and up- and down-stream signaling pathways their dynamic exchange might not only alter the channel properties, but also their association and functional interaction with any of these signaling pathways (Table 1). For example channels associated with $\beta_2$ will be protected from AA inhibition whereas channels associated with $\beta_3$, $\beta_4$, or $\alpha_2$ are not. CaM1.3 channels bound to $\beta_{1b}$, but not to $\beta_2$, will undergo CDF mediated by erbin. Or upon $\alpha$-adrenergic stimulation CaM1.2 channels bound to $\beta_{1a}$ will be more strongly modulated channels associated with $\beta_2$. On contrary, CaM1.3 channels bound to $\beta_2$ will be more strongly modulated compared to those associated with $\beta_3$ (Li and Tavalin, 2007). Or channels associated with $\beta_1$ and $\beta_2$ isoforms, but not those associated with $\beta_3$ and $\beta_4$, will undergo CDF mediated by CaMKII. Or presynaptic channels associated with $\beta_3$ and $\beta_4$, but not with $\beta_{4b}$, will bind synaptotagmin I and be located in proximity of the synaptic release machinery, whereas only channels associated to $\beta_3$ will be able to modulate transcriptional regulation. In addition to dynamic exchange between two isoforms of the same protein channel, channels devoid of any $\beta$ subunit lose the ability to undergo RGG and Gp$_1$-protein interaction, and, in the case of CaM2.2 channels, also to undergo PKC modulation.

Whereas the calcium channels are very stable in the plasma membrane, the auxiliary $\beta$ and $\alpha_\omega$ subunits can dynamically interact with the channel complex. This further increases the molecular diversity of VGCC at the membrane. Exchange of one auxiliary subunit isoform with another can result in differential modulation of the channel activity, in different protein–protein interactions, and in altered down-stream signaling.

Conclusion

Whereas expression studies in heterologous cells highlighted the effects of auxiliary subunits on channel functions, in their native context the auxiliary subunits appear to be involved in orchestrating macromolecular signaling complexes involved in a multitude of calcium-regulated cell functions. The great molecular diversity endowed to VGCC by the auxiliary channel subunits and other associated proteins enables them to specifically interact with different up-stream regulators and down-stream targets localized within the confinement of the calcium micro- or nanodomain. Whereas some auxiliary subunits are stably associated with a particular VGCC signaling complex, others are capable of dynamically exchanging with VGCCs. Whether such dynamic exchange of auxiliary subunits and interacting proteins indeed differentially regulates native cell functions remains to be demonstrated. New experimental approaches and sophisticated cell systems pave the way for experiment to address this pending problem in calcium channel research.

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