Cell. Mol. Life Sci.
DOI 10.1007/s00018-011-0843-y

Cellular and Molecular Life Sciences

REVIEW

Trafficking and stability of voltage-gated calcium channels

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Received: 16 July 2011 / Revised: 15 September 2011 / Accepted: 19 September 2011

Abstract Voltage-gated calcium channels are important mediators of calcium influx into electrically excitable cells. The amount of calcium entering through this family of channel proteins is not only determined by the functional properties of channels embedded in the plasma membrane but also by the numbers of channels that are expressed at the cell surface. The trafficking of channels is controlled by numerous processes, including co-assembly with ancillary calcium channel subunits, ubiquitin ligases, and interactions with other membrane proteins such as G protein coupled receptors. Here we provide an overview about the current state of knowledge of calcium channel trafficking to the cell membrane, and of the mechanisms regulating the stability and internalization of this important ion channel family.

Keywords Calcium channels · Beta subunit · Alpha2-delta subunit · Calmodulin · Ubiquitin · ER

Introduction

Voltage-gated calcium channels are one of the major sources of depolarization-evoked calcium entry into electrically excitable cells. The ensuing increase in intracellular calcium concentrations gives rise to a wide range of physiological responses, including calcium-dependent gene transcription, calcium release from internal stores, excitation contraction coupling, and secretion/neurotransmitter release [1–8]. Physiological roles of voltage-gated calcium channels are well summarized in other reviews [9]. On the other hand, excessive entry of calcium ions into the cytosol is toxic [10], and therefore calcium entry and handling must be under precise cellular control. With regard to calcium channels, this can be achieved in two principal manners: (1) regulation of calcium channel activity, and (2) the active number of channels that are embedded in the plasma membrane. While much attention has focused on the former mechanism, the molecular determinants that underlie calcium channel trafficking to and from the plasma membrane remain incompletely understood. That said, there have been a number of recent advances in our understanding of calcium channel trafficking and their targeting to specific subcellular loci, as well as mechanisms that determine the lifetime of channel complexes in the plasma membrane. Here we provide a detailed overview of this current state of knowledge.

Subtypes, structure, and subunit composition of voltage-gated calcium channels

Voltage-gated calcium channels are classified based on their biophysical properties, into high-voltage-activated (HVA) and low-voltage-activated (LVA) channels [11, 12]. The HVA channels include multiple subtypes, including N-, P/Q-, L-, and R-type channels that can be distinguished both by their gating characteristics and their pharmacological profiles [13]. They are multi-subunit complexes that contain a pore-forming Cavα1 subunit, plus ancillary Cavβ (hereafter referred to as the β subunit), Cav$\alpha 2\delta$ (hereafter referred to as $\alpha 2\delta$) and in some cases Cavγ subunits (hereafter referred to as the γ subunit), which co-assemble into a functional channel in a 1:1:1:1 stoichiometry [14–16] for review, see [13, 17] (Fig. 1). Furthermore, the calcium-

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Published online: 02 October 2011

binding protein calmodulin is now considered part of the HVA calcium channel complex [18]. The mammalian genome expresses seven different types of HVA Cavα1 subunits (falling into Cav1 and Cav2 families), four different types of β subunits, and four different types of $\alpha 2\delta$ subunits for review, see [13]. The α1 subunit is the major determinant of calcium channel subtype, such that L-type channels are encoded by one of four different Cavα1 subunits (Cav1.1, Cav1.2, Cav1.3, or Cav1.4), P/Q-type channels contain Cav2.1 subunits, N-type channels are defined by Cav2.2 and finally R-type channels contain Cav2.3 subunits [13]. The α1 subunit is comprised of four homologous transmembrane domains, each of which contains six transmembrane helices plus a re-entrant p-loop structure (Fig. 1a). The domains are flanked by intracellular N- and C-terminus regions, and connected by large cytoplasmic linkers. While the Cavα1 subunit contains all of the molecular machinery necessary to form a functional channel, including voltage sensors [19], inactivation gate [20], and selectivity filter [21], the coexpression of ancillary β and $\alpha 2\delta$ subunits regulates the basic functional properties of the channels [22, 23], altering their regulation by intracellular messengers [24, 25], and most importantly, greatly increasing cell surface density (for review see [26, 27]). The β subunit is a cytoplasmic protein that binds to a highly conserved region within the domain I–II linker region of HVA Cavα1 subunits, which has been termed the *A*lpha *I*nteraction *D*omain (AID) (Fig. 1b) [28, 29]. Crystal structure data have revealed that the β subunit contains SH3 and guanylate kinase (GK) domains that are highly conserved among the different β subunits [30–32]. Amino acid residues in the GK domain participate in the high-affinity binding of the AID region [31, 33, 34]. Among the four major types of β subunits, β2a is unique in that its N-terminal region contains two cysteine residues that are palmitoylated and thereby anchored to the plasma membrane [35]. Each of the four $\alpha 2\delta$ subunits are transcribed and translated from a single gene, post-translationally cleaved into α2 and δ fragments, which are then re-linked via disulfide bonds. For a review, see [36]. The δ subunit was originally thought to span the plasma membrane, but may in fact be linked to the extracellular leaflet of the membrane via a GPI anchor [37, 38], whereas the α2 subunit is purely extracellular. The physiological role of GPI-anchoring is still up for debate however, as $\alpha 2\delta$ subunits lacking a GPI-anchor sequence still target to lipid rafts with the addition of an inert transmembrane sequence [39]. Because most calcium channel subunits, including $\alpha 2\delta$, are subject to alternate splicing, a tremendous diversity of calcium channel complexes and phenotypes is possible [40–43]; for reviews see [44–46].

Mammals express three different types of LVA α1 subunits (Cav3.1, Cav3.2, and Cav3.3), all of which encode T-type calcium channels [47]. Unlike HVA channels, these subunits do not appear to co-assemble with ancillary subunits, and expression of just the Cav3 α1 subunit gives rise to large currents with typical T-type current waveforms [48]. Consistent with the absence of β subunit interactions, T-type channels lack the AID region in the domain I–II linker.

Cavβ subunit regulation of HVA channel membrane trafficking

The ability of β subunits to promote membrane expression of HVA calcium channels is well established. Early experiments revealed that antisense knockdown of β subunits reduced HVA plasma membrane expression in dorsal root ganglion neurons [49], and that whole cell currents carried by various HVA calcium channels in transient expression systems such as *Xenopus* oocytes and tsA-201 cells were dramatically reduced in the absence of co-expressed β subunits, albeit not completely eliminated [22, 50]. This β subunit-dependent increase in plasma membrane expression levels could be in principle due to a number of different mechanisms, including an upregulation of Cavα1 subunit transcription, enhanced export of the channels from the ER, or an increase in channel stability once the channels have reached the plasma membrane. Bichet et al. [51] showed that CD8 receptor fusion constructs of the Cav2.1 I–II linker region were retained in the ER unless coexpressed with a β subunit. Furthermore, deletion of part of the I–II loop in the full-length channel resulted in increased ER export in the absence of the β subunit. Based on these findings, the authors suggested that one of the major roles of the β subunit is to occlude an ER retention motif on the I–II loop of the channel. The ER retention mechanism is also supported by experiments showing that a single point mutation in the AID region can prevent the plasma membrane translocation of Cav1.2 and Cav2.2 channels even in the presence of β subunits [52, 53] and it is therefore unlikely that the β subunit acts as a transcription factor for Cavα1.

However, the molecular details by which β subunits promote ER export appear to vary with calcium channel subtype. Using an approach similar to that used by Bichet et al., our lab showed that the domain I–II linkers of Cav1.2 and Cav2.2 are not retained in the ER, but that instead the C-terminus region of these two channel subtypes may be responsible for the ER retention of N-type and L-type channels [54]. Because the β subunit does not bind to the C-terminus region of these channels, this suggests that the β subunit could perhaps indirectly (i.e., sterically or allosterically) mask these retention motifs when β is bound to the AID region. Such a mechanism was explored recently in an elegant study by Fang and Colecraft [55]

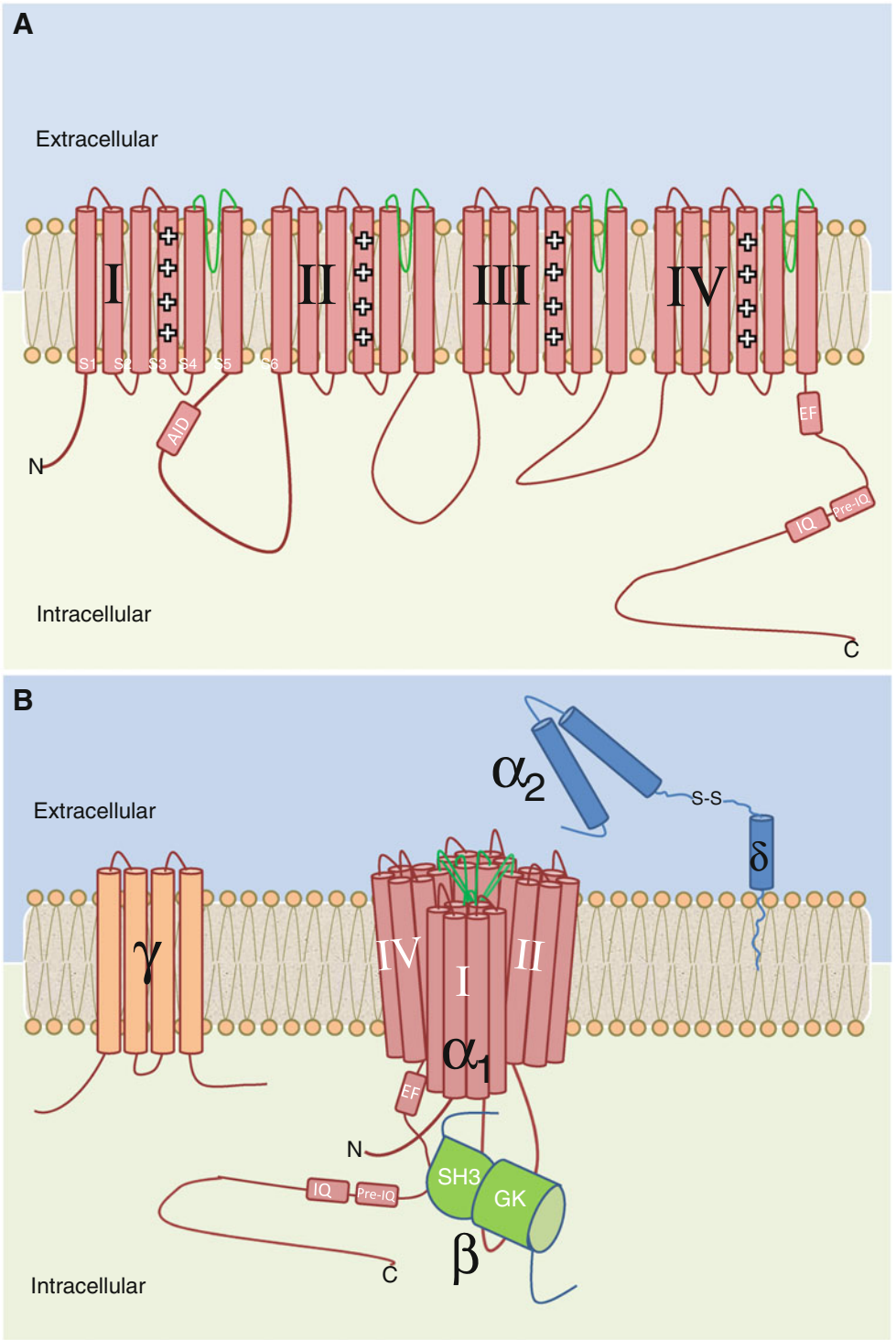

Fig. 1 Structure of Cavα1 and the auxiliary subunits of voltage-gated calcium channels. **a** Structure of the pore-forming Cavα1 subunit is composed of four transmembrane domains, each of which contains six membrane spanning segments. Segment four of each domain contains a positively charged voltage sensor, while calcium selectivity is provided by re-entrant pore loops (*green*) between segments five and six of each domain. The intracellular loops of HVA Cavα1 subunits contain sequences vital to trafficking and function, such as the AID (alpha-interaction-domain) in the I–II loop, as well as the EF-hand motif, pre-IQ and IQ domains in the C-terminus. **b** Structure of the assembled HVA calcium channel complex consists of 1:1:1:1 stoichiometry of the pore-forming Cavα1, cytoplasmic Cavβ, membrane anchored Cavα2δ, and in some instances, transmembrane Cavγ subunit. Note: the AID domain of Cavα1 is occluded by binding of Cavβ (via the Guanylate Kinase domain), and that the δ portion of the α2δ subunit is anchored to the cell membrane by a GPI anchor. The II–III and III–IV linkers have been omitted for clarity

who swapped intracellular regions between L-type and T-type calcium channels to examine structural determinants of L-type channel membrane expression. Based on the expression of these constructs in the absence and the presence of the β subunit, the authors concluded that the ancillary subunit promotes rearrangements of the major intracellular loops of the channel, which ultimately result in the obfuscation of multiple ER retention motifs contained within these linkers. Interestingly, the authors also showed that the domain I–II linker in fact contains an ER-export motif whose function is compromised by β subunit coexpression. While the net effect of β subunit coexpression is an overall enhancement of channel trafficking, this ER export motif may perhaps explain the ability of some

β-free channels to escape the ER. Altogether, it appears as if the β subunit is able to overcome intrinsic ER retention properties of at least three of the major HVA channel subtypes, albeit perhaps by channel subtype specific mechanisms.

What happens to channels that remain associated with the ER? We recently showed that Cav1.2 channels are tonically ubiquitinated, and that the degree of both mono and poly-ubiquitination is increased in the absence of the β subunit (Fig. 2) [54]. Furthermore, we were able to show that RFP2, an ER associated ubiquitin ligase, was responsible for this effect. We were then able to demonstrate that in the absence of the β subunit, an interaction occurs between the channel and p97/Derlin-1, two proteins associated with the ER Associated Protein Degradation (ERAD) system. The end result is channel retrotranslocation and proteasomal degradation in the cytosol [56, 57]. Consistent with this model, treatment of cells with the proteasomal inhibitor MG132 resulted in increased cell surface expression of Cav1.2 channels lacking the β subunit. The proteasomal inhibitor MG132 has also been reported to enhance trafficking of N-type calcium channels expressed in sympathetic neurons [58].

Altogether, these findings suggest that the β subunit acts as a switch that diverts channels away from a proteasomal degradation pathway to allow channel export to the plasma membrane via reduced ER retention.

Cav$\alpha 2\delta$ regulation of HVA channel membrane trafficking

Coexpression of HVA channels with $\alpha 2\delta$ also promotes the surface expression of channels, and this effect appears to be synergistic with that of the β subunit [59]. The $\alpha 2\delta$ subunit is of particular interest as it is the principal target for gabapentin and pregabalin [60, 61], which are widely prescribed analgesics for patients with chronic pain [62, 63]. Effects of $\alpha 2\delta$ coexpression on HVA channel density have been observed in various expression systems such as *Xenopus* oocytes, tsA-201 and Cos7 cells [59, 64, 65]. A role of $\alpha 2\delta$ in channel expression is also supported by experiments in *Ducky* mice—a mouse line in which the $\alpha 2\delta$-2 subunit carries a premature truncation mutation. These mice show severe ataxia and a dramatic reduction in Cav2.1 channel current densities in cerebellar Purkinje neurons [66]. On the other hand, the phenotypes of the

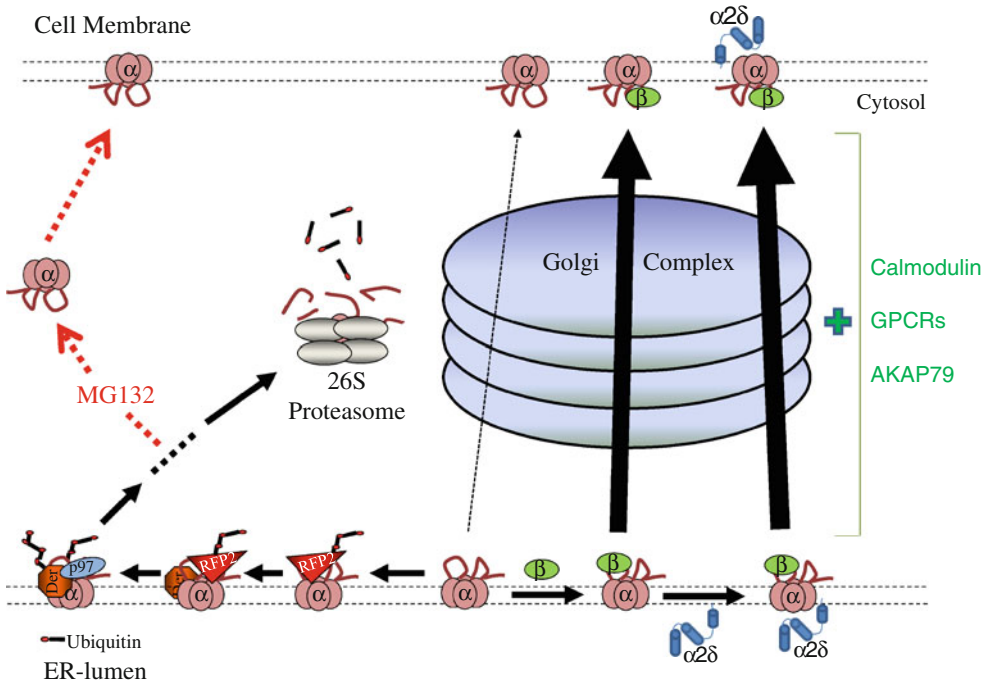

Fig. 2 Forward trafficking of Cav1 and Cav2 channel complexes. The Cav$\alpha 1$ subunit can traffic to the surface without auxiliary subunits, but β and $\alpha 2\delta$ subunits dramatically and additively increase the proportion of channels in the cell membrane. This increased surface expression is achieved by promoting ER export and increasing overall channel stability. Calmodulin, AKAP79, and GPCRs such as, nociceptin, D1 and D2 dopamine receptors can augment forward trafficking of calcium channel complexes. The β subunit protects Cav1.2 and Cav2.2 from ER associated proteasomal degradation (ERAD). The β subunit protects Cav1.2 from ERAD by interfering with the ubiquitin ligase activity of RFP2. RFP2 ubiquitination leads to Derlin and p97 association with the calcium channel complex and retrotranslocation of the poly-ubiquitinated channels. Poly-ubiquitinated channels in the cytosol move on to the 26S proteasome for degradation. Proteasomal inhibitors such as MG132 are able to rescue some of the retrotranslocated channels

recently published α2δ-1 and α2δ-3 knock-out mice are relatively mild, perhaps due to compensation by other α2δ isoforms [67, 68].

The α2δ subunit contains a metal ion-dependent adhesion site in a region that is homologous to a specific domain (i.e., A-domain) found in the glycoprotein "*von Willebrand factor*". This putative metal adhesion site is localized in the proximal third of the α2 portion of the subunit [65], and when this site was mutated, the α2δ subunit still trafficked to the cell surface, however, it lost the ability to mediate membrane targeting of Cav1.2, Cav2.1 and Cav2.2 subunits. The authors suggested that the Cavα1 subunit may act as a ligand for α2δ, and once the two partners are in a complex, a functional von Willebrand domain is needed to trigger ER export (Fig. 2).

As noted above, gabapentin serves as a potent analgesic, and these properties are lost in mice carrying a single point mutation in α2δ [69]. The mechanism by which gabapentin inhibits pain signalling is controversial. Although there are some reports of acute effects of gabapentin on channel function (for review, see [70]), recent evidence indicates an effect of gabapentin on N-type calcium channel trafficking. First, trafficking of transiently expressed Cavα2 channels appears to be impeded by gabapentin [71]. Second, the enhancement of N-type calcium channel surface expression associated with chronic pain states is abrogated by gabapentin treatment [72]. Finally, gabapentin has been shown to inhibit Rab11-dependent recycling of N-type calcium channels from the plasma membrane [73], suggesting that the α2δ subunit may under normal circumstances be involved in the regulation of calcium channel re-insertion into the cell membrane. Altogether, these observations fit with a mechanism by which gabapentin interferes with normal α2δ-mediated promotion of channel trafficking to the cell surface, akin to what is observed in von Willebrand factor mutants. It is, however, important to note that (with the exception of α2δ-2), the regulation of calcium channel membrane targeting by α2δ subunits has been examined primarily in expression systems, and it is possible that the in vivo trafficking function of α2δ in native cells may depend on calcium channel subtype and/or the cellular environment [74–76].

Cavγ subunits—regulators of function but not trafficking

It is well established that skeletal muscle L-type calcium channel complexes contain a γ subunit. It was subsequently shown that neurons express multiple homologues of skeletal muscle γ, most notably γ2, which is also known as "*stargazin*" [77]. The primary physiological function of *stargazin* is to mediate AMPA receptor trafficking to synaptic sites [78–80], while γ4 appears to be even more effective than *stargazin* at this function [81]. Furthermore, γ7 appears to be localized to signalling endosomes in neurons [82]. Hence, it may be reasonable to expect a similar trafficking function for neuronal calcium channels. However, most coexpression studies involving different types of γ subunits have revealed relatively subtle effects on channel function [77, 83–85]. One notable exception is γ7, which dramatically reduces N-type current density in transient expression systems [86]. The authors of this study showed that these effects were not mediated by alterations in channel trafficking, but instead appeared to occur as a result of altered channel expression. Along these lines, γ6 inhibits endogenous Cav3.1 T-type channels in an atrial cell line [87], however, once again, this effect does not appear to be mediated by alterations in channel trafficking, but instead by functional inhibition of channel activation [88, 89]. Altogether, it appears that γ subunits of neuronal voltage-gated calcium channels are not involved in membrane trafficking of either HVA or LVA calcium channels.

Calmodulin

Although calmodulin is a ubiquitous calcium sensing protein with numerous cellular functions, the fact that it is associated with every type of HVA calcium channel has led to suggestions this protein is a de facto calcium channel subunit. In the context of voltage-gated calcium channels calmodulin is recognized mainly for contributions to calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF) [90, 91]. These calcium/calmodulin-dependent processes regulate calcium channel gating and open probability so that channel flux is reduced (CDI), or increased (CDF) in response to changing intracellular calcium [92]. Calcium/calmodulin can bind HVA, but not LVA Cavα1 subunits at multiple C-terminal sites, although a region referred to as the IQ domain is absolutely necessary for binding of calcium free Apo-calmodulin and for promoting CDI [93–96]. All Cav1 and Cav2 family members bind calcium/calmodulin and exhibit CDI, with the exception of Cav1.4, which binds calcium/calmodulin but is not functionally regulated [91, 97–101]. This insensitivity to CDI has been explained by the presence of a C-terminal modulator region in Cav1.4 that acts as an inhibitory domain (termed ICDI) [99, 102, 103]. The ICDI region does not disrupt calmodulin binding to Cav1.4, and if anything, calmodulin bound Cavα1.4 channels display a greater current density than IQ domain mutants unable to associate with calmodulin [104]. This suggests calmodulin can either increase conductance of Cav1.4 when bound to the IQ motif, or help traffic Cavα1.4 to the cell surface in an IQ-dependent manner.

The contribution of calcium/calmodulin to trafficking of other HVA calcium channels is controversial. Data from HEK-293 cells suggest calmodulin has no additive effect on Cav1.2 surface expression in either the presence, or the absence of ancillary β and/or $\alpha 2\delta$ subunits [105]. On the other hand, it has been shown in tsA-201 cells, that deleting the PreIQ3 region—a second sequence important for calcium/calmodulin binding just upstream of the IQ domain—in combination with an IQ deletion, is sufficient to completely abolish surface expression of Cav1.2 in the presence of β2a [106]. In COS1 cells, which are believed to be devoid of all VGCC auxiliary subunits, exogenous calmodulin increased surface expression and recovered activity of $\alpha 2\delta$-free, and functionally silent Cav1.2/β2d channels [107]. Previous studies have shown that mutations in the EF-hand domain of Cav1.2 channels can drastically alter current density [2, 100, 108]. While it is possible that these mutations affect channel function rather than cell surface targeting [109, 110] it is probable that the lack of whole cell currents carried by some of these mutants arises from disruption of a calmodulin-dependent cell surface trafficking mechanism. In hippocampal neurons, Cav1.2 trafficking to the distal dendrites is accelerated by the presence of calcium/calmodulin, but not Apo-calmodulin [111]. The idea that calcium/calmodulin can influence the spatial and temporal distribution of channels, rather than just cell surface trafficking, is an intriguing possibility that requires further exploration.

As noted above, our laboratory has identified the C-terminal domains of Cav2.2 and Cav1.2 as putative ER retention regions [54]. The retained region of Cav2.2 contains an EF-hand motif, while the two retained portions of Cav1.2 contain the homologous EF-hand motif as well as a downstream IQ domain. Our observation that this downstream region of Cav1.2 spans the calmodulin-binding IQ domain supports literature that suggests this region of the channel has a role in surface expression [106]. Although it is not understood why the IQ containing region of Cav1.2 is retained in the ER while homologous regions of Cav2.1 and Cav2.2 are not, a possible explanation may lie in the different N and C lobe orientations of calmodulin when it is associated with each channel class [94]. Recent crystal structure data suggest that calcium/calmodulin exists in a 2:1 ratio with the Cav1.2 proximal C-terminus, a result exclusive to this channel [18, 112, 113], and perhaps in itself, can account for differences in ER retention among the channel subtypes. In addition to the C-terminal interaction sites, there is also an N-terminal calmodulin-binding domain that is present in Cav1.2, but which is absent from Cav2 channels [114, 115]. It is interesting to note in this context that Cav1.2 channels lacking the N-terminus appear to have a greater total abundance in COS1 cells [116]. It is thus possible that calmodulin-binding interactions with the N-terminus region contribute to modulation of L-type channel surface expression.

Although there is considerable evidence for functional regulation of HVA calcium channel activity by calcium/calmodulin, a role in regulating cell surface expression of calcium channels remains to be precisely elucidated.

T-type calcium channels

T-type calcium channels do not appear to associate with ancillary β and $\alpha 2\delta$ subunits, and pan-antisense depletion of β subunits from neuroblastoma cell lines does not alter T-type channel current densities [117], in contrast to HVA channels (see above). Yet, coexpression of Cav3 channels with β1b and/or $\alpha 2\delta$-1 subunits in transient expression systems such as *Xenopus* oocytes or tsA-201 cells, results in increased whole cell current densities accompanied by increases in both cell surface and total protein expression [118, 119]. For Cav3.2 channels (but not for Cav3.1 and Cav3.3), the relative fraction of channels in the plasma membrane was increased, hinting at the possibility of either augmented Cav3.2 channel trafficking, or increased stability of these channels in the membrane [118]. It is possible that the formation of endocytic vesicles is reduced when the plasma membrane is overloaded with GPI anchored $\alpha 2\delta$ subunits.

Unlike with HVA calcium channels, the intrinsic trafficking mechanisms of T-type channels have remained relatively unexplored. Some insights into structural determinants of T-type channel trafficking have however, come from mutations found in Cav3.2 channels of patients with various forms of idiopathic generalized epilepsies (for review see [120]). When introduced into full-length channels, the vast majority of these mutations mediate only subtle effects on channel gating [121–125], raising the possibility that their role in pathophysiology may be linked to altered channel trafficking rather than changes in channel function. Indeed, in tsA-201 cells several of these Cav3.2 epilepsy mutants have been shown to increase cell surface expression of the channel [126], consistent with a gain of function and (in a physiological setting) neuronal hyperexcitability. Several of these mutations were confined to the domain I–II linker, indicating that the domain I–II linker may be an important T-type channel trafficking hotspot. This fits with observations that a specific splice variant of Cav3.1, which lacks a large portion of the I–II loop is more effectively transported to the plasma membrane [127]. Deletion of the central portion of the I–II linkers of Cav3.1 and Cav3.3 has been shown to, respectively, increase, and decrease cell surface expression of these two channels, once again supporting the domain I–II linker as a region that is involved in channel trafficking [128]. At the same time, these findings indicate that the role of this region varies with

T-type channel isoform, and it should be noted that this region of the channel not only affects cell surface expression, but also channel gating (for review, see [129]). In summary, these findings indicate that the domain I–II loop is a regulator of T-type channel trafficking, but at this point it is unclear if this is due to enhanced ER export, or increased stability of the channels in the plasma membrane. Furthermore, it is possible that other regions of the channel may also be involved in the regulation of cell surface expression. Indeed, this may be of importance during pathophysiological states such as cardiac hypertrophy and seizure disorders where a switch of T-type domain III–IV linker channel splice isoforms has been shown to occur, although it remains to be determined if these variants alter channel trafficking in addition to documented effects on channel function [130, 131], for review see [132].

Two recent studies have identified the actin-binding protein Kelch-like 1 as a regulator of T-type channel expression [133, 134]). Coexpression of Cav3.1 and Cav3.2 T-type calcium channels with Kelch-like 1 was shown to enhance cell surface expression of these channels, in addition Kelch-like 1 could be co-immunoprecipitated with the channels [134]. While disruption of the actin cytoskeleton by itself did not affect T-type channel activity, the effects of Kelch-like 1 were lost under these circumstances, indicating that Kelch-like 1 regulates T-type channels in an actin F-dependent manner. The authors also showed that the mechanism of action involved an increased rate of re-insertion of T-type calcium channels from recycling endosomes [133]. It remains to be determined if Kelch-like 1 mediates its effect via binding to the domain I–II linker of the channel.

Trafficking of calcium channels to specific subcellular loci

Investigations of the subcellular distributions of voltage-gated calcium channels have primarily focused on neurons. Initial studies using channel subtype specific antibodies revealed that L-type calcium channels were expressed predominantly on cell bodies, whereas N-type and P/Q-type calcium channels were targeted to presynaptic sites [135–138]. While this general principle holds true, different types of calcium channel $\alpha 1$ subunits can be more broadly distributed depending on cell type. For example, in hippocampal neurons, Cav1.2 channels can be targeted to synaptic (both pre and post) and extrasynpatic compartments [139–141]. In cultured hippocampal neurons, Cav1.3 channels can be found at postsynaptic sites where they associate with the postsynaptic adaptor protein Shank [142]. In photoreceptors, Cav1.4 subunits are expressed at ribbon synapses where they are colocalized with bassoon [143]. The Cav1.3 isoform is also found at presynaptic sites in cochlear hair cells where it interacts with the protein Ribeye [144]. It is likely that interacting proteins such as Shank, Ribeye, and Bassoon are responsible for targeting the channels to these specific synaptic sites. Along these lines, N-type calcium channels are not just found presynaptically, as a dendritic co-localization of these channels with D1 dopamine receptors in prefrontal cortex neurons has been reported [145], with D1 receptors apparently defining the localization of the channels at these non-synaptic sites. A similar heterogeneity of calcium channel distribution has been reported for T-type channels, where different T-type calcium channel isoforms show distinct expression patterns depending on neuron subtype [146]. These issues are complicated further through alternate splicing of Cav$\alpha 1$ subunits, which can alter the subcellular targeting of a given calcium channel subtype, as illustrated for N-type calcium channels [147–149].

The mechanisms that define the subcellular trafficking of voltage-gated calcium channels are not well understood. Perhaps the most detailed investigations have focused on the synaptic targeting of N-type calcium channels. By using epitope tagged Cav2.2 constructs, as well as CD8 fusion constructs of intracellular Cav2.2 linkers, Maximov and Bezprozvanny showed that the C-terminus of the channel was a key structural region involved in the synaptic targeting of these channels, and that the formation of synaptic contact was a key requirement for channel trafficking [147]. The authors also showed that interactions of this region of the channel with Mint-1 and CASK were involved in synaptic targeting. Similar observations were obtained with an invertebrate homolog of Cav2 channels [150]. However, other regions of the channel also appear to be involved in synaptic targeting. Cav2.2 and Cav2.1 calcium channels contain a synaptic protein interaction (*synprint*) domain within the II–III linker region that interacts with synaptic proteins such as syntaxin-1, SNAP-25, and synaptotagmin 1 [151, 152]. These interactions may help couple channels to active release sites in addition to providing feedback regulation of channel activity [153–158]. Interestingly, transfer of the synprint region of Cav2.2 channels to Cav1.2 resulted in synaptic targeting of Cav1.2 [159], but see [160], suggesting that interactions of the channel with presynaptic proteins may act as a second synaptic targeting mechanism in addition to the CASK and Mint-1 interactions. Splice variants of N-type channels, which lack the synprint site have been identified in human brain [161]. When fused to green fluorescent protein and expressed in cultured hippocampal neurons, these splice variants were targeted to axonal sites, but failed to be effectively transported to presynaptic loci [160]. On the other hand, the aforementioned invertebrate Cav2.2 homolog lacks the synprint region altogether, yet targets to presynaptic sites [150]. Moreover, channels such as Cav1.4

and Cav1.3 do not contain a synprint motif, yet can be targeted to specialized synapses. These observations suggest that multiple regions and (perhaps redundant) protein interactions of the channel may be involved in defining its trafficking to synaptic sites. This issue is further complicated by the observation that different types of β subunits are targeted to distinct loci in autaptic hippocampal neurons. Specifically, β4 and β2a appear in clusters at synaptic sites, whereas other types of β subunits show a more diffuse distribution [162]. It is therefore possible that the association of channels with specific types of β subunits modulates trafficking contributions of the synprint and C-terminus regions.

Most of the targeting studies rely on overexpression of channel constructs in cultured neurons. The fact that presynaptic nerve terminals are relatively small and have a defined number of "slots" for N-type and P/Q-type calcium channels [163] must be taken into consideration when interpreting such overexpression studies. This issue was highlighted in a recent study by Cao and Tsien [164]. These authors showed that there are specific slots for N-type and P/Q-type channels in presynaptic terminal, and that N-type channels could displace P/Q-type channels from their slots, whereas P/Q-type channels could not be inserted in to N-type preferring slots. In practical terms, this means that the limited ability of synapses to accommodate overexpressed channels together with a channel preference of certain presynaptic sites could confound experimental results, under conditions of excessive overexpression of calcium channel subunits.

Calcium channel internalization and recycling

The total level of calcium channels protein in the plasma membrane is not only determined by export of channels and trafficking from the ER, but equally by the life time/stability of channels in the plasma membrane, and their recycling after internalization. Some hints at calcium channel stability come from antisense knockdown experiments in cultured cells and in vivo. For example, antisense depletion of β subunits in cultured dorsal root ganglion neurons produces maximal effects 4–5 days after injection of the oligonucleotides [49]. In vivo antisense knockdown of T-type or N-type calcium channels in dorsal root ganglion neurons produces physiological effect within days after intrathecal delivery [165, 166]. Altogether, this suggests that the stability of channel protein occurs on the order of tens of hours. This is also supported by kinetic studies investigating the rate of internalization of N-type channels containing Cav2.2 + β1b + $\alpha 2\delta$, or just Cav2.2 + β1b [167]. Notably, the presence of $\alpha 2\delta$ slowed the internalization of the complex, suggesting that this subunit contributes to membrane stability of the channel. At this point it is unclear whether the Cavα1 subunit contains specific sequence motifs that are responsible for its internalization, and precisely how this process may be regulated by ancillary subunits. For example, the SH3 domain of the β subunit is able to enhance dynamin-dependent internalization of Cav1.2 [168]. In tsA-201 cells this process appears to result from dimerization of SH3 domains on opposing β subunits [169] (Fig. 3). As for in-built Cavα1 internalization signals, insights can perhaps be gleaned from a specific C-terminus splice variant of Cav2.2, which appears to be more readily internalized due to the existence of a YXLL internalization motif contained within the alternately spliced region [170].

Like mechanisms of internalization, processes that govern calcium channel recycling are poorly understood. A recent set of studies has identified collapsin response mediator protein 2 (CRMP-2) as a possible regulator of N-type channel recycling [171] (Fig. 3). This protein (which is best known for its role in microtubule assembly) associates with the domain I–II linker and C-terminus region of the channel, and its coexpression augments N-type currents. Conversely, disrupting these interactions results in reduced N-type channel density in the plasma membrane, and thereby mediates analgesic effects in vivo [172]. CRMP-2 has been shown to provide a link between endocytic proteins and the dynein motor complex [173], while its interactions with the N-type channels may suggest a mechanism for selective N-type channel insertion into the plasma membrane. Considering that the β subunit binds to the I–II linker, this may also open the possibility of a β subunit-dependent regulation of the recycling process. To our knowledge, this has not been explored.

Internalization of N-type channels can be facilitated by their association with G protein coupled receptors (Fig. 3). Our laboratory has shown that Cavα2.2 calcium channels physically associate with nociceptin receptors, as well as with D1 and D2 dopamine receptors [145, 174, 175]. Coexpression of the channel with these receptors appears to increase trafficking of the channels to the plasma membrane, but more strikingly, prolonged application of receptor agonist results in the internalization of channel receptor complexes, and in the case of nociceptin receptors, into lysosomes for possible degradation [176].

L-type calcium channels have been shown to undergo depolarization-induced internalization, while KCl-induced depolarization of hippocampal neurons results in a calcium-dependent decrease in Cav1.2 cell surface expression [177]. This effect was mediated by a recruitment of the tumor-repressor protein eIF3e to the domain II–III linker region of the channel, followed by trafficking of the channel complex to an endosomal compartment (Fig. 3). This mechanism may on the one hand serve to protect neurons from calcium overload during periods of intense activity. It is also possible that some of the internalized

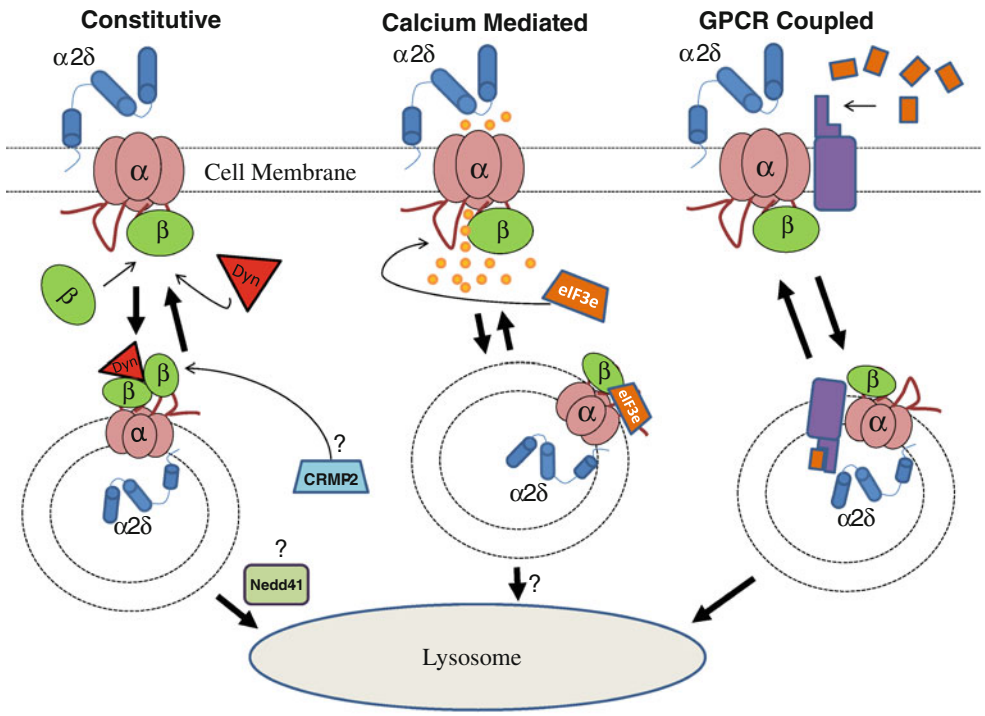

Fig. 3 Internalization and recycling of Cav1 and Cav2 channel complexes. Cav1 channels undergo constitutive internalization via a β subunit-dependent interaction with dynamin. Internalization is regulated by Cavβ dimerization, and involves the SH3 domain of the β subunit. The β subunit protects Cav1.2 from Nedd4-1-mediated lysosomal degradation; however, it is unclear when Nedd4-1-mediated degradation occurs. Nedd4-1 could promote lysosomal degradation following channel internalization, or alternatively, during forward trafficking, post-ER. A protein traditionally associated with microtubule assembly, CRMP-2, has been shown to associate with the I–II linker of Cav2.2 and augment channel surface density. CRMP-2 is thought to be involved in Cav2.2 recycling, although this has yet to be shown. Activity driven internalization by sustained calcium influx leads to the recruitment of eIF3e, and subsequent internalization of L-type channels. Cav2.1/2.3 channels can also associate with eIF3e suggesting this means of internalization may be used by multiple, but not all channel types. Whether eIF3e internalization leads directly to lysosomal degradation has yet to be determined. Cav2.2 channels are internalized by prolonged activation of GPCRs, such as nociceptin, D1, and D2 dopamine receptors. GPCR coupled internalization can lead to lysosomal degradation, or recycling of the channel complex to the cell surface

channels move into lysosomal compartments for degradation. Given that the isolated C-terminus of Cav1.2 may act as a transcription factor [178], this then may lead to the downstream activation of genes [179]. It should be noted, however, that depolarization-induced internalization was not observed in mature hippocampal neurons, suggesting the possibility that this process may be developmentally regulated [180].

In a related matter, it has been reported that prolonged activation of NMDA receptors via glutamate results in L-type channel internalization via a pathway controlled by the phosphoinositide kinase, PIKfyve [181]. Similar to the KCl-evoked internalization, such a mechanism may protect neurons from excessive calcium entry, but at this point it is not clear if this effect is modulated by the presence of ancillary calcium channel subunits. A possible role of β subunits in regulating channel stability may be supported by recent findings showing that the β subunit can protect Cav1.2 from proteasomal and lysosomal degradation by the ubiquitin ligase Nedd4-1, a process that occurs post-ER, and without detectable ubiquitination [182]. The Nedd4 family of ubiquitin ligases is known to modulate internalization of other ion channel proteins, and although this has not been directly tested for Cav1.2 it is a likely possibility [183–185]. Finally, a recent study reported that β4 subunits are targeted to the nucleus of cerebellar granule cells and Purkinje neurons [186]. The authors of this study used dysgenic myotubes to show that nuclear targeting was modulated by L-type channel activity, but unlike L-type channel internalization in hippocampal neurons, activity and calcium inhibited nuclear targeting of the β4 subunit. Nuclear targeting of β subunits, which is dependent on calcium channel activity may provide yet another pathway for L-type channel-mediated regulation of gene transcription.

Concluding remarks

The trafficking of voltage-gated calcium channels to and from the plasma membrane is an important means for regulating calcium entry into electrically excitable cells. In order to fulfill specialized cellular functions, different types of calcium channels are trafficked to particular subcellular loci where they can shape electrical activity, or provide local calcium signaling events, which are linked to specific physiological outputs such as neurotransmitter release. Trafficking of channels involves interactions with ancillary subunits and a host of other regulatory proteins that are localized in various intracellular and plasma membrane compartments. While we have highlighted many of the known mechanisms that control calcium channel trafficking, there are a number of additional interacting proteins, which are known to positively or negatively affect the membrane expression of channels, including A-Kinase Anchoring Proteins and small GTPases [187–189], and it is likely that many more will be discovered. This altogether underlines the complexity of calcium channel trafficking, and the difficulty in studying the underlying mechanism under physiological conditions. Detailed knowledge of these mechanisms can potentially identify novel agents that regulate cell surface density of calcium channels in neurons and ultimately give rise to therapeutic agents, as evidenced by the analgesic effects of CRMP-2 interfering peptides [172].

Acknowledgments Work from our laboratory that we discussed here was supported by the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council, and the Heart of Stroke Foundation of Alberta and the Northwest Territories. BAS is supported by a studentship from Alberta Innovates-Health Solutions (AI-HS). GWZ is an AI-HS Scientist and a Canada Research Chair.

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