G protein inhibition of Ca\textsubscript{v}2 calcium channels

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Key words: G protein, N-type, P/Q-type, Ca\textsubscript{v}2, calcium channel, inhibition

Voltage-gated Ca\textsuperscript{2+} channels translate the electrical inputs of excitable cells into biochemical outputs by controlling influx of the ubiquitous second messenger Ca\textsuperscript{2+}. As such the channels play pivotal roles in many cellular functions including the triggering of neurotransmitter and hormone release by Ca\textsubscript{v}2.1 (P/Q-type) and Ca\textsubscript{v}2.2 (N-type) channels. It is well established that G protein coupled receptors (GPCRs) orchestrate precise regulation neurotransmitter and hormone release through inhibition of Ca\textsubscript{v}2 channels. Although the GPCRs recruit a number of different pathways, perhaps the most prominent, and certainly most studied among these is the so-called voltage-dependent inhibition mediated by direct binding of G\beta\gamma to the \(\alpha\) subunit of Ca\textsubscript{v}2 channels. This article will review the basics of Ca\textsuperscript{2+}-channels and G protein signaling, and the functional impact of this now classical inhibitory mechanism on channel function. It will also provide an update on more recent developments in the field, both related to functional effects and crosstalk with other signaling pathways, and advances made toward understanding the molecular interactions that underlie binding of G\beta\gamma to the channel and the voltage-dependence that is a signature characteristic of this mechanism.

Voltage-gated Ca\textsuperscript{2+} Channels

Voltage-gated calcium channels (Ca\textsuperscript{2+}-channels) play pivotal roles in a diverse array of cellular functions, in large part by translating the electrical inputs of excitable cells into biochemical outputs due to influx of the ubiquitous second messenger Ca\textsuperscript{2+}. Calcium triggers neurotransmitter and hormone release, muscle contraction, modulates ion channels to control excitability, the activity of many enzymes, gene expression, cellular differentiation and motility. Clearly there must be precise spatiotemporal regulation of calcium signaling in order to manifest these many downstream events in a controlled manner. In part this is accomplished by the rich functional diversity of Ca\textsuperscript{2+} channels mediated by distinct subunit combinations and complex modulation by a variety of second messengers and protein-protein interactions.

Initially, biophysical differences suggested the existence of multiple channel subtypes: low voltage-activated (LVA) channels require only modest membrane depolarization to open whereas high voltage-activated (HVA) channels require stronger depolarization. Differences in channel kinetics, and pharmacological sensitivity led to further distinction within the HVA class of channels: L-type channels were blocked by dihydropyridines, N-type channels blocked by \(\omega\)-conotoxin GVIA, P/Q-type channels by \(\omega\)-agatoxin IV A and R-type channels were resistant to these previously mentioned blockers but sensitive to SNX-482 (reviews of the history of Ca\textsuperscript{2+} channels\textsuperscript{3,4}). To date, ten different mammalian genes that encode the pore forming \(\alpha\) subunit of Ca\textsubscript{v}2 channels have been identified. Based on sequence homology these are grouped into three major families (reviewed in refs. 4 and 5): four Ca\textsubscript{v}1 members (Ca\textsubscript{v}1.1–Ca\textsubscript{v}1.4; all L-type channels), three Ca\textsubscript{v}2 members (Ca\textsubscript{v}2.1, P/Q-type; Ca\textsubscript{v}2.2, N-type; Ca\textsubscript{v}2.3, R-type channels) and three Ca\textsubscript{v}3 members (Ca\textsubscript{v}3.1–Ca\textsubscript{v}3.3, all T-type channels). The functional diversity of \(\alpha\) subunits is even greater due to splice variants that can alter the biophysical properties, change the context and impact of other mutations, or even introduce novel regulatory sites.\textsuperscript{6–9}

The \(\alpha\)-subunit consists of four homologous repeats (domains I–IV) each with six transmembrane spanning \(\alpha\)-helical segments (S1–S6) and a ‘P-loop’ between S5 and S6 (Fig. 1). Each domain is reminiscent of a voltage-gated K\textsuperscript{+} channel subunit and it is thought that the channel adopts a similar tetrameric architecture with the S5–S6 and P-loop segments forming the pore domain and the S1–S4 segments (in particular the S4 helix that has multiple charged residues) forming the voltage-sensor domain. The intracellular N- and C-termini and the cytoplasmic loops connecting domains I–IV are all important for interaction with other proteins including the auxiliary \(\beta\) subunit of the channel, SNARE proteins (syntxin, SNAP25), synaptotagmin, calmodulin and related Ca\textsuperscript{2+}-binding proteins, various adapter proteins and kinases, and of note for this review, G protein \(\beta\)\(\gamma\) heterodimers (G\(\beta\)\(\gamma\)) (Fig. 1).

High voltage-activated channels (Ca\textsubscript{v}1.1 and Ca\textsubscript{v}2.2) are hetero-oligomeric protein complexes and additionally contain a cytoplasmic \(\beta\) subunit (Ca\textsubscript{v}1.3) and a membrane associated \(\alpha\)\(\delta\) subunit\textsuperscript{5} (Fig. 1). Four genes (each with multiple splice variants) encode Ca\textsubscript{v}3 subunits (\(\beta\) through \(\delta\)), and four genes encode \(\alpha\)\(\delta\) subunits (\(\alpha\)\(\delta\)-1 through \(\alpha\)\(\delta\)-4). Both the \(\alpha\)\(\delta\) and \(\beta\) subunits increase trafficking/plasma membrane expression of the \(\alpha\) subunit and modulate channel kinetics. The \(\alpha\)\(\delta\) subunit is the product of a single gene that undergoes posttranslational cleavage and subsequent formation of a disulphide bond between the \(\alpha\) and \(\delta\) components. The \(\alpha\) component is extracellular and until recently it was considered that \(\delta\) traversed the membrane giving rise to a type 1 transmembrane topology.\textsuperscript{10,11} However, in contrast to this assumption, recent evidence suggests that \(\alpha\)\(\delta\) is glycerophosphatidylinositol (GPI)-anchored, and does not span the plasma membrane.\textsuperscript{12,13} Gabapentin and pregabalin, two
recently evidence suggests this CaV overlaps with one of the binding sites for Gβγ channels, in particular CaV2.1 (P/Q-type) and CaV2.2 (N-type) effects. CaV channels (discussed further below). Skeletal muscle (CaV1.1) imparting voltage-dependence to Gβγ channels, in particular CaV2.1 (P/Q-type) and CaV2.2 (N-type) effects. CaV light onto the structural elements that mediate these functional effects on channel trafficking and gating and particularly since the crystal structures of several subtypes were reported and shed light onto the structural elements that mediate these functional effects. CaVβ is modular in nature with src-homology-3 (SH3) and guanylate kinase-like (GK) domains, placing it in the larger family of MAGUK proteins (reviewed in refs. 18–20). The GK domain binds to the so-called α-interaction domain (AID) on the cytoplasmic I-II linker (Fig. 1). Notably, the AID partially overlaps with one of the binding sites for Gβγ heterodimers, and recent evidence suggests this CaVβ-AID interaction is critical for imparting voltage-dependence to Gβγ-mediated inhibition of CaV2 channels (discussed further below). Skeletal muscle (CaV1.1) channels also contain a γ-subunit but it remains unclear if there are direct interactions of γ with other CaV channel complexes.

The remainder of this review will focus on the CaV2 family of channels, in particular CaV2.1 (P/Q-type) and CaV2.2 (N-type) that are widely expressed in the mammalian nervous system and trigger neurotransmitter release at most synapses and also some neuroendocrine cells.

G Protein Coupled Receptors and Heterotrimeric G Proteins

Almost 800 human genes encode G protein coupled receptors (GPCRs) and over 30% of drugs on the market today target GPCRs. GPCRs are characterized by seven transmembrane spanning alpha helices with extracellular N- and intracellular C-termini. The receptors couple to heterotrimeric G proteins which act as molecular switches to transduce extracellular ligand binding into intracellular signaling cascades. In human, sixteen genes encode Gα subunits, five genes encode Gβ subunits and twelve genes encode Gγ subunits (reviewed in refs. 23–26). In addition to transducin (Gαt) in the retina, there are four major families of Gα, and GPCRs are generally characterized according to the family of Gα subunit to which they couple: Gα coupled receptors typically activate adenyl cyclase to elevate cAMP; Gβ family receptors inhibit cAMP production, Gα family receptors activate phospholipase C signaling, and Gβγ receptor signaling is less well defined. Agonist binding to the GPCR catalyzes the exchange of GDP to GTP on the G protein α-subunit (Gα) causing conformational changes/dissociation of the Gα and Gβγ heterodimer. Subsequently, both the liberated Gα and Gβγ signal to multiple downstream effectors, including voltage-gated Ca2+-channels (see below). Intrinsic GTPase activity of the Gα subunit and subsequent reassociation of the Gα-GDP subunit with the βγ heterodimer terminates signaling. The GTPase activity can be accelerated by regulator of G protein signaling (RGS) proteins which consequently can influence the extent and duration of downstream events. Conversely, activator of G protein signaling (AGS) proteins can stimulate nucleotide exchange and lead to signaling independent of GPCR activation. Termination of signaling in the continued presence of agonist can also occur due to receptor desensitization. Desensitization is complex and for some receptors (e.g., the μ-opioid receptor) can be mediated by distinct signaling pathways depending upon the identity of the bound agonist. Phosphorylation by PKA, PKC or G protein coupled receptor kinases (GRKs) is involved and can result in uncoupling of the receptor from the downstream G proteins. Endocytic removal of the GPCR from the plasma membrane can also occur: GRKs, recruited by Gβγ, phosphorylate the C-terminus of the GPCR leading to recruitment of arrestins and subsequently the

Figure 1. Schematic representation of voltage–gated Ca2+ channel topology and subunit composition. (A) Topology of the pore forming α1 subunit. The four homologous repeats (domain I through domain IV) each with six transmembrane spanning α-helices (S1–S6) (blue or orange cylinders) and a P-loop between S5 and S6. The pore domain (orange) comprises the S5-S6 and P-loop segments, and the voltage-sensor domain (blue) consists of the S1–S4 segments (in particular S4 that has multiple charged residues). The intracellular N- and C-termini and the cytoplasmic loops connecting domains I–IV are all important for interaction with other proteins including the auxiliary β subunit of the channel that binds to the AID on the I-II linker, synaptic proteins that interact at the “synprint site”, and G protein βγ heterodimers (Gβγ) that interact at three sites on the N-terminus, I-II linker and C-terminus (see text for more details). (B) Representation showing the 3D topology with the intracellular β subunit that interacts through its GK domain with the α1 subunit. The α1 subunit is largely extracellular and likely GPI-anchored to the plasma membrane.
endocytic machinery. All of these mechanisms terminate G protein signaling and thus channel modulation, but in some cases, direct GPCR-Ca,2 interactions have been demonstrated that result in endocytic removal of the channel from the plasma membrane upon GPCR desensitization.

**Inhibition of Ca\(^{2+}\) Channels by G Protein βγ Subunits—Functional Effects**

Since the first demonstration that norepinephrine could reduce action potential duration and Ca\(^{2+}\) current amplitude in chick sensory neurons, it has become clear that inhibition of Ca\(^{2+}\) channels by GPCRs is widespread and important for controlling neurotransmitter and hormone release (reviewed in refs. 38–42). The diversity and complexity of GPCR/G protein signaling is huge and several distinct pathways converge on Ca\(^{2+}\) channels. Perhaps the most prominent, and certainly most studied among these is the so-called voltage-dependent inhibition mediated by direct binding of G\(βγ\) to the α subunit of Ca,2 channels. Voltage-independent inhibition is generally less well characterized and likely comprised of several distinct mechanisms including channel trafficking, phosphorylation and lipid signaling pathways (reviewed in refs. 34, 35, 40, 43–48). The primary focus of this review is the direct, voltage-dependent inhibition of Ca,2 channels.

Direct inhibition by GPCRs predominantly targets Ca,2.2 (N-type) and Ca,2.1 (P/Q-type) channels, although Ca,2.3 channels are also inhibited by similar mechanisms. There are several characteristic features of the inhibition that have given rise to the voltage-dependent moniker: the peak amplitude of the whole-cell Ca\(^{2+}\)-channel current \(I_{Ca}\) is reduced but this inhibition is diminished at depolarized membrane potentials; activation kinetics are slowed; the voltage-dependence of activation is shifted to more depolarized potentials; a conditioning prepulse to depolarized potentials can relieve most of the inhibition and normalize channel kinetics (termed prepulse relief or prepulse facilitation). These characteristics of the inhibition have been incorporated into models in which the channels exhibit two functional gating states, “willing” and “reluctant.” Perhaps the most prominent, and certainly most studied among these is the so-called voltage-dependent inhibition mediated by direct binding of G\(βγ\) to the α subunit of Ca,2 channels. Voltage-independent inhibition is generally less well characterized and likely comprised of several distinct mechanisms including channel trafficking, phosphorylation and lipid signaling pathways (reviewed in refs. 34, 35, 40, 43–48). The primary focus of this review is the direct, voltage-dependent inhibition of Ca,2 channels.

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**Figure 2.** Functional effects of voltage-dependent inhibition. (A) Current records from an adrenal chromaffin cell in the absence (black traces) and presence (red traces) of extracellular ATP. Chromaffin cells express P2Y receptors that are activated by ATP co-released with catecholamines. Hallmark features of voltage-dependent inhibition are shown and include reduced peak amplitude with prominent slowing of the activation kinetics (left pair of current traces). A conditioning prepulse to +100 mV applied just before the test pulse to +10 mV reversed most of the inhibition and normalized the activation kinetics of \(I_{Ca}\), but had no effect under control conditions (black trace) (right pair of current traces). (B) Voltage-dependent relief of inhibition (facilitation) can also occur during stimuli that mimic brief trains of action potentials. The example is taken from an adrenal chromaffin cell stimulated with a 45 Hz train of ten action potential-like stimuli (from -60 mV to +35 mV) in the presence of ATP to produce inhibition (red) or in control conditions (black traces). Currents were normalized to the first pulse of the train and mean data is shown on left. In control conditions there was a small decrease in current amplitude (probably due to inactivation), but in the presence of ATP there was a robust increase in current, reflecting partial reversal of the G protein mediated inhibition.

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In turn this might contribute to short term synaptic plasticity, at least at some synapses.

The general consensus is that voltage-dependent relief of the inhibition reflects a shift of the channels to the “willing” state due to transient dissociation of G\(βγ\) (Fig. 3). Perhaps the most convincing evidence for this comes from kinetic analyses of prepulse relief as a function of agonist or G\(βγ\) concentration. As the concentration of G\(βγ\) was increased the rate of relief following the prepulse was not altered but the rate of re-inhibition following the prepulse was accelerated. This is the predicted
Shocks in activation gating are the dominant features of the “willing”-“reluctant” models and additional evidence for this comes from recordings of “gating currents” that reflect movement of the voltage-sensor domain of the channels in response to membrane potential changes. In recombinant Ca2,2,2 channels expressed in HEK cells these gating currents can be recorded in isolation as the cells lack endogenous voltage-gated channels. G proteins were found to reduce the amplitude and shift the voltage-dependence of gating currents to more depolarized potentials.73 G proteins also produced a significant separation in the voltage-dependent activation of gating current and ionic current.73 These data are consistent with the idea that Gβγ binding slows movement of the voltage-sensor and uncouples or disrupts this movement from opening of the activation gate. More recently, gating current modulation by G proteins has also been reported in rat sympathetic neurons.74,75

Gβγ can also reduce inactivation of Ca2,2,2 channels,76-77 although this is somewhat more subtle and can be masked by concomitant voltage-dependent reversal of Gβγ-mediated inhibition (Gβγ unbinding). Inactivation of Ca2+ channels is complex and mediated by several voltage-dependent and Ca2+-dependent mechanisms.78-80 The precise molecular correlates of inactivation remain somewhat unclear, but fast voltage-dependent inactivation might involve a “hinged lid” type mechanism, with the intracellular loop connecting domains I and II of the α-subunit serving the role of the “inactivation gate” (reviewed in ref. 79, 81 and 82). The I-II loop is also an important binding site for Gβγ on the channel83-86 (Fig. 1) (see below for more discussion). It is conceivable that binding of Gβγ disrupts movement of this putative inactivation gate, or its interaction with other channel domains, but this remains to be determined. It has also been proposed that Ca2,2,2 channels inactivate preferentially from intermediate closed state(s) favored during trains of brief depolarizing pulses.81 If Gβγ were to reduce the probability that the channels populate this state (from which inactivation is preferred) it might reduce the cumulative inactivation throughout a stimulus train.

Ca2+-dependent inactivation is mediated by calmodulin bound to the C-terminus of the channel α subunit.87,91 It can be blocked in Ca2,2,2 channels, but not Ca1,1 channels, by strong intracellular Ca2+ buffering (EGTA or BAPTA in the patch pipette solution) indicating that it is mediated by a “global” elevation rather than a “local” microdomain of Ca2+. As such, the reduction of Ca2+-dependent inactivation by Gβγ76 might result from fewer channels opening and a diminished “global” Ca2+ signal. Consistent with this, blocking ~50% of Ica by ω-conotoxin GVIA (i.e., by decreasing global Ca2+ entry) reduced calcium dependent inactivation, but a similar block of Ica by Cd did not reduce inactivation.76 This highlights the complexity of Ca2+-dependent regulation of Ca2,2,2 channels and perhaps hints that the effects of Gβγ involve more than simply reducing global Ca2+ entry. A recent study proposed that allosteric hindrance of channel activation underlies Ca2+-dependent inactivation of Ca1,2,3,81 although it is not known if this holds true for Ca2,2 channels. Calmodulin might also facilitate interaction between the N and C terminal tails of Ca2+ channels,82-84 and Ca4,β binding to I-II linker appears to play a role in Ca2+-dependent inactivation.82 As

outcome for voltage-dependent dissociation and rebinding of Gβγ. Furthermore, the kinetics of reinnibition were consistent with binding and unbinding of a single Gβγ dimer.68

Single channel studies provided early evidence that the inhibition was “direct” and “membrane delimited”—in other words it did not involve a diffusible second messenger. In cell attached single channel recordings, bath application of agonist did not inhibit the channels whereas agonist in the patch pipette did.66,69,70 “Reluctant gating” has also been demonstrated in single channel recordings. Upon membrane depolarization, the delay before the channel first opens (latency) was increased during inhibition (i.e., the channels were “reluctant” to open), but there was little impact on other single channel parameters.51,71 Thus, the inhibited channels appeared essentially silenced, unable to open until Gβγ dissociated and the channels shifted to the “willing” state. However, two other studies reported that very brief channel openings from the “reluctant” state can occur in N-type channels (i.e., without Gβγ unbinding), although the probability of such events was low.52,53 In contrast, no such “reluctant openings” were observed for single P/Q-type channels. Overall, the dominant effects observed in all studies is the shift in activation and prolonged latency to first channel opening. The slow activation kinetics seen in whole cell recording (Fig. 2A) and delayed latency in single channel recordings reflect the conformational changes and subsequent dissociation of Gβγ from the channel upon membrane depolarization. Another functional manifestation of this voltage-dependence is that rapid application of neurotransmitter during a depolarizing voltage-step produced little inhibition of whole cell Ica,72 presumably because binding of Gβγ is not favored by the conformational changes that accompany channel opening.

Figure 3. Voltage-dependent relief reflects transient dissociation of Gβγ from the channel at depolarized membrane potentials. Recombinant Ca2,2,2 channels expressed with β,δ and α in HEK293 cells. Gβγ was also co-expressed and produced tonic inhibition of Ica that could be reversed by a step to +100 mV (lower panel shows a series of superimposed currents. The interval between the conditioning prepulse to +100 mV and the following test pulse to +10 mV was increased from 10 ms to 200 ms. With longer intervals the increase in current amplitude becomes less as Gβγ rebinds to the channel. The blue line shows an exponential fit to the peak current amplitudes.
discussed below, these regions of the channel are also intimately involved in voltage-dependent, Gβγ-mediated inhibition of CaV2 channels. Direct in vitro binding of Ca2+-calmodulin to Gβγ heterodimers has also been demonstrated.95 At present this is merely a list of coincident observations, but it is interesting to speculate that there might be crosstalk between Gβγ-mediated and calmodulin-mediated regulation of CaV2 channels.

**Differential Voltage-Dependent Inhibition within the CaV2 Family**

Initially it was thought that CaV2.3 channels were not inhibited, but this depends on truncation of the α1 subunit N-terminus, whereas a splice variant with a longer N-terminus can be inhibited.96-98 However, the majority of studies have focused on N- and P/Q-type channels in part because these are prominently inhibited and in part because they underlie the Ca2+ entry that triggers neurotransmitter release at most synapses and some neuroendocrine cells. Although very similar, there are subtle differences in the inhibition of CaV2.1 and CaV2.2 channels that might have important physiological consequences. The relative expression levels of the two types of channels differ between neurons and even between neighboring synapses arising from the same neuron. Thus, differential modulation of the two channels could lead to cell and/or synapse specific alterations in neuromodulation by GPCRs.

As already mentioned, differences in the inhibition of these channels are apparent at the single channel level: very brief duration, low probability “reluctant openings” have been observed in CaV2.2 but not CaV2.1 channels.52-55 In whole cell recordings the peak amplitude of \( I_{\text{Ca}} \), is reduced substantially more for CaV2.2 than CaV2.1 \( I_{\text{Ca}} \).99,100 Moreover, trains of action potential-like stimuli relieve a greater proportion of CaV2.1 inhibition than CaV2.2 inhibition.102 These differences likely reflect the affinity of Gβγ binding to the channels. Indeed the apparent affinity of Gβγ (inferred from prepulse relief and reinforcement) is quite similar for the two channels at hyperpolarized or very depolarized potentials, but diverges at moderately depolarized levels (<+30 mV) within the physiologically relevant range of action potentials.52 Other evidence also suggests subtle differences in the binding of Gβγ heterodimers to CaV2.1 and CaV2.2 channels. For example, the rank order of inhibition produced by the five different Gβ subunits (Gβ₁,β₃), all paired with the same Gγ₁ subunit, differs for CaV2.1 and CaV2.2 channels.103 In another study, point mutations on the Gβ₁ subunit had distinct effects on the inhibition of CaV2.1 and CaV2.2 channels.104 Functional differences might also arise from variable interactions or crosstalk with other signaling pathways. It has been shown that activation of PKC can reduce voltage-dependent inhibition of CaV2.2 channels (N-type) \( I_{\text{Ca}} \).105-107 Phosphorylation of Thr⁴³² located in the I-II linker region of the rat α₁ subunit underlies this effect and is close to one of the Gβγ binding sites.108,109 Of note, phosphorylation of Thr⁴³² only disrupts inhibition mediated by Gβ₁, but not other Gβ subunits.110 Thus the nature of the Gβγ heterodimer as well as the channel subunits will shape the functional impact of inhibition and its modulation by PKC. There are several other phosphorylation sites on CaV2.2 that might also contribute to channel regulation111 and there are also phosphorylation sites on CaVβ,20 although it is not known if these influence Gβγ-mediated inhibition. In some cases, the antagonistic effects of PKC might be mediated indirectly through phosphorylation of the GPCR.112

Three distinct regions of CaV2.2 α₁ subunits seem to participate in binding and/or transduction of the inhibition by Gβγ: the cytoplasmic linker between domains I and II (I-II linker), the N-terminus and the C-terminus (Fig. 1). Given that voltage-dependent inhibition appears to involve a single Gβγ, these regions may contribute to a multi-site binding pocket.85 The C-terminus was identified as being essential for modulation of CaV2.3,113 but other studies suggest it plays more of a modulatory role in CaV2.2 channels, perhaps by increasing the affinity of binding and hence responses to low concentrations of free Gβγ.100,114,115 A variety of other signaling proteins bind to the C-terminus including calmodulin, CaMII, PKC and Gα subunits.81,116 This might facilitate crosstalk with other signaling pathways to fine tune Gβγ-mediated inhibition, as suggested for binding of Gqα subunits and perhaps PKC.107,108 Splice variants of the CaV2.2 C-terminus have also been demonstrated to introduce a novel regulatory site for tyrosine kinases that underlies one form of voltage-independent inhibition.44

Studies investigating the role of the I-II linker in voltage-dependent inhibition have at times come to somewhat differing conclusions about the relative importance of this region (reviewed in refs. 38 and 39). Two distinct binding sites have been identified within the I-II linker.84,103,109 The first partially overlaps with the binding site for the Caγβ subunit, the AID,16,114 and contains a QXXER consensus sequence for Gβγ found in other binding partners including phospholipase C β₁ and type 2 adenylly cyclase (QQIER in all three CaV2 family members). The second binding site for Gβγ identified downstream from the AID is termed the G protein interaction domain (GID).85 In vitro assays demonstrate high affinity (20–60 nM) interaction of Gβγ with the I-II linker,4,103,115 although partial masking of the first site by the Caγβ subunit reduces the affinity somewhat.120 Peptides based on both regions of the I-II linker diminish voltage-dependent inhibition of the channels and mutation to the sites can either reduce or enhance inhibition. Recently, a scanning mutagenesis approach systematically examined the involvement of each amino acid in these regions in voltage-dependent inhibition of rat CaV2.2 channels.117 Residues from 372–389 and 410–428 of the rat CaV2.2 channel were mutated to either alanine or cysteine (except for six residues in the 372–389 stretch shown to be buried by Caγβ binding). Two-out-of-thirty mutations (Arg⁵⁷⁶ and Val⁵⁸⁶ to alanine) significantly reduced the extent of voltage-dependent inhibition. Moreover, mutation of Arg⁵⁷⁶ to phenylalanine increased inhibition, further implicating this residue in binding of Gβγ and/or transduction of Gβγ binding into inhibition. It should be noted that some other studies have found a less prominent role.
for the I-II linker in voltage-dependent inhibition. For example, chimeric channels in which the I-II linker of CaV1.2 was introduced into the CaV2.2 backbone conferred modest inhibition onto these channels.100,121 However, recent studies would seem to support a central role for the I-II linker, at least for imparting voltage-dependence to the inhibition.120,123,124 The N-terminus has also emerged as essential in voltage-dependent inhibition.121,122,125,126 Some of the first evidence for this came from the finding that CaV2.3 channels with a truncated N-terminus lacked inhibition, whereas the full-length N-terminus was permissive for inhibition.97 Likewise, truncating the N-terminal 55 amino acids of CaV2.2 prevented voltage-dependent inhibition and introducing the CaV2.2 N-terminus into the CaV1.2 backbone conferred modest inhibition onto these normally resistant channels.76,122,134,135 The N-terminus has a glycine-rich stretch of amino acids (9–40) that is followed by eleven amino acids (45–55) that are predicted to form an α-helix.127 This eleven amino acid stretch is critical for Gβγ-mediated inhibition of the channels, in particular residues S48, R52 and R54, with I49 also involved to a lesser extent.121 The functional importance of this region was confirmed and direct interaction with Gβγ demonstrated by the Yue group.122 Moreover, Agler et al.122 also demonstrated another role for the N-terminus: residues 56–95 directly bind to the N-terminal one-third of the I-II linker (from CaV2.2 but not CaV1.2). In the context of the full length channel this interaction is "gated" by Gβγ, and appears to be crucial for transducing the inhibition. Thus, the N-terminus contributes both to binding of Gβγ, and as an "inhibitory module" that through intra-molecular interaction with the I-II linker mediates the functional shift from willing to reluctant gating states. This finding might complicate interpretation of some studies investigating the role of the I-II linker. For example, point mutations within the proximal I-II linker region or peptides based on this sequence disrupt inhibition. This was interpreted as being due to disrupted binding of Gβγ to the I-II linker, but it is also possible that the perturbations disrupted binding of the N-terminus to the I-II linker. That being said, Gβγ can also bind to the I-II linker at the downstream GID site, and phosphorylation within this region disrupts Gβγ-mediated inhibition.110 This is consistent with disrupted binding of Gβγ to this region, but again, subtle allosteric effects that alter a somewhat distant binding site, or interaction of the upstream I-II linker with the N-terminus are also possible.

To summarize, current data suggest that the binding site for Gβγ is comprised from multiple sites on the N-terminus, I-II linker and probably C-terminus of the channel. Binding of Gβγ causes a conformational shift that facilitates interaction of the N-terminus (residues 56–95) with the initial one-third of the I-II linker (Fig. 5). This (and perhaps other interactions) shifts gating charge movement to more depolarized potentials and uncouples voltage-sensor movement from channel activation.

**Structural Determinants on Gβγ**

Several studies have also investigated the molecular/structural determinants on Gβγ that are responsible for interaction with CaV2 channels. Unlike the channel, there are high resolution crystal structures of Gβγ available both in isolation and bound to Gα, GRK2 and phosducin128-132 (Fig. 4). Gβ adopts a toroidal, seven blade β-propeller structure with an α-helical N-terminal domain that binds to the α-helical N-terminus of the Gγ subunit. Gα interacts with multiple residues on the top face of Gβ and the side aspect of propeller blade 1 (Fig. 4). Many effectors bind to the same surface of Gβ that interacts with Gα and a protein interaction “hot spot” on this face has been identified with overlapping subsets of residues that are involved in binding to different effectors.133 Mutation of several residues on the Gα interacting surface disrupt inhibition of CaV2 channels,133,134,135 but residues on the opposite face of Gβ, have also been implicated.133,135 Moreover, Asn19 and Asn16 on Gβγ have been shown to underlie the ability of PKC to antagonize inhibition of CaV2.2.136 Given that Thr122 on the rat CaV2.2 I-II linker has been identified as the phosphorylation site for PKC in this effect,136 it is tempting to speculate that these regions of the channel and Gβγ come into contact...
close proximity with one another. Figure 4 shows a molecular surface rendering of the Gaβγ1i heterotrimer, and the Gaβγ1i heterodimer based on the structure reported by Wall et al.128 Multiple residues that have been reported to disrupt inhibition of CaV channels have been mapped onto this model. Most of these are on the same surface that Ga binds to, and are masked when Ga is present. There are also three residues on the reverse face of Gβγ.

Another study reported that a peptide mimicking the N-terminal 25 amino acids of Gβγ reduced inhibition of CaV2.1 (and activation of GIRK potassium channels).138 The Gβ N-terminal peptide disrupted FRET interaction between the Gβ2 and Gγ1 subunits suggesting a conformational shift or reorientation of the heterodimer that could disrupt interaction with the channels. Other studies have shown that the identity of Gγ within the Gβγ heterodimer can influence the extent of inhibition, with Gγ generally eliciting greater inhibition than Gγ1 or Gγ2.139,140 It remains unclear how Gγ exerts this influence, but it is interesting to note that the II-III linker of CaV2.1 contains a G-gamma-like (GGL) domain.141

Taken together, it is clear that multiple sites on both the CaV2.1 α subunit and the Gβγ heterodimer are involved in mediating voltage-dependent inhibition. However, the precise nature of these interactions remains unclear, including whether they represent direct protein-protein interactions or perhaps more subtle allosteric effects on the binding sites. Recently, small molecules and peptides that target the protein interaction “hot spot” on Gβγ have been shown to selectively disrupt interaction with some effectors (e.g., GRK2 and phospholipase Cβ).142 Some of these compounds have even demonstrated in vivo efficacy, for example to reduce the progression of heart failure through block of Gβγ-GRK2 interaction, or to reduce acute morphine induced anti-nociceptive tolerance perhaps through block of phospholipase Cβ.143,144 Thus, selective targeting of Gβγ-effector interactions is a viable proposition, so it is possible that compounds that enhance or disrupt modulation of calcium channels could be identified. Given that some point mutations on Gβ differentially altered inhibition of CaV2.1 and CaV2.2 channels,145 in principle it might even be possible to preferentially target the inhibition of one channel type over the other.

**Why is the Inhibition Voltage-Dependent?**

As already discussed, the consensus view is that voltage-dependent relief of the inhibition reflects transient dissociation of Gβγ from the channel at depolarized potentials. Recent data have identified some of the underlying molecular interactions that confer this voltage dependence to the inhibition. In particular, there appears to be an absolute requirement for CaV2 subunit binding to the AID in the I-II linker.120,123,124,145

The role of CaV2 subunits in voltage-dependent inhibition has been controversial with several seemingly opposing data sets and interpretations that have been reviewed in depth elsewhere.20,38,39 Briefly, one of the central questions that arose was whether or not CaV2 and Gβγ can both bind to the channel at the same time, or whether they compete for binding to the I-II linker in a mutually exclusive manner. Overlapping binding sites for the two proteins have been identified on the I-II linker, and initial observations showed that the functional effects of Gβγ generally oppose those of CaV2. Contradictory FRET data suggested either competition146 or synergistic binding.147 Various other pieces of contradictory data were also obtained, and at least part of the explanation for this might stem from the presence of endogenous CaV2 subunits in some heterologous expression systems (including Xenopus oocytes) or confounding shifts in the voltage-dependence of activation by some CaV2 subunits (reviewed in ref. 38). Closer examination of functional effects at both the whole-cell and...
single-channel level strongly suggest that both proteins can and indeed must interact with the channel simultaneously to mediate voltage-dependent inhibition. Recently, several papers from the Dolphin and Yang labs have provided evidence that a major role for the Ca\(\beta\) subunit is to confer voltage-dependence to G\(\beta\gamma\)-mediated inhibition of CaV2.2,123,124,145 and CaV2.1 channels.120

The general approach used by both groups was to disrupt the Ca\(\alpha\beta\) interaction with the AID to record currents from channels that lack a \(\beta\) subunit. The Dolphin lab mutated the AID of CaV2.2 channels (W391A) to reduce \(\beta\) subunit binding affinity by ~1,000 fold.123 This resulted in a dramatic decrease in plasma membrane expression of the channels (due to disrupted trafficking) and alterations in gating kinetics indicating that the channels did indeed lack a \(\beta\) subunit. Activation of co-expressed D2 dopamine receptors still inhibited the mutant (W391A) channels to a similar degree as wild type channels, as did coexpression of G\(\beta\gamma\). However, prepulse reversal of the inhibition was almost abolished. Mutation of an additional two N-terminus residues shown to be essential for G\(\beta\gamma\)-mediated inhibition (R52A and R54A-discussed above) abolished this voltage-independent inhibition in W391A channels. A follow up study demonstrated essentially the same findings when wild type CaV2.2 \(\alpha\) \(\beta\) subunit was expressed with \(\alpha\beta\) but without CaV\(\beta\).124 Also in this study, the voltage-independent inhibition in the absence of the CaV\(\beta\) was blocked by overexpression of transducin which acts to scavenge free G\(\beta\gamma\) subunits. Thus, in the absence of CaV\(\beta\), inhibition of the channels by G\(\beta\gamma\) was still present, but voltage-dependent reversal of the inhibition was abolished. The \(\beta\) subunit was used in the experiments outlined above, but when \(\beta\) \(\omega\) was expressed with the W391A channels voltage-dependent relief of the G\(\beta\gamma\) mediated inhibition was restored. Palmitoylation of two N-terminal cysteine residues promotes plasma membrane targeting of \(\beta\) \(\omega\), and mutation of these residues led to loss of voltage-dependent relief (i.e., the data resembled \(\beta\) \(\omega\)). Thus, the authors proposed that palmitoylation enabled the local plasma membrane concentration of \(\beta\) \(\omega\) to reach high enough levels that low affinity interaction with \(\alpha\) could take place and permit voltage-dependent relief of the inhibition. In this context it is pertinent to note that previous work has shown the subtype of CaV\(\beta\) can influence the extent and kinetics of G\(\beta\gamma\)-mediated inhibition.148,149 Moreover, the particular effects of the CaV\(\beta\) subunits are dependent on the identity of the G protein \(\beta\) subunit involved in producing the inhibition.140 Thus, the inhibition is precisely tuned by the identity of both the CaV\(\beta\) and G protein \(\beta\) subunits.

Similar findings for CaV2.1 channels have also been reported recently in reference 120. In this study the authors used mutated CaV\(\beta\) subunits that had reduced affinity for the AID. These were coexpressed in Xenopus oocytes with CaV2.1. Giant inside-out patches that contained many channels were pulled from the oocytes and macroscopic currents were recorded. Due to the reduced binding affinity of the mutant CaV\(\beta\) subunit, washing the cytoplasmic face of the patches resulted in dissociation of CaV\(\beta\) and this was confirmed by the expected shifts in channel kinetics compared to wild type. In the absence of CaV\(\beta\), application of purified G\(\beta\gamma\) still inhibited the currents but prepulse reversal was abolished.

As outlined above, the CaV\(\beta\) subunit has SH3 and GK domains that are separated by a variable HOOK region.38,20 The GK domain is solely responsible for binding to the AID on the I-II linker, although other regions including the SH3 and HOOK domains might interact elsewhere on the \(\alpha\) subunit to modulate functional properties. For example, the HOOK domain has been reported to modulate inactivation kinetics of CaV2.2 channels.120 The Dolphin and Yang groups both demonstrated that binding of the isolated GK domain of CaV\(\beta\) to the AID was sufficient to restore voltage-dependence to the G\(\beta\gamma\)-mediated inhibition120,124 (see below for further discussion). However, it is possible that the SH3 and variable HOOK domains of CaV\(\beta\) might contribute in other ways. Deleting the HOOK domain resulted in tonic inhibition of CaV2.2 channels, perhaps due to increased affinity for the basal level of free G\(\beta\gamma\) in the cells.124 Furthermore, at least some of the distinct effects of CaV\(\beta\) on modulation are linked to its palmitoylation.124,149 In part this might be due to an effective concentration of the subunit at the plasma membrane, but other actions might also contribute. For example, the effects of CaV\(\beta\) on channel kinetics are dependent on its orientation relative to the \(\alpha\) subunit.151 It is possible that palmitoylation could alter the orientation of CaV\(\beta\) to promote or hinder interactions between the SH3 or HOOK regions with as yet undefined sites on the CaV\(\alpha\) subunit. The palmitate groups on CaV\(\beta\) have also been proposed to interact directly with the CaV2.2 subunit, and thereby mask a site for voltage-independent inhibition mediated by arachidonic acid.39,2,153

The molecular mechanism by which binding of CaV\(\beta\) to the AID enables voltage-dependent relief of G\(\beta\gamma\) mediated inhibition was further investigated by Zhang et al.120 In the absence of CaV\(\beta\) the AID adopts a random coil but when CaV\(\beta\) binds it induces the formation of an \(\alpha\)-helical conformation that extends back to the interface with IS6.15,17,154 Introducing seven glycines between the AID and IS6 to disrupt the \(\alpha\)-helical structure prevented the ability of CaV\(\beta\) to confer voltage-dependence to the inhibition. In contrast, introducing seven alanines (not expected to disrupt the \(\alpha\)-helix) maintained the ability of CaV\(\beta\) to confer voltage-dependence to G\(\beta\gamma\)-mediated inhibition. Thus, it is possible that binding of CaV\(\beta\) to the AID induces a rigid \(\alpha\)-helical link with the upstream IS6 and this transmits movement of the voltage-sensor and activation gate (including IS6) to shift the I-II linker and alter the G\(\beta\gamma\) binding pocket at depolarized potentials. It is also worth noting that G\(\beta\gamma\)-mediated inhibition was still present (although slightly reduced) in both channel types lacking CaV\(\beta\), and in the CaV2.1 channels containing the seven glycine insert upstream of the AID.120,124 Thus, it would appear that the rigid \(\alpha\)-helical link to the upstream activation gate and voltage-sensor is not required per se to transduce binding of G\(\beta\gamma\) into functional inhibition.

**Modulation of G\(\beta\gamma\)-Mediated Inhibition by Other Proteins**

Skeletal muscle L-type calcium channels (Ca\(\lambda\)1.1) have been shown to associate with a \(\gamma\) subunit in addition to a \(\beta\) and an \(\alpha\) \(\beta\) subunit. Although several neuronal \(\gamma\) subunits have been identified that can alter expression of CaV2.2 channels, it remains uncertain that these proteins constitute bona fide channel subunits and the \(\gamma\)2
isoform (also called stargazin) and related proteins (γ3-7) associate with and modulate glutamatergic AMPA receptors. However, a recent study reported that stargazin binds Gβγ in vitro and that it scavenges Gβγ in Xenopus oocytes to reduce inhibition of CaV2.2 channels or activation of GIRK channels. Thus, the γ subunit, although not covalently bound to the channel complex, can modulate its properties by virtue of disrupting Gβγ-mediated inhibition.

CaV2.2 channels also interact with a variety of synaptic proteins. Syntaxin, SNAP25 and synaptotagmin all bind to the synaptic protein interaction site (synprint site) on the II-III linker of CaV2.2 channels (Fig. 1). The functional consequences of these interactions are manyfold and reviewed in reference 157 and 158, but include targeting of the channels to synaptic release sites and modulation of channel kinetics. Syntaxin 1A (but not syntaxin 1B) enhanced voltage-dependent inhibition of CaV2.2 channels and in particular promotes tonic inhibition perhaps through a scaffolding role to colocalize the channels and G proteins. Cleavage of syntaxin with botulinum neurotoxin C diminished inhibition of calcium channels in neuronal preparations supporting the notion that this interaction is physiologically important. Cysteine string protein also interacts with the synprint site and produces tonic inhibition of CaV2.2 channels perhaps due to colocalization of Gβγ and the channel and/or direct activation of GDP-GTP exchange (GEF activity) on the Go subunit to liberate Gβγ.

In recent years considerable attention has been given to regulator of G protein signaling (RGS) proteins which accelerate the intrinsic GTPase activity of Go subunits. There are over 20 members of the RGS family and several of these modulate G protein inhibition of calcium channels. In part this involves the well documented acceleration of intrinsic GTPase activity to limit the availability of Gβγ. However, RGS proteins have several other modes of action including some that dimerize with Gαi subunits and some (e.g., RGS12) that directly interact with CaV2.2 channels. Experimental manipulation of RGS expression has been demonstrated to alter calcium channel inhibition and it has also been shown that this in turn can alter short term synaptic plasticity. Expression of several RGS proteins can be dynamically regulated by physiological and pathophysiological situations. For example, dopamine depletion (as occurs in Parkinson’s disease) upregulated RGS4 in striatal interneurons resulting in diminished autoinhibition of calcium channels by M4 muscarinic receptors. Thus, RGS proteins have emerged as important modulators of Gβγ-mediated inhibition (among other pathways) and there is considerable interest in targeting these proteins for therapeutic development.

Conversely, activator of G protein signaling (AGS) proteins can stimulate nucleotide exchange and lead to signaling independent of GPCR activation. Coexpression of AGS1 or the related Rhes protein with CaV2.2 in HEK293 cells induced tonic inhibition due to liberation of Gβγ from Gi/o-type heterotrimers. Thus it is possible that such signaling can occur, but this remains to be demonstrated in native channels under a physiological (or pathophysiological) context.

As outlined above, even though the voltage-dependent inhibition is mediated by Gβγ, in most cases the GPCR couples to pertussis toxin sensitive Gi/o-type G proteins. This functional selectivity might be achieved in several ways. For example, Gq-coupled receptors have been shown to couple (although not exclusively) with Gβγ, which in general is less efficacious at producing inhibition. The Goq subunit might also associate directly with the channel to blunt the inhibition through PKC mediated phosphorylation. Voltage-dependent inhibition is membrane delimitated so the GPCR and channel must be in close proximity. In part this might be mediated through adapter proteins like Homer or NHERF2. Direct interaction of GPCRs and CaV2.2 channels has also been demonstrated for opioid-like receptors (ORL1). Constitutive activity of the receptors leads to tonic voltage-dependent inhibition, and upon prolonged agonist application the channel is internalized as a consequence of desensitization of the coupled ORL1 receptor. More recently it has been shown that ORL1 and classic opioid receptors (e.g., μ-opioid receptors) can heterodimerize. One notable consequence of this is that μ-opioid receptor agonists lead to internalization of the GPCR-channel complex, which does not occur in the absence of the ORL1 receptor. D1 and D2 dopamine receptors also bind directly to CaV2.2 channels and modulate channel trafficking and GABAb receptors also lead to channel internalization although the mechanism is somewhat different.

It is also worth noting that point mutations linked to familial hemiplegic migraine (FHM mutations) have been identified in CaV2.1 and lead to complex alterations in channel function. Many of these seem to exert “gain-of-function” effects, including three (R192Q, S218L, Y1245C) that diminish Gβγ-mediated inhibition. It is also worth noting that point mutations linked to familial hemiplegic migraine (FHM mutations) have been identified in CaV2.1 and lead to complex alterations in channel function. Many of these seem to exert “gain-of-function” effects, including three (R192Q, S218L, Y1245C) that diminish Gβγ-mediated inhibition.

Overall, it is clear that a multitude of protein-protein interactions and second messengers can fine tune the inhibition of CaV2 channels by G proteins. Membrane potential, firing patterns, channel subunit composition/splice variants and Gβγ heterodimer composition all modulate the extent and/or kinetics of inhibition. Indeed, the complexity of calcium channel inhibition is even greater because there are also several mechanisms leading to voltage-independent inhibition, including multiple kinases, phosphatases and lipid signaling pathways. These mechanisms are outside the scope of this review, but it is worth mentioning that some also involve Gβγ. Direct (but voltage-independent) inhibition of CaV3.3, T-type channels by Gβγ has also been reported. Ultimately, this complexity is linked to the overriding need to precisely control the calcium entry that plays such pivotal roles in cellular physiology, including the triggering and modulation of neurotransmitter release.

Acknowledgements
I would like to thank Mark Jewell (currently a graduate student in my lab) for help with the molecular graphics images in Figure 4 that were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081). Work in my lab is supported by the National Institutes of Health National Institute Of Neurological Disorders And Stroke [Grant R01-NS052446].
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