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Regulation of Ca_V2 calcium channels by G protein coupled receptors

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Abstract

Voltage gated calcium channels (Ca^{2+} channels) are key mediators of depolarization induced calcium influx into excitable cells, and thereby play pivotal roles in a wide array of physiological responses. This review focuses on the inhibition of Ca_V2 (N- and P/Q-type) Ca^{2+}-channels by G protein coupled receptors (GPCRs), which exerts important autocrine/paracrine control over synaptic transmission and neuroendocrine secretion. *Voltage-dependent* inhibition is the most widespread mechanism, and involves direct binding of the G protein βγ dimer (Gβγ) to the α1 subunit of Ca_V2 channels. GPCRs can also recruit several other distinct mechanisms including phosphorylation, lipid signaling pathways, and channel trafficking that result in *voltage-independent* inhibition. Current knowledge of Gβγ-mediated inhibition is reviewed, including the molecular interactions involved, determinants of voltage-dependence, and crosstalk with other cell signaling pathways. A summary of recent developments in understanding the voltage-independent mechanisms prominent in sympathetic and sensory neurons is also included.

Keywords

Calcium channel; G protein coupled receptor; inhibition; Gβγ; PKC; tyrosine kinase; PiP2; arachidonic acid; splice variant; SNARE

1. Voltage gated calcium channels

Voltage gated calcium channels (Ca^{2+} channels) are key mediators of depolarization induced calcium influx into excitable cells, which in turn mediates a wide array of physiological responses including the activation of calcium dependent enzymes, smooth muscle contraction, pacemaker activity and neurotransmitter release [1-8]. Ca^{2+} channels are also associated with a wide range of pathologies, including pain, epilepsy, migraine, cardiac arrhythmias and autism [9-14]. It is widely known that there are subtypes of Ca^{2+} channels with different pharmacological and biophysical properties, and distinct cellular and physiological functions [15-17]. In neurons, certain L-type Ca^{2+} channel isoforms are

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expressed at cell bodies and dendrites, and one of their key functions is the initiation of calcium dependent gene transcription events [18-22]. Other L-type channel subtypes are expressed in cochlear hair cells and photoreceptor nerve terminals where they regulate neurotransmitter release at ribbon synapses [23, 24]. T-type calcium channels are expressed in cell bodies as well as dendrites and one of their key functions is to regulate cellular excitability and neuronal firing properties [25-27], in addition to participating in secretion [28-30]. N-type and P/Q-type calcium channels are expressed at synaptic nerve terminals where their opening results in the release of neurotransmitters [1, 19, 31-34].

All Ca^{2+} channels are comprised of a pore forming Cavα1 subunit that contains the major structural features required for permeation, activation, and inactivation. The mammalian genome encodes ten different Cavα1 subunits that fall into three major families - Cav1 (L-type channels), Cav2 (N, P/Q- and R-types), and Cav3 (T-types) [17, 35]. The Ca_V1 and Ca_V2 families are high voltage activated (HVA) channels, and are heteromers comprised of a pore forming Cavα1 subunit as well as Cavα2-δ and Cavβ subunits [36-38] (Fig 1). In addition, these channels associate with calmodulin which is now considered part of the HVA channel macromolecular complex [39-44]. The Cavα1 subunit determines the Ca^{2+} channel subtype and is a large (~175-225 kDa) protein with four homologous transmembrane domains that are connected by cytoplasmic loops and bracketed by cytoplasmic N- and C-termini [37] (Fig 1). These cytoplasmic regions are key targets for second messenger regulation including protein kinases and G proteins, as we discuss here in detail. The Cavβ subunits are cytoplasmic proteins that associate with HVA α1 subunits at a highly conserved region within the domain I-II linker (termed the Alpha Interaction Domain – AID) [45-47]. These subunits are encoded by four different genes (for review see [48, 49]). The Cavα2-δ subunits are transcribed from one of four different Cavα2-δ genes, proteolytically cleaved and then reconnected via a disulfide bond (for review, see [50]). The α2 portion is located at the extracellular side of the channel, whereas the δ portion either spans the membrane or may be linked to the extracellular leaflet of the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor [51]. The function of these ancillary subunits is to regulate channel properties and promote Cavα1 subunit trafficking to and stabilization at the plasma membrane [52-54] (for reviews see [48, 49, 55-57]. As we will outline below, Cavβ subunits also alter second messenger regulation the channel complex [58-61]. Finally, it should be noted that most Ca^{2+} channel subunits are subject to alternate mRNA splicing, thus greatly increasing the functional diversity of calcium channels [62-65]. Recently described RNA editing events that alter channel function add further complexity [66]. This then makes it challenging to precisely reconstitute all specific features of native calcium currents in transient expression systems. In this review, we focus on the Ca_V2 family of channels, and in particular their regulation by G protein coupled receptors.

2. G protein coupled receptors and heterotrimeric G proteins

G protein coupled receptors (GPCRs) are a large family of membrane proteins encoded by almost 800 human genes, and represent an important class of therapeutic targets [67, 68]. GPCRs are characterized by an extracellular N-terminus, seven transmembrane spanning alpha helices, and an intracellular C-terminus which couples to heterotrimeric G proteins. Extracellular ligand binding to the receptor leads to activation of the G proteins and a myriad of downstream intracellular signaling cascades. In human, sixteen genes encode G protein α subunits (Gα), and these are classified into four major families: G_s, G_i, G_q, and G_{12}, in addition to transducin (Gαt) which is found in the retina. Five genes encode Gβ subunits, and twelve genes encode Gγ subunits (for reviews see[69-72]. Binding of agonist to the GPCR catalyzes the exchange of GDP to GTP on Gα causing conformational changes/dissociation of the Gα and Gβγ heterodimer [71, 73]. The liberated Gα and Gβγ are both capable of signaling to multiple downstream effectors, including voltage-gated

Ca^{2+}-channels as discussed in this article. Signaling is terminated by intrinsic GTPase activity of Gα and subsequent reassociation of the Gα-GDP subunit with the Gβγ heterodimer. This GTPase activity can be accelerated by a family of RGS proteins (regulator of G protein signaling) which thus influence the extent and duration of downstream events [74]. Receptor desensitization in the continued presence of agonist can also terminate signaling. Desensitization is complex, involving phosphorylation by PKA, PKC, or G protein coupled receptor kinases (GRKs) and 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 the endocytic machinery [75, 76]. As discussed below (section 10), direct interaction of GPCRs and Ca^{2+} channels might result in co-internalization adding another dimension to channel modulation.

4. Inhibition of Ca_V2 channels by G protein coupled receptors

Neurotransmitter mediated inhibition of Ca^{2+} channels was first demonstrated ~30 years ago by Dunlap and Fischbach who reported that norepinephrine reduced the duration of action potentials [77] and the amplitude of I_{Ca} [78] in chick sensory neurons. It is now apparent that a variety of different neurotransmitters/neuromodulators acting on their cognate GPCRs inhibit I_{Ca}, and that this is important for controlling neurosecretion (for reviews see [79-85]). It is also known that GPCRs can recruit several distinct signaling pathways that converge on Ca^{2+} channels. The most widespread and intensively studied of these involves direct binding of Gβγ to the α1 subunit of Ca_V2 channels. As detailed below (section 5), Gβγ-mediated inhibition shifts the voltage-dependence of channel activation, is less prominent at depolarized membrane potentials, and is transiently relieved by large depolarizing voltage steps. Consequently, this mechanism is often termed *voltage-dependent inhibition*. GPCRs can also elicit *voltage-independent inhibition* of I_{Ca} which is mediated by several other distinct and generally less well characterized pathways including phosphorylation, lipid signaling pathways, and channel trafficking (see sections 10-12). While voltage-dependent inhibition is widespread throughout the nervous system, voltage-independent inhibition is more variable in extent and mechanism but seems particularly prominent in sensory and sympathetic neurons. In this review we first consider Gβγ-mediated inhibition, including recent developments and crosstalk with other cell signaling pathways. Then we outline some of the voltage-independent mechanisms prominent in sympathetic and sensory neurons.

5. Voltage-dependent inhibition mediated by Gβγ

Voltage-dependent inhibition primarily targets $Ca_V2.1$ (P/Q-type) and $Ca_V2.2$ (N-type) channels, although $Ca_V2.3$ channels are also inhibited by similar mechanisms (see section 5.4 below). The voltage-dependent nature of the inhibition was first demonstrated by Bean [86], who showed that the decrease in current amplitude was not due to a loss of channels *per se*, but rather a shift in the gating properties that could be overcome by strong depolarization. Several hallmarks are characteristic of this *voltage-dependent* mechanism: In whole cell recordings, the inhibition of peak I_{Ca} amplitude 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 relieves most of the inhibition and normalizes channel kinetics (termed prepulse relief or prepulse facilitation). Figure 2 shows an example of voltage-dependent inhibition of I_{Ca}. Prominent slowing of activation kinetics and prepulse relief of the inhibition is clearly seen. Voltage-dependent relief of the inhibition can also occur at least to some extent during more physiologically relevant stimuli such as high frequency trains of action potential-like waveforms [87-92]. In turn, this might contribute to short term synaptic plasticity at some synapses [93].

Bean also introduced the "willing and reluctant" model to explain these functional effects [86], a framework that persists to this day [94-97]. The channels exhibit two functional gating states, "willing" and "reluctant". In the absence of Gβγ, the "willing" state predominates, whilst binding of Gβγ favors the "reluctant" state which displays the shifts in channel gating noted above. Voltage-dependent relief of the inhibition is thought to reflect a shift of the channels from "reluctant" to "willing" due to transient dissociation of Gβγ (Fig 2B). This was supported by kinetic analyses of prepulse relief as a function of agonist or Gβγ concentration. Increasing the concentration of Gβγ did not alter the rate of relief during the prepulse, but did accelerate the rate of reinhibition following the prepulse [98-101], as expected for voltage-dependent dissociation and rebinding of Gβγ. Further investigations revealed that the kinetics of reinhibition were consistent with binding and unbinding of a single Gβγ dimer with the channel [101].

5.1. Single channel investigations

Single channel studies provided early evidence that the inhibition did not involve a diffusible second messenger. In the "cell-attached" ("on-cell") recording configuration, bath application of agonist did not inhibit the channels whereas agonist in the patch pipette did [99, 102, 103]. This led to the conclusion that the inhibition was "direct" or "membrane delimited". Single channel recording also directly revealed "reluctant" gating of inhibited channels. Upon membrane depolarization, the latency (delay) to first channel opening was increased during inhibition whereas there was little impact on other single channel parameters [95, 104]. As a result, the inhibited ("reluctant") channels appeared essentially silenced, unable to open until Gβγ dissociated and the channels shifted to the "willing" state. Subsequently it has been reported that $Ca_V2.2$ (N-type) but not $Ca_V2.1$ (P/Q-type) channels can display very brief channel openings from the "reluctant" state (i.e. without Gβγ unbinding), although the probability of such events was low [96, 97].

Overall, the dominant effects of inhibition observed in all studies are the shift in activation and prolonged latency to first channel opening. The slow activation kinetics seen in whole cell recording (Fig 2) and longer latency in single channel recordings reflect the conformational changes and subsequent dissociation of Gβγ from the channel upon membrane depolarization. This diminished binding of Gβγ at depolarized potentials also results in little inhibition of whole cell I_{Ca} when neurotransmitter agonists are rapidly applied *during* a depolarizing voltage-step [105].

5.2. Alteration of gating currents by Gβγ

Further evidence for altered activation comes from recording of channel "gating currents". Gating currents are not due to ionic flux through the channel pore, but rather reflect movement of the charged voltage-sensor domain of the channels in response to membrane potential changes. Expression of recombinant $Ca_V2.2$ in HEK293 cells enables recording of these gating currents in isolation as the cells lack other endogenous voltage-gated channels. G proteins were found to reduce the amplitude, and shift the voltage-dependence of gating currents to more depolarized potentials [106]. G proteins also produced a significant separation in the voltage-dependent activation of gating current and ionic current [106]. These data suggest that Gβγ binding slows movement of the voltage-sensor and uncouples this movement from opening of the activation gate. Modulation of gating currents by G proteins has also been reported in rat sympathetic neurons [107, 108].

5.3. Gβγ and channel inactivation

In addition to these dominant effects on channel activation, evidence supports the idea that Gβγ can also modulate *inactivation* of $Ca_V2.2$ channels [109, 110]. Inactivation of Ca^{2+} channels is complex and mediated by several voltage-dependent and Ca^{2+}-dependent

mechanisms [111-113]. The precise molecular correlates remain somewhat unclear, but fast voltage-dependent inactivation might involve a “hinged lid” type mechanism in which the intracellular loop connecting domains I and II of the α_1-subunit serves as the “inactivation gate” [112, 114] (but see [115]). The I-II loop is also an important binding site for Gβγ on the channel [116-119] (Fig 1) (see section 7 for more discussion). Therefore, it is possible that binding of Gβγ disrupts movement of this putative inactivation gate, or its interaction with other channel domains. Inactivation of $Ca_V2.2$ can also occur from intermediate closed state(s) of the channel favored during trains of brief repetitive stimuli [104]. 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. Further investigations are needed to determine quite how G protein modulation and channel inactivation interact.

Ca^{2+}-dependent inactivation is mediated by calmodulin bound to the C-terminus of the channel α1 subunit [42, 120-123]. Strong intracellular Ca^{2+} buffering (EGTA or BAPTA in the patch pipette solution) blocks Ca^{2+}-dependent inactivation of Ca_V2 channels indicating that it is mediated by a “global” elevation rather than a “local” microdomain of Ca^{2+}. The reduction of Ca^{2+}-dependent inactivation by Gβγ [109] might therefore result from fewer channels opening and a diminished “global” Ca^{2+} signal, although more complex interactions are also possible, and direct *in vitro* binding of Ca^{2+}-calmodulin to Gβγ has been reported [124].

5.4. Differential inhibition of Ca_V2 channels by Gβγ

Originally demonstrated for N-type channels ($Ca_V2.2$) in sensory and sympathetic neurons (for example, [78, 125-127]), it subsequently became clear that $Ca_V2.1$ (P/Q-type) channels are also modulated by Gβγ in a similar manner [128]. Initially it was thought that $Ca_V2.3$ channels were insensitive to G proteins [129-131], although other studies did find some degree of inhibition [132-135]. Chimeric approaches suggested that the lack of (or poor) responsiveness of $Ca_V2.3$ resided in several regions within the N-terminus, domain I, and I-II linker of the channels [136-138]. Subsequently it was discovered that alternative splicing of the N-terminus conferred G protein sensitivity to the channels [139]. Truncation of 50 amino acids abolished inhibition, whereas a splice variant with full length N-terminus did display inhibition, albeit to a lesser extent than $Ca_V2.2$ channels [139]. While inhibition of $Ca_V2.3$ channels can occur (depending on splice variation), it is generally to a lesser extent and remains less well understood than for $Ca_V2.1$ and $Ca_V2.2$ channels. In part this might be due to difficulty in isolating these channels in neuronal cell types. The focus on $Ca_V2.1$ and $Ca_V2.2$ channels is also driven by their prominence in triggering neurotransmitter release, and most of the following discussion revolves around those two channels.

Although the basic mechanism of inhibition is similar for $Ca_V2.1$ and $Ca_V2.2$ (direct binding of Gβγ to the channel), subtle differences have emerged. As noted above, single channel recording showed that $Ca_V2.2$ but not $Ca_V2.1$ channels display very brief duration, low probability “reluctant openings” [96, 97]. Differences are also apparent with macroscopic (whole-cell) recordings: activation of GPCRs or expression of Gβγ reduces the peak amplitude of I_{Ca} to a significantly greater extent for $Ca_V2.2$ than $Ca_V2.1$ [129, 140, 141]. Moreover, trains of action potential-like stimuli reverse a greater proportion of $Ca_V2.1$ inhibition than $Ca_V2.2$ inhibition [92]. These effects can be explained by differences in the affinity of Gβγ binding to the channels. The apparent affinity of Gβγ for the channel can be inferred from prepulse relief and re-inhibition experiments, and is quite similar for the two channels at hyperpolarized or very depolarized potentials. However, at moderately depolarized potentials (< +30mV), within the physiologically relevant range of action potentials, there is a significant divergence in the affinity of Gβγ binding to the two channel types [96]. Subtle differences in binding of Gβγ to the channels is also suggested when

comparing the inhibition produced by different Gβ subunits ($G\beta_{1-5}$), all paired with the same $G\gamma_2$ subunit. Such experiments revealed a different rank order of inhibition for $Ca_V2.1$ and $Ca_V2.2$ channels [142]. Point mutations on the $G\beta_1$ subunit also have distinct effects on the inhibition of $Ca_V2.1$ and $Ca_V2.2$ channels [143].

It would appear that subtle differences in the binding affinity of Gβγ to the $Ca_V2.1$ and $Ca_V2.2$ channels results in differential inhibition: $Ca_V2.2$ I_{Ca} is inhibited to greater extent and this inhibition is more resistant to reversal by high frequency bursts of action potentials. The relative expression level of the two channel types varies between neurons, and even between neighboring synapses arising from the same neuron. Therefore, differential inhibition of $Ca_V2.1$ and $Ca_V2.2$ could lead to cell and/or synapse specific neuromodulation by GPCRs. Functional differences might also arise from variable interactions or crosstalk with other signaling pathways such as PKC (see section 9).

6. Structural determinants on Gβγ that govern modulation of Ca_V2 channels

Gβγ is thought to be an obligate heterodimer and there are several high resolution crystal structures in isolation or bound to interacting proteins including Gα, GRK2, and phosducin [144-148]. Figure 3 shows a rendering of the heterotrimer ($G\alpha_i\beta_1\gamma_2$) (Fig 3A), and heterodimer (the $G\beta_1\gamma_2$) (Fig 3B) based on the structure reported by Wall et al [144] (PDB ID: 1GP2). Gβ adopts a seven blade β-propeller structure with an α-helical N-terminal domain that binds to the α-helical N-terminus of the Gγ subunit (Fig 3B). In the heterotrimeric complex, Gα interacts with multiple residues on the top face of Gβ and the side aspect of propeller blade 1 (Fig 3A). Gβγ interacts with multiple downstream effectors and mutagenesis approaches have been used to map the interaction sites important for binding to these targets. Many effectors bind to a protein interaction “hot spot” on the surface of Gβ that interacts with Gα, with overlapping subsets of residues involved in binding to different effectors [149]. A number of residues identified in mutagenesis studies to contribute to inhibition of Ca^{2+} channels are highlighted in figure 4. Most of these are on the Gα interacting surface (Fig 3C) and are masked when Gα is present [85, 109, 150-152], although residues on the reverse face of $G\beta_1$ have also been implicated [152-154] (Fig 3D). Also of note, Asn^{35} and Asn^{36} on $G\beta_1$ mediate the ability of PKC to antagonize inhibition of $Ca_V2.2$ [153]. Thr^{422} on the rat $Ca_V2.2$ I-II linker has been identified as the phosphorylation site for PKC that mediates this effect [155], so it is tempting to speculate that this region of the channel and Gβγ come into close proximity with one another (see section 7).

Another study reported that a peptide mimicking the N-terminal 25 amino acids of $G\beta_2$ reduced inhibition of $Ca_V2.1$ [156]. The Gβ N-terminal peptide disrupted FRET interaction between the $G\beta_2$ and $G\gamma_3$ subunits suggesting a conformational shift or reorientation of the heterodimer that could disrupt interaction with the channels. A few studies have also shown that the subtype of Gγ within the Gβγ heterodimer can influence the extent of inhibition, with $G\gamma_2$ generally eliciting greater inhibition than $G\gamma_1$, $G\gamma_3$ or $G\gamma_{13}$ [157, 158]. The molecular basis for why the Gγ subtype influences inhibition of I_{Ca} is not clear, but it is interesting to note that the II-III linker (of the channel α1 subunit) contains a G-gamma-like (GGL) domain [159].

7. Structural determinants on the channel α1 subunit that govern modulation by Gβγ

Although there is currently no crystal structure for voltage-gated calcium channels that could be used to visualize their interactions with G proteins, site directed mutagenesis, chimeric, and biochemical approaches have been used to elucidate channel structural determinants involved in modulation. The first investigations involved chimeras between

Cav2.1 and Cav2.2 channels [140]. These chimeras were expressed in *Xenopus* oocytes and their sensitivities to G proteins assessed via two electrode voltage clamp. These experiments identified domain I as a key determinant of G protein inhibition, along with the C-terminus of the channel. Subsequent biochemical studies using in vitro translated Gβγ subunits revealed two spatially distinct regions on the I-II linker of CaV2.1 as possible Gβγ targets [117]. The existence of two separate Gβγ binding domains in the domain I-II linker was also observed in functional assays. Zamponi et al. [160] showed that intracellular dialysis of tsA-201 cells with ~20 amino acid peptides directed against different regions of the I-II linker of both Cav2.1 and Cav2.2 channels prevented the ability of exogenously delivered Gβγ subunits to mediate voltage dependent inhibition of the channels. The first site contains a QXXER consensus sequence (QQIER in all three Ca_V2 family members) found in other Gβγ binding partners. This site also overlapped partially with the putative Cavβ subunit binding domain on the channel (the AID). Subsequent co-crystal structures of the Cavβ subunit bound to its interaction site on the isolated domain I-II linker revealed that only part of the 20 amino acid stretch forming the putative Gβγ interaction site is likely to be accessible in the presence of a bound Cavβ subunit [46]. This may suggest two possibilities: Either the Cavβ subunit partially dissociates from regions involved in Gβγ binding, or alternatively Gβγ interacts with those residues that remain exposed after Cavβ docking.

Further support for the involvement of the I-II linker came from scanning mutagenesis of the amino acids in each of the two binding regions in rat $Ca_V2.2$ channels [161]. Mutation of two residues (Arg^{376} and Val^{416} to alanine) out of thirty tested significantly reduced the magnitude of voltage-dependent inhibition while mutation of Arg^{376} to phenylalanine *increased* inhibition. Irrespective of the precise nature of the Gβγ interaction on the domain I-II linker, this general region has been implicated as being important for functional channel inhibition by a number of other groups [116, 131, 136]. These studies contrast with work from Qin and colleagues [134] whose data implicated the C-terminus rather than the domain I-II linker as the critical element for G protein modulation. While likely playing an auxiliary role, the C-terminus region does not appear to be essential for N-type channel inhibition as large parts can be deleted with only small consequences on the extent of receptor mediated voltage-dependent modulation [155, 162, 163]

Several other groups attributed an important role to the N-terminus of the channel based on site directed mutagenesis work [136, 138, 151, 164]. The Dolphin lab identified the N-terminal 55 amino acids of $Ca_V2.2$, and in particular an eleven amino acid stretch (45-55) that is predicted to form an α-helix [165], to be critical for Gβγ-mediated inhibition of the channels. The Yue group demonstrated direct interaction of the N-terminus with Gβγ [151] and that the N-terminus (residues 56-95) also binds directly to the I-II linker. Thus, the N-terminus contributes both to binding of Gβγ, and as an “inhibitory module” which binds the I-II linker to perhaps mediate the shift from willing to reluctant gating states. Finally, a recent study revealed that a point mutation (S218L) in the domain I S4-S5 linker of Cav2.1 that is found in patients with familial hemiplegic migraine (FHM) facilitates recovery of the channels from Gβγ inhibition, perhaps by facilitating the dissociation of the G protein dimer [166]. Two other FHM mutations (R192Q, Y1245C) have also been reported to diminish Gβγ-mediated inhibition [167, 168].

Taken together, several sites on both the Ca_V2 α_1 subunit and the Gβγ heterodimer have been implicated in voltage-dependent inhibition. On the Cav2 α1 subunit, the domain I-II linker and N-terminus are essential structural elements (Fig 4). Ultimately crystal structure data will be needed to precisely determine how G protein subunits interact with these channel loci in the presence and the absence of the Cavβ subunit.

8. Contribution of the $Ca_V\beta$ subunit to voltage-dependent inhibition

The subtype of $Ca_V\beta$ can influence the extent and kinetics of $G\beta\gamma$-mediated inhibition and this depends on the subtype of Gβ involved [169, 170]. However, the precise role of $Ca_V\beta$ subunits in voltage-dependent inhibition of I_{Ca} has been unclear (for reviews see [49, 79]). Overlapping binding sites for the two proteins have been identified on the I-II linker, and one fundamental question that arose was whether $Ca_V\beta$ and $G\beta\gamma$ can bind to the channel at the same time, or whether they compete in a mutually exclusive manner. Seemingly contradictory data including FRET analyses suggested either competition [171] or synergistic binding [172]. Some of this confusion might stem from endogenous $Ca_V\beta$ subunits found in some heterologous expression systems (including Xenopus oocytes), or confounding shifts in the voltage-dependence of activation by some $Ca_V\beta$ subunits (see [79]). Evidence from the Dolphin and Yang labs outlined below now suggest that both proteins can interact with the channel simultaneously, and that binding of the $Ca_V\beta$ subunit is required to confer voltage-dependent reversal to $G\beta\gamma$-mediated inhibition (Fig 4) [173-176].

The Dolphin lab introduced a mutation (W391A) into the AID on the I-II linker of $Ca_V2.2$ channels which reduces CaVβ subunit binding affinity by ~1000 fold. [174]. While the extent of $G\beta\gamma$-mediated inhibition was similar for mutant (W391A) and wild-type channels, prepulse reversal of the inhibition was almost abolished in the mutant. Expression of wild type $Ca_V2.2$ along with $\alpha_2\delta$ but without $Ca_V\beta$ resulted in similar findings, and the voltage-independent inhibition in the absence of the $Ca_V\beta$ was blocked by overexpression of transducin which acts to scavenge free $G\beta\gamma$ subunits [175]. Thus, in the absence of $Ca_V\beta$ binding to the I-II linker, $G\beta\gamma$-mediated inhibition of the channels was still present but could no longer be reversed in a voltage-dependent manner. The experiments outlined above used the β_{1b} subunit, but when β_{2a} was expressed with the W391A channels voltage-dependent relief of the $G\beta\gamma$ mediated inhibition was restored. Unlike β_{1b}, the β_{2a} subunit is palmitoylated at two N-terminal cysteine residues, and mutation of these residues led to loss of voltage-dependent relief (i.e. the data resembled β_{1b}). The authors proposed that palmitoylation increased the local plasma membrane concentration of β2a such that low affinity interaction with α_1 could still take place and permit voltage-dependent relief of the inhibition.

The Yang lab came to similar conclusions for $Ca_V2.1$ channels [176]. In this case the authors mutated $Ca_V\beta$ to reduce the affinity for the AID. The channels were expressed in Xenopus oocytes and macroscopic currents were recorded from giant inside-out patches that contained many channels. Washing the cytoplasmic face of the patches resulted in dissociation of $Ca_V\beta$ (due to the reduced binding affinity of the mutant), and this was confirmed by the expected shifts in channel kinetics compared to wild type. In these channels lacking $Ca_V\beta$, purified $G\beta\gamma$ still inhibited the currents but prepulse reversal was abolished.

The $Ca_V\beta$ subunit consists of SH3 and GK domains separated by a variable HOOK region [48, 49]. Binding to the AID on the I-II linker of α_1 is mediated by the GK domain, although interaction between the SH3 and HOOK domains elsewhere on the α_1 subunit might also modulate functional properties. In terms of $G\beta\gamma$ effects, voltage-dependent reversal was restored even by binding of the isolated GK domain of $Ca_V\beta$ to the AID [175, 176]. In the absence of such binding the AID adopts a random coil, but the presence of $Ca_V\beta$ induces an α-helical conformation that extends back to the interface with IS6 [46, 47, 177, 178]. The Yang lab introduced seven glycines between the AID and IS6 to disrupt this α-helical structure and found that this prevented the ability of $Ca_V\beta$ to confer voltage-dependence to the inhibition [176]. Conversely, introducing seven alanines (not expected to

disrupt the α-helix) maintained the ability of $Ca_V\beta$ to confer voltage-dependence to Gβγ-mediated inhibition. It is possible that binding of $Ca_V\beta$ to the AID induces a rigid α-helical link with domain IS6, and this transmits movement of the voltage-sensor and activation gate (including IS6) to the I-II linker to alter the Gβγ binding pocket at depolarized potentials. It is also worth noting that Gβγ-mediated inhibition was still present in both channel types lacking $Ca_V\beta$, and in the $Ca_V2.1$ channels containing the seven glycine insert [175, 176]. Apparently the rigid α-helical link to the upstream activation gate and voltage-sensor is not required *per se* to transduce binding of Gβγ into functional inhibition. $Ca_V\beta$ might also influence Gβγ-mediated inhibition in other ways. For example, deletion of the HOOK domain promoted tonic inhibition of $Ca_V2.2$ channels, perhaps due to increased affinity for the basal level of free Gβγ in the cells [175].

9. Crosstalk between N-type channels, Gβγ, kinases and synaptic proteins

9.1. Protein kinase C

Most cell signaling events do not occur in isolation, but instead in an integrated fashion. G protein regulation of voltage-gated calcium channels is no exception. This is exemplified by the modulation of voltage dependent Gβγ inhibition of N-type channels by protein kinase C (PKC). In peripheral neurons, activation of PKC was shown to reduce the extent of subsequent G protein modulation by a number of different receptor pathways, including GABA-B, adenosine and muscarinic receptors [179-181]. Such an interference with G protein inhibition could be due to PKC dependent phosphorylation of the G protein interaction site on the channel, the G protein coupled receptor, or the G protein itself. The first hint supporting the first mechanism came from experiments showing that in vitro phosphorylated domain I-II linker peptides could no longer effectively interact with Gβγ peptides [160]. Subsequent work showed that a threonine residue within the putative I-II linker Gβγ interaction site was responsible for this effect. When phosphorylated, or substituted for glutamic acid, this residue destabilizes the interaction of the channel with Gβγ, and its substitution for alanine precludes the antagonistic effects of PKC [155]. Interestingly, only Gβ1 mediated signaling (not other Gβ isoforms) was subject to this type of PKC crosstalk [182], and this was attributed to a single locus unique to Gβ1 [153] (Fig 3C). This observation suggests that activation of Gq-coupled receptors can modulate signaling of certain types of Gβ1 linked receptors to N-type calcium channels. It should also be noted that PKC activation not only results in antagonistic effects on Gβγ-mediated inhibition but, depending on N-type channel splice variant, can also promote direct enhancement of current activity [155]. This is mediated by phosphorylation of both the above noted threonine residue and an adjacent serine and adds further complexity to the PKC-G protein signaling crosstalk.

9.2. Synaptic proteins

The two types of calcium channels that are most susceptible to the effects of Gβγ also control neurotransmitter release at CNS synapses [1]. Both Cav2.1 and Cav2.2 channels physically associate with proteins that are involved in synaptic vesicle release, such as syntaxin 1A and SNAP25. These SNARE proteins bind directly to a synaptic protein interaction (synprint) site on the II-III linker (Fig 1) which serves to bring the channels into close proximity of the synaptic vesicle release sites [183-189]. RIMs (rab3 interacting molecules) have also emerged as important organizers of the presynaptic active zone [190], and bind Ca_V2 channels both directly and through RIM binding proteins to control their density and localization at release sites [191-195]. Binding of syntaxin 1A to both Cav2.1 and Cav2.2 also results in a hyperpolarizing shift in the voltage dependence of channel inactivation [185, 196-198] (for review see [199]). In addition to this effect on channel gating, syntaxin 1A modulates G protein regulation of the channels. Coexpression of

syntaxin 1A with N-type channels in tsA-201 cells induces tonic inhibition mediated by Gβγ [200]. Syntaxin 1A physically associates with Gβγ at a site distinct from that involved in binding to the N-type channel [198, 201], suggesting the possibility that syntaxin 1A serves to colocalize the channels and Gβγ to ultimately promote a form of tonic inhibition. In contrast, syntaxin 1B does not mediate such an effect, even though it is capable of binding to both the channel and Gβγ [202]. This may suggest that the spatial orientation of the syntaxin/G protein complex relative to the N-type channel complex is critical for functional modulation. Gβγ interaction with SNARE proteins might also serve to directly regulate neurotransmitter release in both lamprey and mammalian neurons and neuroendocrine cells [203-206].

Several other types of synaptic proteins have been shown to alter G protein regulation of N- and P/Q-type channels. Cysteine string protein (CSP) interacts with G proteins and the synprint site and mediates an effect similar to that seen with syntaxin 1A [207]. In addition, CSP appears to stimulate Gα subunit activity by promoting the exchange of GTP for GDP in a receptor independent manner [208]. In contrast to these enhancing effects of syntaxin 1A and CSP, coexpression of Rim1 with Cav2.2 in HEK293 cells promotes “deinhibition” (recovery from Gβγ-mediated inhibition during depolarization) in addition to substantially slowing channel inactivation [209].

9.3. Calcium channel γ subunits

Another protein of note is stargazin, a member of the calcium channel γ subunit family. Skeletal muscle $Ca_V1.1$ channels have been shown to associate with a γ1 subunit in addition to β and α2δ subunits. Several neuronal γ subunits have been identified although it remains uncertain that these constitute *bona fide* channel subunits. Indeed the γ2 isoform (also called stargazin) and related proteins (γ3-7) associate with and modulate glutamatergic AMPA receptors [210]. However, it has been shown that stargazin can bind Gβγ *in vitro*, and acts to scavenge Gβγ and reduce inhibition of $Ca_V2.2$ channels in *Xenopus* oocytes [211]. Altogether, these findings highlight the notion that Gβγ modulation of calcium channels does not occur in isolation, but is tightly controlled by a wide range of cellular processes and signalling pathways.

10. Direct GPCR/N-type calcium channel interactions

Efficient signaling necessitates close proximity between GPCRs and effectors such as ion channels. This can be accomplished through the formation of large macromolecular signaling complexes between receptors, channels, G proteins, and kinase anchoring proteins [212-215]. In addition, a physical association between receptors and channels provides for a possible mechanism by which receptors can control channel function in an agonist independent manner. This was first shown by Kitano and colleagues [216] who identified a physical association of Cav2.1 channels with metabotropic glutamate receptors that results in altered P/Q-type channel function. For N-type channels, the formation of a signaling complex between Cav2.2 and the NOP (a.k.a. nociceptin) receptor was demonstrated in dorsal root ganglion neurons, and shown to promote tonic voltage-dependent modulation in the absence of receptor ligand, presumably reflecting constitutive receptor activity [217]. Similar observations have been reported for δ- and μ-opioid receptors coexpressed with N-type channels in tsA-201 cells [218, 219].

Association of channels with receptors also provides for an additional level of control through regulation of channel density in the plasma membrane. NOP receptors coexpressed with N-type channels not only promote the cell surface expression of the channels (Fig 5A), but also trigger an agonist mediated co-internalization of the channel/receptor complex into lysosomes [219, 220], thus giving rise to a new form of voltage-independent inhibition (Fig

5B). The extent to which this occurs in neurons is up to some debate. While imaging studies show a clear NOP receptor mediated internalization of channels in cultured DRG neurons, and reduced calcium entry in response to prolonged activation of receptors [220], there do not appear to be clear effects on whole cell current densities in nociceptin treated neurons [221]. It is possible that receptor mediated internalization is offset by kinase pathways that augment the activities of channels remaining in the plasma membrane. D1 and D2 dopamine receptors also associate with N-type calcium channels [222, 223], but while NOP receptors and Cav2.2 channels interact via their C-termini, D1 and D2 receptors also interact with other regions of the Cav2.2 channel α1 subunit such as the domain II-III linker. As with the NOP receptor, D1 or D2 receptor coxpression facilitates trafficking of the channels to the plasma membrane, and allows for receptor-channel co-internalization. In prefrontal cortex neurons, the D1 receptor appears to target N-type channels to dendritic sites [222]. It is likely that other types of GPCR may form complexes with N-type and perhaps P/Q-type calcium channels, however this will need to be confirmed experimentally. Altogether, the formation of macromolecular signaling complexes between receptors and channels provides for previously unrecognized means for controlling channel activity/density.

11. Voltage-independent inhibition of Ca_V2 channels by Gq-coupled GPCRs

Sympathetic neurons have been used extensively to investigate modulation of $Ca_V2.2$ (N-type) channels, and at least two distinct pathways have been identified: the “fast” pathway, mediated by pertussis toxin sensitive Gi/o-coupled GPCRs, is due to direct, voltage-dependent inhibition by Gβγ; a “slow”, voltage-independent pathway takes tens of seconds to develop, is mediated by Gq-coupled GPCRs, involves a diffusible second messenger, and is sensitive to intracellular [BAPTA] (for reviews see [224, 225]). Typical Gq-coupled signaling pathways downstream of phospholipase Cβ including IP3/Ca^{2+}, diacylglycerol, and PKC were shown not to mediate the inhibition, and the pathway remained elusive for quite some time. In the past decade evidence has mounted implicating depletion of plasma membrane PIP_2 and/or generation of arachidonic acid as possible mediators of this inhibition [226-229]. Here we outline the proposed mechanisms along with some recent developments.

PIP_2 is required for a variety of ion channels to function (for reviews see [229-232]). The first evidence that this included Ca^{2+} channels was the demonstration that time-dependent “rundown” (loss) of $Ca_V2.1$ channel activity in excised membrane patches was slowed by application of PIP_2 and accelerated by depleting or sequestering PIP_2[233]. Similar effects were subsequently reported for N-type ($Ca_V2.2$) channels, along with evidence that the “slow” inhibition by Gq-coupled GPCRs in sympathetic neurons was due to phospholipase C mediated PIP_2 hydrolysis [234]. For example, inhibition by muscarinic receptors was blunted by including PIP_2 in the patch pipette, whereas recovery from inhibition was slowed by blocking PI-4 kinases which replenish the depleted PIP_2. The overall picture that has emerged is that PIP_2 is required for channels to open in response to membrane potential changes. This may involve dynamic low affinity interaction of PIP_2 with the channels and perhaps additional higher affinity binding to a distinct channel domain. It has been postulated that such interactions might “crosslink” hydrophobic and hydrophilic domains and favor protein conformations conducive to active channel states. Similar to Gq-mediated modulation of M-type potassium channels [226, 230], this model proposes that depletion of local PIP_2 by phospholipase C mediated hydrolysis removes this permissive interaction and is both necessary and sufficient to inhibit channel activity.

An alternative, although related, lipid signaling pathway has been proposed by the Rittenhouse lab [227] who reported that arachidonic acid elicits bidirectional modulation of N-type channels; I_{Ca} was enhanced at relatively hyperpolarized test potentials and inhibited

at more depolarized potentials [235, 236]. The enhancement seems to involve extracellular actions of arachidonic acid [237], whereas the inhibition is mediated at the cytoplasmic face of the membrane. Arachidonic acid can be produced either by the action of phospholipase A2 on PIP_2 and other membrane phospholipids, or by the action of diacylglycerol-lipase on diacylglycerol [227]. It is postulated that activation of these lipases by muscarinic receptors cleaves PIP_2 and generates arachidonic acid which binds to the channel. This binding has the opposite effect to PIP_2 such that it stabilizes closed/inactivated states of the channel and thus leads to inhibition. The involvement of arachidonic acid is a matter of some debate, in part due to conflicting reports on the ability of DAG-lipase inhibitors to block channel inhibition [226, 227, 234, 236].

A recent study from the Hille lab has provided evidence in support of the PIP_2 depletion model [238]. To avoid downstream and parallel signaling pathways subsequent to Gq-coupled receptor activation, the authors used controlled activation of exogenous polyphosphoinositide 5-phosphatases which convert PIP_2 into PI(4)P [239, 240]. In one approach, cells were transfected with a voltage-sensitive phosphatase (VSP) that enabled rapid (~1s) and reversible depletion of PIP_2. Another approach involved chemical dimerization to translocate transfected yeast INP54p 5-phosphatase to the membrane and irreversibly deplete PIP_2. In both cases I_{Ca} was inhibited by PIP_2 depletion, and the rate/extent of recovery from this inhibition tracked the rate/extent of PIP_2 resynthesis [238]. These data support the idea that depletion of PIP_2 in of itself is sufficient to inhibit I_{Ca}. However, it was noted that the magnitude of I_{Ca} inhibition was less than that produced by muscarinic receptors, even though the predicted depletion of PIP_2 is comparable. Therefore, it is possible that another signal, perhaps arachidonic acid, also contributes to the Gq-mediated inhibition, perhaps through synergistic actions with PIP_2 depletion [226, 227].

11.1. $Ca_V\beta$ and intracellular Ca^{2+} modulate Gq-mediated inhibition

Notably, it has been reported that the Ca_V β2a subunit opposes inhibition of I_{Ca} by arachidonic acid or PIP_2 depletion. The β2a subunit is palmitoylated at two N-terminal residues and it is this lipidation that diminishes the inhibition. Indeed, N-type channels containing β2a are enhanced rather than inhibited by Gq-coupled GPCRs and arachidonic acid [60, 227, 241]. Rittenhouse and colleagues postulated that the palmitoyl groups interact directly with the α1 subunit of the channel and thereby mask an inhibitory binding site for arachidonic acid. Inhibition of I_{Ca} by PIP_2 depletion (using a voltage-sensitive phosphatase) is also diminished in channels containing a palmitoylated β2a subunit [61]. It is speculated that the palmitoyl groups of $Ca_V\beta2a$, the lipid tail of PIP_2, and arachidonic acid compete for binding to a site(s) on the α1 subunit of the channel. Binding of the palmitoyl groups or lipid tails of PIP_2 favors active channel conformations whereas loss of this interaction and/or binding of arachidonic acid favors closed/inactive channel conformations.

Highlighting the complexity of neuronal Ca^{2+} channel regulation, it is noteworthy that even in the same cell, not all Gq-coupled GPCRs elicit inhibition of I_{Ca} [234]. This correlates with the ability of the different receptors to elicit significant IP_3 mediated release of intracellular Ca^{2+} stores which likely relates to the proximity of the GPCR, IP3 receptors, and other components of a macromolecular signaling complex [226, 242]. In turn, such Ca^{2+} elevations are postulated to promote phosphatidylinositol 4-kinase activity thereby preventing local PIP_2 depletion and inhibition of I_{Ca} [226, 234, 243, 244]. Thus, the extent of this voltage-independent inhibition depends on the subunit composition of the channels ($Ca_V\beta$ isoform), and colocalization of GPCRs and various phosphoinositide and Ca^{2+} signaling components in macromolecular signaling complexes. Adding further to this complexity, another type of “fast”, voltage-independent inhibition of N-type I_{Ca} mediated by a distinct pathway(s) perhaps involving both Gα and Gβγ signaling has also been described in these same sympathetic neurons [245, 246].

12. Kinase-mediated, voltage-independent inhibition of Ca_V2 channels in sensory neurons

Voltage-independent inhibition that appears to involve channel phosphorylation has also been described. For example, PKC has been implicated in the inhibition of N-type I_{Ca} in chick sensory neurons [247, 248] In frog and mammalian neurons PKC can also potentiate I_{Ca}, might target multiple phosphorylation sites with opposing actions, antagonize Gβγ mediated inhibition, or modulate channel trafficking (see sections 9, 10) [155, 180, 181, 249, 250]. It was also reported that rapid activation of a tyrosine kinase by $GABA_B$ receptors resulted in voltage-independent inhibition of N-type I_{Ca} in chick sensory neurons [251]. More recently, the Lipscombe lab demonstrated that manifestation of this tyrosine kinase mediated inhibition in mammalian neurons is controlled by alternative splicing of the $Ca_V2.2$ C-terminus (Fig 6) [252, 253]. There are two mutually exclusive forms of exon 37 (e37a and e37b). Gβγ-mediated, voltage-dependent inhibition is identical in recombinant channels containing either e37a or e37b. However, channels containing exon 37a are also inhibited by another voltage-independent pathway. This second pathway involves rapid activation of pp60c-src tyrosine kinase and requires a tyrosine residue (Y1747) present in exon 37a that is replaced by phenylalanine in exon 37b. Of particular note, expression of exon 37a is restricted to dorsal root ganglia, and preferentially expressed in capsaicin sensitive, nociceptive neurons [254]. The gene encoding chicken $Ca_V2.2$ only has one exon 37 which is similar to e37a and includes a tyrosine residue [252]. This then explains the restriction of this pathway to nociceptive neurons in mammals and its prevalence in chick neurons. This expression pattern also suggests that the e37a splice variant might be tailored to play a role in pain transmission [255]. Recently, using an exon replacement strategy in mice, it was shown that basal nociceptive transmission was unaltered by loss of e37a, but the analgesic effects of intrathecal morphine were diminished [253].

13. Concluding remarks

In this review we have highlighted the complex inhibition of Ca_V2 channels by G protein coupled receptors. Voltage-dependent inhibition, mediated by direct binding of Gβγ to the Ca^{2+} channel α1 subunit, is the most common and best understood mechanism. Membrane potential, firing patterns, channel subunit composition/splice variants, and Gβγ heterodimer composition all modulate the extent and/or kinetics of voltage-dependent inhibition. Although less well understood and perhaps less widespread, there are also several mechanisms leading to voltage-independent inhibition of Ca_V2 channels. These include direct interaction with GPCRs, inhibition through lipid signaling pathways, and channel phosphorylation. Ca_V2 channels are also subject to a variety of other regulatory mechanisms, notably Ca^{2+}-dependent feedback (both inactivation and facilitation). Thus, GPCRs in combination with Ca^{2+} channels sense and integrate a complex array of inputs in order to fine tune the spatiotemporal aspects of Ca^{2+} entry that play such pivotal roles in cellular physiology and synaptic transmission.

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Highlights

- Ca_V2 channels play pivotal roles in neurotransmitter and hormone release
- G protein coupled receptors orchestrate precise control of Ca_V2 channels
- *Voltage-dependent* inhibition is mediated by direct binding of Gβγ to the channels
- *Voltage-independent* inhibition is mediated by several other distinct pathways
- Current understanding of these important mechanisms is provided in this review

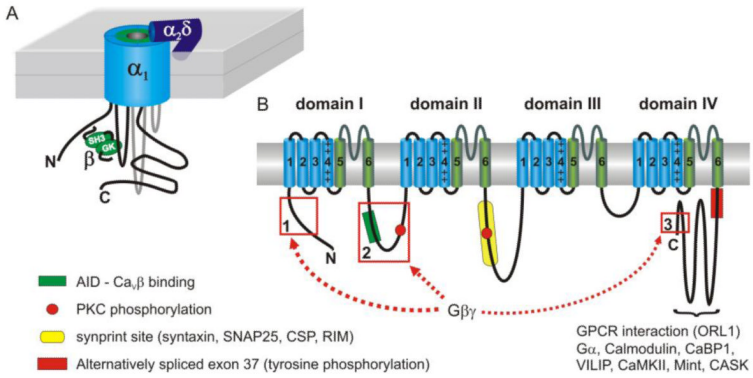

Figure 1.

Schematic depiction of the topology and subunit composition of Ca_V2 voltage-gated Ca^{2+} channels. (**A**) Cartoon showing the 3D topology along with channel auxiliary subunits. The intracellular β subunit interacts through its guanylate kinase-like domain (GK) with the I-II linker of the α1 subunit (at the α-interaction domain or AID). The α2δ subunit is largely extracellular and likely GPI-anchored to the plasma membrane. (**B**) Topology of the pore forming α_1 subunit. Four homologous repeats (domain I through domain IV) each consist of six transmembrane spanning α-helices (S1-S6) (blue or green cylinders) and a 'P-loop' between S5 and S6. The S5-S6 helices and P-loop comprise the pore domain of the channel (colored green), while S1-S4 (in particular S4 that has multiple charged residues) comprises the voltage sensor (colored blue). The intracellular N- and C-termini and the cytoplasmic loops connecting domains I-IV are important for interaction with other proteins including the auxiliary β subunit, synaptic proteins, Gβγ, GPCRs, calmodulin and other Ca^{2+} binding proteins (CaBP1, VILIP). These cytoplasmic domains are also targeted by second messenger pathways including phosphorylation by PKC, CaMKII, and tyrosine kinases. Alternative splicing greatly increases the functional diversity of the channels. For example, alternative splicing of exon37 on the proximal C-terminus controls inhibition of $Ca_V2.2$ channels by GPCRs in sensory neurons (see section 12 for more details).

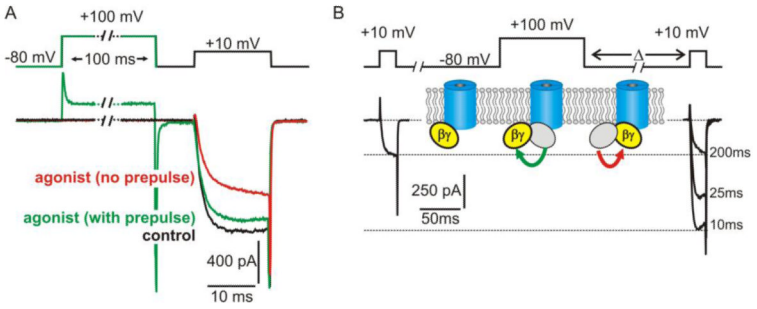

Figure 2.

Functional effects of voltage-dependent inhibition on Ca_V2 channels. (**A**) "Whole cell" patch clamp recording of I_{Ca} from an adrenal chromaffin cell which express purinergic P2Y autoreceptors. Application of a P2Y receptor agonist (red trace) inhibited I_{Ca} compared to control conditions (black trace) with the hallmark features of voltage-dependent inhibition. Peak amplitude was reduced with prominent slowing of the activation kinetics, and both of these effects were reversed by a conditioning prepulse to +100mV (green trace). (**B**) Voltage-dependent relief of inhibition reflects transient dissociation of Gβγ from the channel. Shown is an example of "whole cell" I_{Ca} recorded from recombinant $Ca_V2.2$ channels expressed with β1b, α2δ and Gβγ in HEK293 cells. Gβγ produced tonic inhibition of I_{Ca} that was reversed by a conditioning prepulse to +100 mV. The magnitude of this reversal (prepulse facilitation) diminished as the interval between prepulse and test pulse (Δ) was increased (examples shown are with Δ = 10 ms, 25 ms, and 200 ms). As illustrated by the inset cartoon, prepulse facilitation is thought to reflect dissociation of Gβγ from an inhibitory binding site on the channel at the depolarized membrane potential. Upon return to the hyperpolarized membrane potential, Gβγ rebinds to (and re-inhibits) the channel. This re-inhibition of I_{Ca} is monoexponential, and the rate depends on the local concentration of Gβγ.

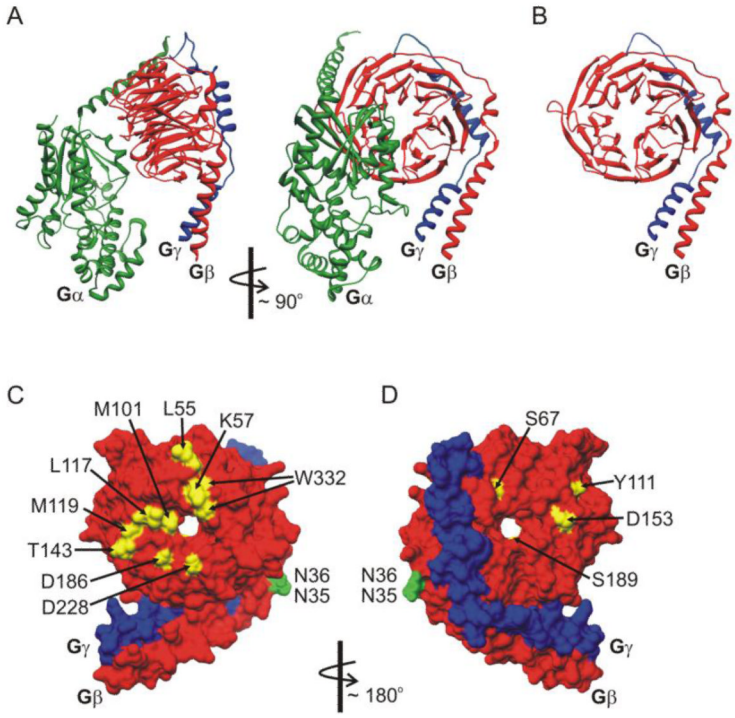

Figure 3.

Structural determinants on Gβγ that govern modulation of Ca_V2 channels. (**A, B**) Ribbon diagram renderings of the heterotrimeric G protein structure in panel A, and the Gβγ dimer in panel B ($G\alpha_i$ - green; $G\beta_1$ - red; and $G\gamma_2$ blue). Gβ adopts a seven blade β-propeller structure with an α-helical N-terminal domain that binds to the α-helical N-terminus of Gγ. Gα interacts with multiple residues on the top face of Gβ and the side aspect of propeller blade 1. Many effectors bind to a protein interaction "hot spot" on the surface of Gβ that is masked by Gα in the heterotrimer. (**C, D**) Molecular surface rendering of the Gβγ dimer (Gβ - red; Gγ - blue). Panel C shows the Gα interacting face of Gβγ, and panel D is rotated ~180° to show opposite face of Gβγ. Residues marked in yellow have been reported to disrupt inhibition of Ca_V2 channels. Residues marked in green are involved in crosstalk between Gβ1 and PKC phosphorylation of $Ca_V2.2$. Molecular graphics images based on data reported by Wall et al [144] (PDB ID: 1GP2) were produced using the UCSF Chimera package [256, 257] from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

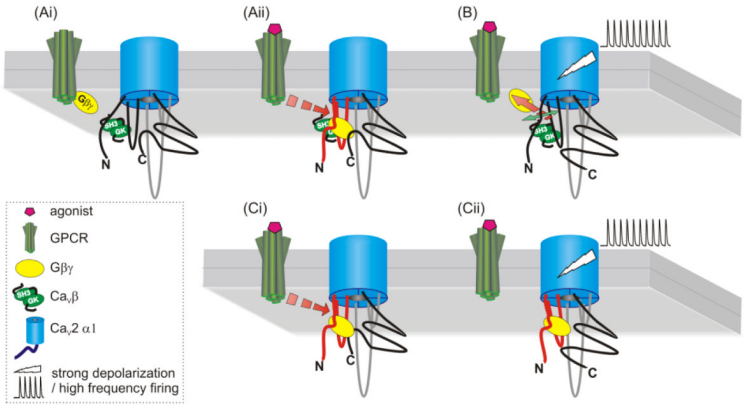

Figure 4.

Model depicting the molecular interactions that underlie Gβγ-mediated inhibition of Ca_V2 channels. Panels A and B (upper three images) depict a channel with a $Ca_V\beta$ subunit, while panel C (lower images) depicts the situation in which the $Ca_V\beta$ subunit is absent. Currently, data suggest 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 promotes interaction of the N-terminus "inhibitory module" with the initial one-third of the I-II-linker (panel Aii). This (and perhaps other interactions) shifts gating charge movement to more depolarized potentials and uncouples voltage-sensor movement from channel activation. Strong membrane depolarization (panel B) leads to conformational changes that result in unbinding of Gβγ and loss of interaction between the N-terminus and I-II linker. This depends upon binding of a $Ca_V\beta$ subunit to the AID on the I-II linker that induces a rigid α-helical connection to the upstream IS6 region of the pore and voltage-sensor. In the absence of $Ca_V\beta$ subunit binding, inhibition still occurs (panel Ci) but cannot be reversed by strong depolarization (panel Cii).

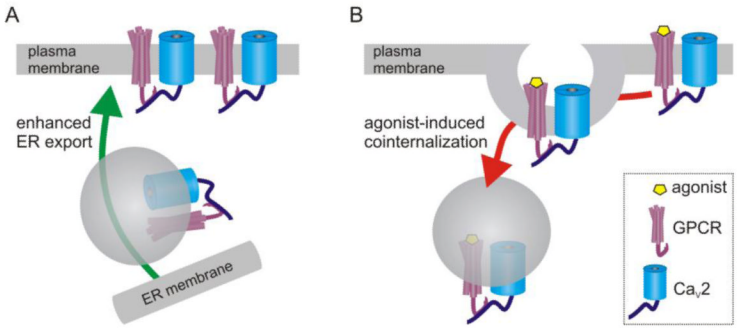

Figure 5.

Trafficking-mediated modulation of $Ca_V2.2$ channels due to direct interaction with GPCRs. **(A)** Nociceptin receptors (NOP) interact directly with the Cav2.2 α1 subunit via their C-termini. D1 and D2 receptors also interact with additional regions of the α1 subunit such as the domain II-III linker. Coexpression of these GPCRs with $Ca_V2.2$ facilitates trafficking of the channels to the plasma membrane, and the D1 receptor appears to target N-type channels to dendritic sites in prefrontal cortex [222]. (**B**) Prolonged NOP agonist application has been reported to promote co-internalization of the receptor/channel complex into lysosomes in cultured sensory neurons, thus giving rise to a new form of voltage-independent inhibition [220] (but see [221]).

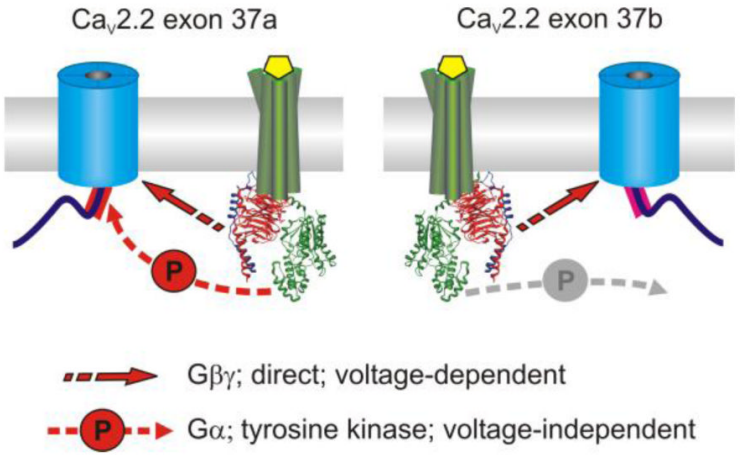

Figure 6.

Alternative splicing of $Ca_V2.2$ controls voltage-independent inhibition of N-type I_{Ca} in sensory neurons. Two mutually exclusive forms of exon 37 encode the proximal C-terminus of $Ca_V2.2$. Expression of exon 37a is restricted to dorsal root ganglia, preferentially in nociceptive neurons, while exon 37b is widely expressed throughout the nervous system [254]. Gβγ-mediated, voltage-dependent inhibition of I_{Ca} is identical in channels containing either isoform of exon 37. An additional Gα-mediated, voltage-independent pathway involving pp60c-src tyrosine kinase inhibits channels containing exon 37a but not exon 37b. Thus alternative splicing of $Ca_V2.2$ results in cell-type specific alteration in the magnitude and mechanisms of GPCR-mediated inhibition.