# Modulation of voltage-dependent calcium channels by G proteins

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Voltage-gated calcium channels are found in all excitable cells, in which they regulate many important physiological functions, including excitability, gene transcription, muscle contraction, and neurotransmitter and hormone release. The differential modulation of calcium channels by intracellular second messengers constitutes a key mechanism for controlling calcium influx. Recent advances have provided important clues to the underlying molecular mechanisms involved in the inhibition of N-type and P/Q-type calcium channels by a membrane-delimited G-protein-dependent pathway.

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**Abbreviations**

| | |
|---|---|
| **AID** | $\alpha_1$ interaction domain |
| **GABA** | γ-aminobutyric acid |
| **PKC** | protein kinase C |

## Introduction

Nearly two decades ago, Dunlap and Fischbach [1] first reported the rapid inhibition of calcium currents in chick dorsal root ganglia (DRG) neurons by noradrenaline and serotonin. In the intervening years, many hormones and neurotransmitters, acting via seven transmembrane G-protein-coupled receptors (including muscarinic acetylcholine, GABA, opioid, somatostatin, prostaglandin, adrenergic and adenosine receptors), have all been linked to the inhibition of native calcium currents ([2–12]; reviewed in [13]).

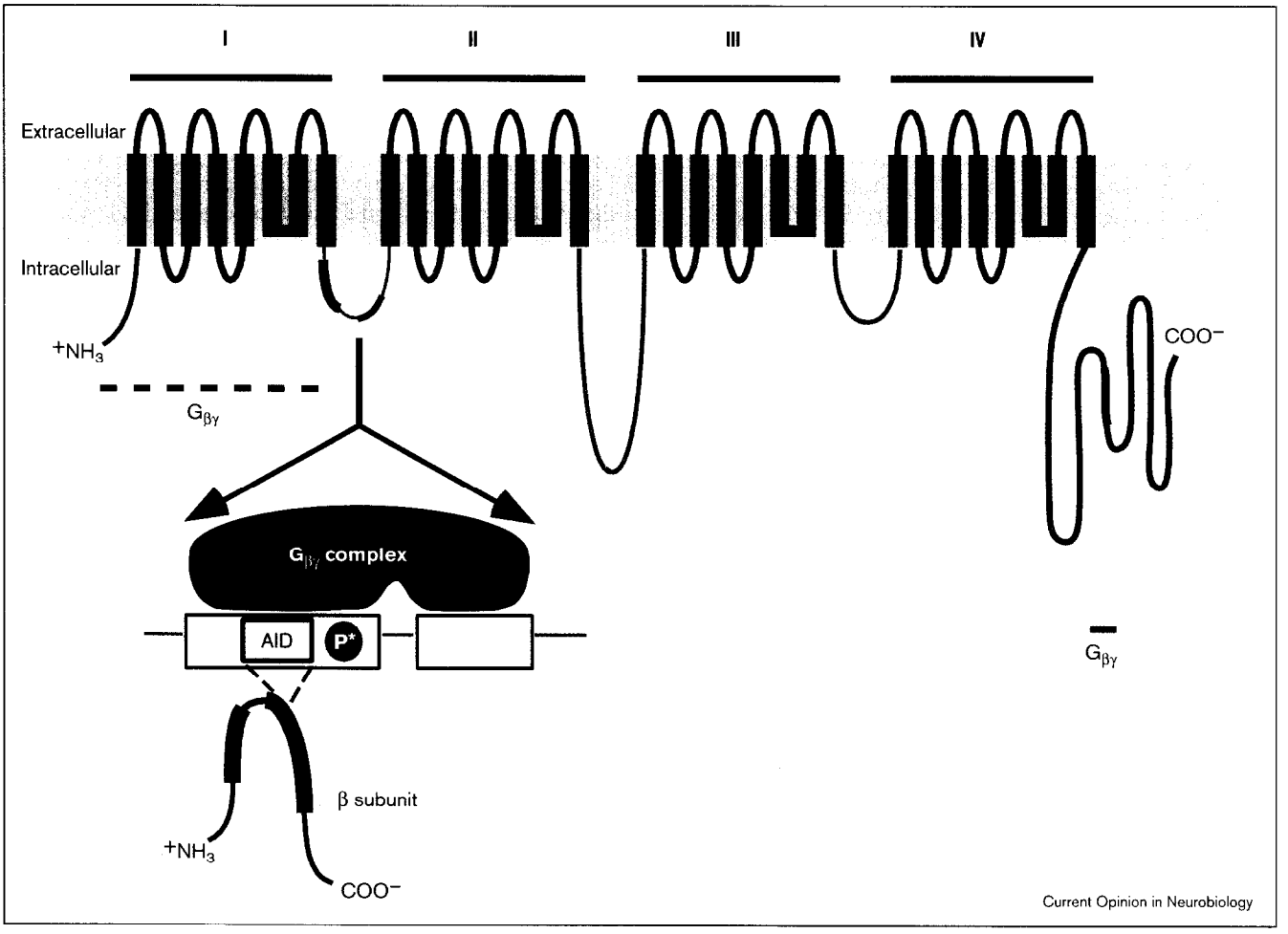
There are a number of defining characteristics common to the rapid, transmitter-induced inhibition of high-threshold calcium currents. First, the activation of G-protein-coupled receptors results in the reduction of peak current amplitude, which is accompanied by an apparent slowing of activation and inactivation kinetics. Second, treatment with pertussis toxin abolishes transmitter-induced inhibition in most cases. In many neuronal and endocrine preparations, inhibition is predominantly linked to the activation of the $G\alpha_o$ class of heterotrimeric G-proteins, although in some instances $G\alpha_i$ and $G_q$ have also been implicated (see, for example, [3–9,14–18]). Third, calcium current inhibition appears to occur via a membrane-delimited pathway rather than via diffusible cytoplasmic messengers ([19]; reviewed in [13]). Finally, membrane delimited G-protein-dependent inhibition is strongly voltage dependent and can be temporarily relieved by the application of strong depolarizations that result in an apparent prepulse-dependent facilitation (a voltage-dependent unblock of the channel (see, for example, [5,6,20]).

At the single-channel level, it has been proposed that G-protein-dependent inhibited channels are less likely ('reluctant') to open in response to membrane depolarization [6]. Within this proposed framework, strong membrane depolarizations may mediate a transition from the 'reluctant' (G-protein inhibited) to a 'willing' (noninhibited) gating state [5,6,12,13,20,21]. On the basis of the independence of the prepulse effect on neurotransmitter concentration, it has been suggested that the prepulse might induce a conformational change in the channel and thus a change in gating behavior that does not involve the complete unbinding of the G protein [5]. In contrast, others have proposed that the prepulse might mediate a complete physical dissociation of the G protein(s) from the channel, and that recovery from facilitation over time may be due to a reassociation of the channel with the G protein(s) (reviewed in [13]). The exact stoichiometry underlying G-protein inhibition and recovery from the prepulse effect has been the subject of some discussion. Models have been proposed in which one, two or four G-protein molecules interact simultaneously with the channel complex [12,20,21].

## Which types of calcium channels are inhibited by G proteins?

Electrophysiological and pharmacological characterizations define multiple classes of native calcium channels: T, N, L, P/Q and R types (reviewed in [22,23]). In most neurons, the *Conus geographus* toxin ω-conotoxin GVIA blocks the G-protein-sensitive current component, indicating that N-type channels represent a major target for inhibition [13]. In addition, P/Q-type channels are inhibited by a variety of transmitters, including opioids, GABA and somatostatin [11]. In both native cells and exogenous expression systems, N-type currents are inhibited to a significantly greater extent than P/Q-type currents [24•–26•]. Because both N-type and P/Q-type calcium channels mediate neurotransmission at many central and peripheral synapses [23], their differential modulation by G proteins suggests that these channels may make unique contributions to neurotransmission.

**Figure 1**




Biochemical interaction of the $G_{\beta\gamma}$ complex with non-L-type calcium channel $\alpha_1$ subunits. Activation of heterotrimeric G-protein releases $G_{\beta\gamma}$, which directly binds to the $\alpha_1$ subunit domain I–II cytoplasmic linker [40••,41••] and to 38 amino acid region in the carboxyl terminus [33•]. Functional analyses examining chimeric channels also implicate domain I and possibly the amino terminus as contributing to G-protein-dependent modulation [26•]. Within the domain I–II linker, $G_{\beta\gamma}$ binds to two separate regions, one overlapping the site of interaction of the $\alpha_1$ subunit with the calcium channel β subunit (i.e. the AID site) and a second downstream site [40••,41••]. The PKC-dependent phosphorylation of a site within the first $G_{\beta\gamma}$ binding region (P*) attenuates G-protein-dependent inhibition [40••].

Biochemical studies show that neuronal calcium channels are heteroligomeric complexes composed of $\alpha_1$, $\alpha_2\delta$ and β subunits. Molecular cloning has identified nine calcium channel $\alpha_1$ subunits genes ($\alpha_{1A}$–$\alpha_{1H}$ and $\alpha_{1S}$), four β subunit genes ($\beta_1$–$\beta_4$) and a single gene encoding an $\alpha_2\delta$ subunit (reviewed in [27,28] and also see [29••]). In most instances, the pore-forming $\alpha_1$ subunits have been assigned to a native calcium current counterpart: $\alpha_{1A}$ encodes P/Q-type channels, $\alpha_{1B}$ encodes an N-type calcium channel, $\alpha_{1C}$, $\alpha_{1D}$ and $\alpha_{1S}$ all encode L-type channels, and $\alpha_{1E}$ constitutes a novel channel that exhibits some similarities to both high-threshold and low-threshold channels. The most recently identified, $\alpha_{1G}$ and $\alpha_{1H}$, have been shown to encode two different isoforms of T-type calcium channels [29••]. Exogenous expression of cloned calcium channels reveals that G-protein activation selectively modulates $\alpha_{1A}$ and $\alpha_{1B}$ in a manner identical to that for native P/Q-type and N-type currents, respectively [25•,26•,30•,31•], whereas $\alpha_{1C}$ L-type currents do not exhibit G-protein sensitivity [25•,26•]. In contrast, some studies suggest that $\alpha_{1E}$ currents can be inhibited by G proteins but in others inhibition only occurs in the absence of a coexpressed calcium channel β subunit [30•,31•,32,33•]. Because $\alpha_{1E}$ channels contain high affinity β-subunit binding site(s) and are modulated by β-subunit coexpression, the physiological significance of these results remains to be determined.

## $G_{\beta\gamma}$ subunits inhibit neuronal calcium channels

Over the years, a large number of studies utilizing a variety of approaches including the exogenous expression of $G_\alpha$ subunits and the inhibition of $G_\alpha$ subunits with antisense oligonucleotides and antibodies have lead to the notion that $G_{\alpha o}$ and/or $G_{\alpha i}$ subunits are responsible for calcium channel inhibition (reviewed in [34]); however,

two recent studies [35••,36••] show that in the absence of agonist, the coexpression of $G_{\beta\gamma}$ subunits but not $G_{\alpha}$ mimics the transmitter-induced inhibition of N- and P/Q-type currents. All $G_{\beta\gamma}$ combinations tested ($G_{\beta1\gamma2}$, $G_{\beta1\gamma3}$, $G_{\beta1\gamma7}$) were equally effective and could account for all agonist-induced modulatory properties including: kinetic slowing, reduced peak current magnitude and prepulse-dependent facilitation. Although $G_{\beta\gamma}$ subunits can clearly mimic the effects of receptor-mediated calcium channel inhibition, it remains to be demonstrated whether $G_{\alpha}$ subunits released from the heterotrimeric G-protein complex are inert or whether they have effects on other aspects of channel modulation.

## $G_{\beta\gamma}$ subunits directly target the calcium channel $\alpha_1$ subunit

The first clue that the calcium channel $\alpha_1$ subunit itself is the target of G-protein interaction comes from studies in which antisense oligonucleotides against the calcium channel β subunit enhance the G-protein-dependent inhibition of native N-type currents [37]. In support of this, omission of the calcium channel β subunit results in an increase in the opioid-induced inhibition of exogenous $\alpha_{1A}$ P/Q-type currents [25•]. The result suggests both that the $\alpha_1$ subunit is the target of G-protein interaction and that the β subunit antagonizes G-protein action. Because the calcium channel β subunit has been shown to bind to the calcium channel domain I–II linker [38], and the $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1E}$ I–II linkers also contain a putative consensus binding motif for $G_{\beta\gamma}$ binding (QXXER) [39], this ~140 amino acid region has become a target for intensive investigation.

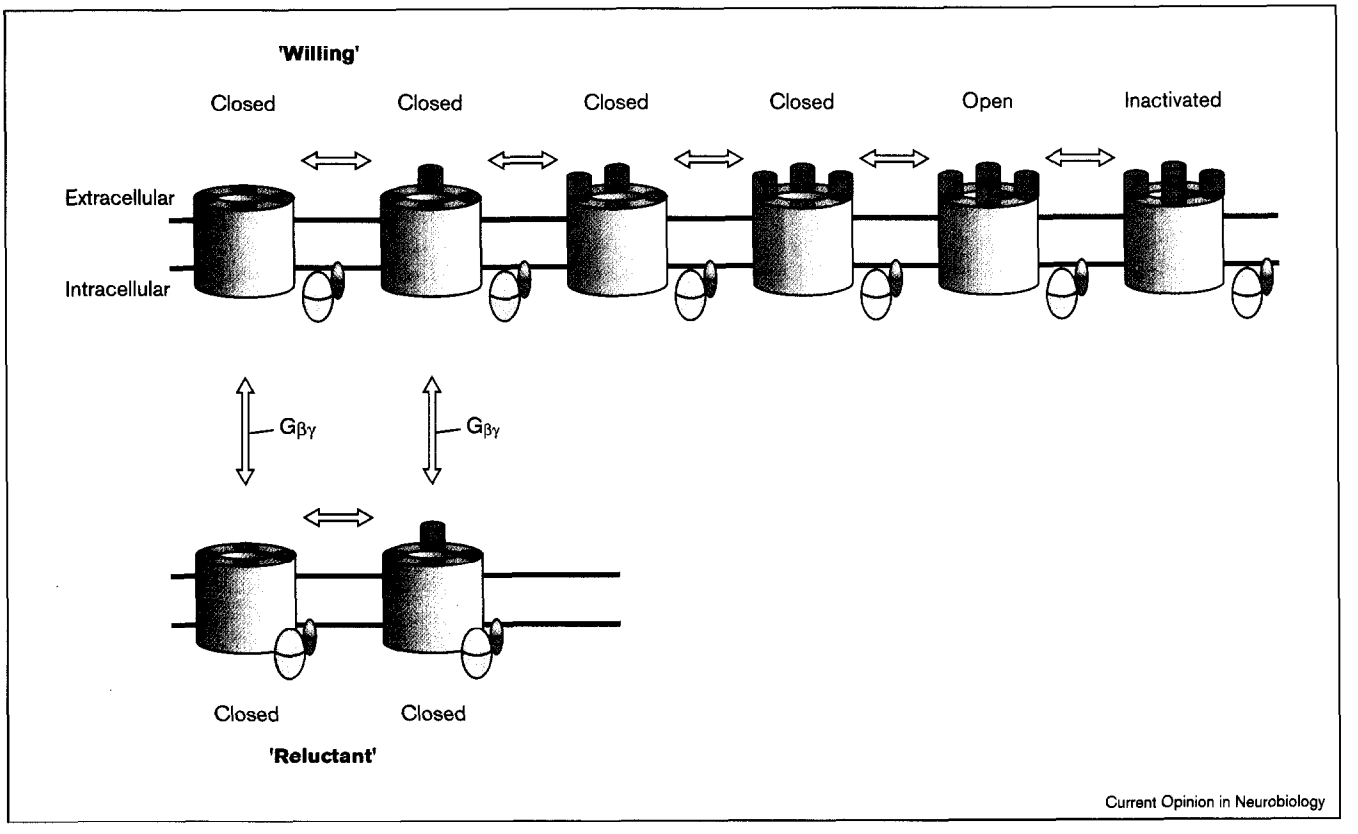
Two groups have recently demonstrated the direct binding of radiolabeled $G_{\beta\gamma}$ to fusion proteins against the $\alpha_{1A}$ and $\alpha_{1B}$ I–II linkers [40••,41••]. There was no apparent binding of $G\alpha_o/G\alpha_i$ subunits; furthermore, using different approaches, both groups found that the I–II linker contains two distinct binding sites for $G_{\beta\gamma}$ (Figure 1). Of particular note, the region of the I–II linker that binds the calcium channel β subunit (AID site) overlaps with one of the two I–II linker $G_{\beta\gamma}$ binding sites, suggesting a possible mechanism of allosteric hindrance underlying the antagonistic action of the calcium channel β subunit on G-protein action [25•,37]. Mutational analysis of the $\alpha_{1A}$ AID site identified QQ–R-L-GY (single letter amino acid code) as one of the motifs required for $G_{\beta\gamma}$ binding. Interestingly, although the R387E mutation in the AID site alone affects $G_{\beta\gamma}$ binding and channel modulation by GTPγS, a similar alteration in the entire I–II linker does not disrupt binding. This suggests that both the AID and downstream $G_{\beta\gamma}$ sites make distinct contributions to channel regulation by $G_{\beta\gamma}$. Two other studies examined chimeric channels and point mutations in the I–II linker and similarly found strong evidence that the domain I–II linker makes functional contributions to the G-protein-dependent modulation of N- and P/Q-type channels [31•,42•].

Is the domain I–II linker the only region involved in G-protein modulation? Two studies [26•,33•] dispute the notion that the calcium channel domain I–II linker contributes to G-protein modulation. Instead, using chimeric $\alpha_1$ subunits, the authors conclude that other regions, specifically domain I and the carboxyl terminus but not the domain I–II linker, are crucial targets for G-protein modulation. The biochemical evidence for a further $G_{\beta\gamma}$ site in the carboxyl terminus of $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1E}$ is convincing [33•]. Together with the notion that two separate regions of the domain I–II linker may contribute to the binding of $G_{\beta\gamma}$ to the calcium channel $\alpha_1$ subunit [40••,41••], the existence of multiple binding sites might provide a basis for the lack of an antagonistic effect of certain I–II linker point mutations on G-protein inhibition. It is possible that the destabilization of only one $G_{\beta\gamma}$ microbinding site may only partially abolish $G_{\beta\gamma}$ action; furthermore, G-protein action is likely to involve two steps: first, the binding of $G_{\beta\gamma}$, and second, the translation of binding into a change in the gating properties of the channel. In this scenario, the domain I and carboxyl regions [26•,33•] may also identify crucial structural components of this translation machinery.

## Stoichiometric aspects of G-protein inhibition

The stoichiometry of the interaction between $G_{\beta\gamma}$ subunits and the calcium channel during reinhibition after a strong depolarizing prepulse has recently been investigated with purified $G_{\beta\gamma}$ subunits and reconstituted N-type channels [43••]. If, as previously proposed [5], prepulses result in a conformational change in the channel, then the reinhibition kinetics should be independent of the $G_{\beta\gamma}$ concentration; however, if one or more G proteins dissociate from the channel, then reinhibition should be concentration dependent. The inclusion of purified $G_{\beta\gamma}$ in the patch pipette showed that the inverse of the prepulse recovery time constant, τ, was linearly dependent on $G_{\beta\gamma}$ concentration, suggesting complete physical dissociation of a single $G_{\beta\gamma}$ molecule during the prepulse, and a 1:1 reassociation reaction between $G_{\beta\gamma}$ and the calcium channel after the prepulse [43••] (Figure 2). The result is consistent with models suggesting the involvement of a single G protein interacting with the calcium channel complex [21]. The results are also consistent with recent single-channel measurements of the mechanism of G-protein modulation of transiently expressed $\alpha_{1B}$ N-type calcium channels [44••]. In this study, the physiological effects of G-protein binding were entirely attributable to an increase in the first latency to (and thus a 'reluctance' of) channel opening upon G-protein binding. The first latency is introduced by the requirement of the G-protein to unbind prior to channel opening, producing slowed activation kinetics as well as late openings that result in an apparent slowing of the rate of inactivation. Upon repolarization, the channels re-enter the closed state ('willing') and reassociate with the G protein in a bimolecular fashion.

**Figure 2**




State dependence and stoichiometry of $G_{\beta\gamma}$ modulation of presynaptic calcium channels. According to the model proposed by Yue and co-workers [44••], the G-protein βγ complex preferentially binds to the deep closed states ($G_{\beta}$, yellow; $G_{\gamma}$, pink). In order for a G-protein-bound channel to undergo transitions from the closed to the open states, the G-protein must first dissociate from the channel. This is reflected as an increase in first latency to opening, which, in turn, is sufficient to account for the slowing of activation, the reduction in peak current amplitude, and the apparent slowing of the time course of activation observed upon stimulation of G protein pathways [44••]. Within the framework of this model, a strong depolarizing prepulse would force all of the channels into the open and thus G-protein-free state. Reinhibition of channels by G proteins after the prepulse would require channel closure prior to high-affinity G-protein reassociation. Recent data [43••] have provided strong evidence that complete dissociation of $G_{\beta\gamma}$ occurs during depolarizing prepulses, and that reassociation occurs with 1:1 stoichiometry between the channel and the $G_{\beta\gamma}$ complex.

## Conclusions

We have recently learned much concerning the molecular and biophysical mechanisms of N-type and P/Q-type calcium channel modulation mediated through the direct interaction of $G_{\beta\gamma}$ subunits; however, in intact cells, it is clear that many types of signaling molecules and pathways act in concert to regulate calcium entry; for example, the activation of protein kinase C (PKC) results in a reduced ability of N-type channels to undergo subsequent G-protein-dependent downregulation [4,45,46]. In a recent study, this cross-talk between signaling pathways was proposed to derive from the PKC-dependent phosphorylation of sites within the I–II linker that bind $G_{\beta\gamma}$ [40••]; furthermore, a recent study by Stanley and Mirotznik [47•] suggests that syntaxin is required for G-protein-dependent modulation. Because syntaxin has been shown to directly bind to the domain II–III linker [48], these results suggest that both the domain I–II and II–III linkers and the proteins that bind to them may all be functionally coupled; for example, the binding of syntaxin is in turn antagonized by the PKC-dependent phosphorylation of sites in the calcium channel II–III linker [49•]. Together with previous reports that N-type calcium channel gating is altered upon coexpression of syntaxin [50], these observations suggest a highly dynamic interaction between calcium influx and subsequent neurotransmitter release.

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- •• of outstanding interest


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A peptide containing the adenylyl cyclase type 2 consensus sequence for $G_{\beta\gamma}$ binding (QXXER) blocks G-protein modulation of P/Q-type channels. The mutational analysis of amino acids in QXXER identifies certain residues crucial for G-protein modulation. Of particular note, the alteration of the $\alpha_{1A}$ sequence QQIER to QQLEE enhances G-protein modulation and is provides strong support for the notion that the I–II linker contributes functionally to channel modulation.

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