G Protein Regulation of Neuronal Calcium Channels: Back to the Future

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ABSTRACT

Neuronal voltage-gated calcium channels have evolved as one of the most important players for calcium entry into presynaptic endings responsible for the release of neurotransmitters. In turn, and to fine-tune synaptic activity and neuronal communication, numerous neurotransmitters exert a potent negative feedback over the calcium signal provided by G protein–coupled receptors. This regulation pathway of physiologic importance is also extensively exploited for therapeutic purposes, for instance in the treatment of neuropathic pain by morphine and other μ-opioid receptor agonists. However, despite more than three decades of intensive research, important questions remain unsolved regarding the molecular and cellular mechanisms of direct G protein inhibition of voltage-gated calcium channels. In this study, we revisit this particular regulation and explore new considerations.

Introduction

Within neurons, calcium ion (Ca2+) represents an essential important signaling molecule, responsible for regulation of a large number of diverse cellular functions (Berridge, 1998). Voltage-gated Ca2+ channels (VGCCs) have evolved as one of the most important players in the initiation of the Ca2+ signal by converting electrical impulses into intracellular Ca2+ elevation (Catterall, 2011). VGCCs are pore-forming multisubunit plasma membrane complexes that are activated upon membrane depolarization (i.e., action potentials) to permit entry of Ca2+ along its electrochemical gradient. To date, 10 genes encoding the pore-forming subunits of mammalian VGCCs have been identified (Fig. 1). Seven genes encode the high-voltage-activated channel subfamily consisting of L-type (Ca1.1 to Ca1.4), P/Q-type (Ca2.1), N-type (Ca2.2), and R-type (Ca2.3), and three genes encode the low-voltage-activated channel subfamily composed exclusively of T-type channels (Ca3.1 to Ca3.3) (Ertel et al., 2000). The Ca2+ pore-forming subunits of VGCCs share a similar transmembrane topology built of four homologous domains, each of them containing six putative transmembrane helices (S1–S6), plus a re-entrant loop (P-loop) that forms the pore of the channel.

The four domains are connected via large cytoplasmic linkers (loops I-II, II-III, III-IV) and cytoplasmic amino- and carboxy-terminal domains, which form interaction sites for various regulatory proteins. In addition to the Ca2+ pore-forming subunit, high-voltage-activated channels contain ancillary subunits (Arikkath and Campbell, 2003): β (β1 to β4, a 55-kDa cytosolic protein of the membrane-associated guanylate kinase family), α2δ (α2δ1 to α2δ4, a 170-kDa highly glycosylated extracellular protein with a single transmembrane domain), and in some cases γ (γ1 to γ3, a 33-kDa transmembrane protein), which control channel trafficking, gating, and function at the plasma membrane.

To make use and regulate the amplitude, duration, and subcellular localization of the Ca2+ signal, VGCCs are under tight regulatory control. One of the most important regulatory mechanisms involves G protein–coupled receptors (GPCRs), also known as seven-transmembrane domain receptors. GPCRs are a large protein family of integral membrane receptors (Vassilatis et al., 2003) that sense extracellular molecules such as neurotransmitters and in turn activate intracellular signaling pathways by regulating the activity of heterotrimeric G proteins. Heterotrimeric G proteins consist of a Gα subunit that binds and hydrolyzes GTP into GDP, and Gβ and Gγ subunits that remain constitutively associated and form the Gβγ dimer (Wettacherech and Offermanns, 2005). In the absence of stimulus, GDP-bound Gα subunit and Gβγ dimer

ABBREVIATIONS: AID, α interaction domain; AP, action potential; CSP, cysteine string protein; DRG, dorsal root ganglion; FHM-1, familial hemiplegic migraine type-1; FRET, fluorescence resonance energy transfer; GPBP, Gβγ protein–binding pocket; GPCR, G protein–coupled receptor; GST, glutathione S-transferase; HEK, human embryonic kidney; M2, muscarinic receptor 2; ORL-1, opioid receptor like-1; PIP2, phosphatidylinositol 4,5-bisphosphate; SNARE, soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptor; VGCC, voltage-gated calcium channel.
are associated with the receptor. Binding of an extracellular ligand onto the GPCR induces a conformational change that promotes the exchange of GDP for GTP from the $G_{\alpha}$ subunit, resulting in the dissociation of the GTP-bound $G_{\alpha}$ and $G_{bg}$ dimer from the receptor. Intrinsic hydrolysis of GTP by $G_{\alpha}$ subunit can be speeded up by GTPase-activating proteins such as regulators of G protein signaling that allows reassociation of GDP-bound $G_{\alpha}$ subunit with the $G_{bg}$ dimer, which terminates G protein signaling (Fig. 2). Although both GTP-bound $G_{\alpha}$ subunit and the $G_{bg}$ dimer mediate intracellular signaling by modulating the activities of neuronal VGCCs, this review is focused on the so-called direct voltage-dependent regulation mediated by the $G_{bg}$ dimer. Interested readers may also refer to the recent review of Zamponi and Currie (2013) for an interesting discussion on the regulation of VGCCs by $G_{\alpha}$- and protein kinase–dependent signaling.

**Description of the Phenomenon**

Interestingly enough, although Ca$^{2+}$ entry at synaptic endings can trigger transmitter release, numerous neurotransmitters released from synaptic contacts and hormones secreted at proximity of the synaptic cleft are in turn able to modulate presynaptic VGCCs via activation of GPCRs to terminate the Ca$^{2+}$ signal and neurotransmitter discharge. This regulation is not only of physiologic importance, but it is also extensively exploited as a therapeutic avenue. For example, one of the most remarkable usages of G protein–mediated inhibition of VGCCs is the management of pain symptoms by specific opioid receptor agonists (e.g., natural opiates like morphine and its synthetic opioid derivatives).

The first observation that synaptic activity is modulated by neurotransmitters goes back to the late 1970s with the pioneer work of Dunlap and Fischbach (1978) on sensory neurons, and later the phenomena was attributed to the inhibition of VGCCs (Dunlap and Fischbach, 1981). To date, up to 20 neurotransmitters and corresponding receptors have been described to modulate VGCCs (Table 1), including noradrenaline (Bean, 1989; Docherty and McFadzean, 1989; Lipscombe et al., 1989; McFadzean and Docherty, 1989), somatostatin (Bean, 1989; Ikeda and Schofield, 1989a,b), GABA (Deisz and Lux, 1985; Dolphin and Scott, 1987; Grassi and Lux, 1989), and acetylcholine (Bernheim et al., 1991; Shapiro et al., 1999).
1986; Scott and Dolphin, 1986). Using an original approach, Forscher et al. (1986) further demonstrated that this regulation is spatially delimited and does not involve diffusible second messengers, suggesting proximity between the Ca\(^{2+}\) channel and the GPCR. The functional importance of channel/GPCR coupling in G protein–mediated inhibition of Ca\(^{2+}\) currents will be further discussed later in this review. A period of intensive work and controversy followed (1989–1996) to determine which of the G protein subunits mediate inhibition of the Ca\(^{2+}\) channel. Using specific antibodies and antisense oligonucleotides to block or knock down G\(_a\) subunits of heterotrimeric G proteins, it was initially proposed that G\(_a\) is the mediator of the inhibition (McFadzean et al., 1989; Baertschi et al., 1992; Campbell et al., 1993; Menon-Johansson et al., 1993). However, during the same period of time, various studies suggested as well an implication of G\(_a\)i (Ewald et al., 1989) or G\(_a\)i and G\(_a\)q (Shapiro and Hille, 1993; Golard et al., 1994; Zhu and Ikeda, 1994). The divergent results led to the hypothesis that GPCR-mediated inhibition of VGCCs is not mediated by G\(_a\) subunits, but rather by a common G protein determinant, and Herlitze et al. (1996) and Ikeda (1996) eventually established that inhibition of Ca\(_{2+}\) channels is mediated by the G\(_\beta\gamma\) dimer concomitantly produced with G\(_a\)-GTP following GPCR activation. Indeed, the overexpression of G\(_\beta_1\gamma_2\) or G\(_\beta_2\gamma_3\) dimers in sympathetic neurons is sufficient to mimic noradrenaline-mediated inhibition of N-type currents, and prevents subsequent inhibition by \(\alpha_2\)-adrenergic agonists. The question is how can we incorporate those results in the observation of other groups suggesting a functional implication of G\(_a\) subunits? Interestingly enough, it appears that even though G\(_\beta\gamma\) dimer is the mediator of the inhibition, G\(_a\) subunits might have an important role in the ability and specificity of GPCRs to functionally couple with VGCCs. Hence, inhibition of neuronal VGCCs is usually mediated by GPCRs coupled to G\(_a\)i or G\(_a\)o (Holz et al., 1986; Scott and Dolphin, 1986) such as \(\alpha_2\) adrenergic receptors, clarifying why depletion of the G\(_a\)o subunit in NG108-15 cells prevents noradrenaline-mediated inhibition of Ca\(^{2+}\) currents (McFadzean et al., 1989).

### Landmarks of GPCR-Mediated Inhibition of VGCCs

Inhibition of VGCCs by GPCRs involves the direct binding of G protein \(\beta\gamma\) dimer onto various structural molecular determinants of the Ca\(_{2+}\) subunit (see next section). At the whole-cell level, this regulation is characterized by various phenotypical modifications of the Ca\(^{2+}\) current properties (Fig. 3). The most obvious is a decrease of the inward current amplitude (Boland and Bean, 1993; Wu and Saggau, 1997) that usually varies from 15 to 80% depending on the Ca\(_{2+}\) channel/GPCR involved. Based on the observation that G\(_\beta\gamma\)-mediated inhibition of VGCCs is less pronounced at depolarized membrane potential, this regulation was named voltage-dependent. In some cases, this inhibition is also accompanied by a depolarizing shift of the voltage-dependence curve of current activation (Bean, 1989) and a slowing of activation.
TABLE 1
Neurotransmitter- and receptor-mediated G protein modulation of Ca\textsubscript{2.2} channels

<table>
<thead>
<tr>
<th>Neurotransmitter</th>
<th>Receptor</th>
<th>Ca\textsubscript{2} Channel</th>
<th>Tissue/Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td>M4</td>
<td>Ca\textsubscript{2},2</td>
<td>SCG/rat</td>
<td>Bernheim et al., 1991</td>
</tr>
<tr>
<td>Adenosine</td>
<td>A1</td>
<td>Ca\textsubscript{2.1} and Ca\textsubscript{2.2}</td>
<td>SCG/mouse</td>
<td>Shapiro et al., 1999</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>P2Y</td>
<td>Ca\textsubscript{2.2}</td>
<td>SCG/rat</td>
<td>Dittman and Regehr, 1996</td>
</tr>
<tr>
<td>Dopamine</td>
<td>D2</td>
<td>HVA</td>
<td>DRG/chicken</td>
<td>Brown et al., 2000; Filippov et al., 2000a</td>
</tr>
<tr>
<td>Endocannabinoids</td>
<td>CB1</td>
<td>Ca\textsubscript{2.1}, Ca\textsubscript{2.2}, and Ca\textsubscript{2.3}</td>
<td>Cerebellum</td>
<td>Garcia et al., 1998a</td>
</tr>
<tr>
<td>GABA</td>
<td>GABA B</td>
<td>Ca\textsubscript{2.1} and Ca\textsubscript{2.2}, and Ca\textsubscript{2.2}, and Ca\textsubscript{2.3}</td>
<td>Hippocampus (CA3—CA1)/rat</td>
<td>Wu and Saggau, 1994</td>
</tr>
<tr>
<td>Galanin</td>
<td>GalR1</td>
<td>Ca\textsubscript{2},2</td>
<td>Hypothalamus/rat</td>
<td>Simen et al., 2001</td>
</tr>
<tr>
<td>Glutamate</td>
<td>mGluR1</td>
<td>Ca\textsubscript{2},2</td>
<td>SCG/rat</td>
<td>Kammermeier and Ikeda, 1999</td>
</tr>
<tr>
<td>LHRH</td>
<td>LHRH-R</td>
<td>Ca\textsubscript{2},2</td>
<td>SCG/bullfrog</td>
<td>Elmslie et al., 1990; Boland and Bean, 1993; Kuo and Bean, 1993</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>a\textsubscript{2}-Adrénergique</td>
<td>Ca\textsubscript{2},2</td>
<td>SCG/bullfrog</td>
<td>Garcia et al., 1998b</td>
</tr>
<tr>
<td>NPY</td>
<td>Y2</td>
<td>Non-L</td>
<td>SCG/rat</td>
<td>Docherty and McFadzean, 1989; McFadzean and Docherty, 1989</td>
</tr>
<tr>
<td>Opioids -</td>
<td>μ</td>
<td>Ca\textsubscript{2.2}</td>
<td>SCG/rat</td>
<td>Plummer et al., 1991</td>
</tr>
<tr>
<td>Enkephalins</td>
<td>κ</td>
<td>Non-L</td>
<td>NG108-15</td>
<td>Toth et al., 1993</td>
</tr>
<tr>
<td>Opioids -</td>
<td>Dyndorphins</td>
<td>Serotonin 5HT-1A</td>
<td>Spinal neuron/Lamprey</td>
<td>Hill et al., 2003</td>
</tr>
<tr>
<td>κ</td>
<td>Serotonin 5HT-1A</td>
<td>Non-L</td>
<td>SPG/rat</td>
<td>Ikeda and Schofield, 1987</td>
</tr>
<tr>
<td>Substance P</td>
<td>NK1</td>
<td>Ca\textsubscript{2.2}</td>
<td>SCG/rat</td>
<td>Shapiro and Hille, 1993</td>
</tr>
<tr>
<td>VIP</td>
<td>VIP-R</td>
<td>Ca\textsubscript{2.2}</td>
<td>SCG/rat</td>
<td>Zhu and Ikeda, 1994</td>
</tr>
</tbody>
</table>

Ach, acetylcholine; LHRH, luteinizing hormone-releasing hormone; NPY, neuropeptide Y; SCG, superior cervical ganglion; VIP, vasoactive intestinal peptide.

(Marchetti et al., 1986) and inactivation kinetics (Zamponi, 2001). In addition, short highly depolarizing voltage step, usually applied about +100 mV before the current eliciting pulse (double-pulse protocol), is sufficient to reverse, at least partially, most of the landmarks of G protein inhibition and produce a so-called prepulse facilitation (Scott and Dolphin, 1990; Ikeda, 1991; Doupnik and Pun, 1994). Current inhibition has been attributed to the direct binding of G\textbeta y dimer to the Ca\textsubscript{2} subunit (referred to as ON landmark), whereas all the other landmarks, including the slowing of current kinetics and prepulse facilitation, can be described as variable time-dependent dissociation of G\textbeta y dimer from the channel (referred to as OFF landmarks) and consequent current recovery from inhibition (Elmslie and Jones, 1994; Stephens et al., 1998; Weiss et al., 2006). It is worth noting that ON and OFF landmarks do not represent two independent regulations, but rather the transition from G\textbeta y-bound channels to G\textbeta y-unbound channels, and vice versa. Furthermore, although the dissociation of G\textbeta y dimer was previously defined as voltage-dependent, it was then proposed that channel opening after membrane depolarization and associated conformational changes of the Ca\textsubscript{2} subunit was most likely the trigger for G\textbeta y dissociation from the channel (Patil et al., 1996). More recently, this concept was further analyzed, and it was shown that the voltage dependence of the kinetic of G\textbeta y dissociation correlates to the voltage dependence of the channel activation (Weiss et al., 2006). Hence, it is likely that the trigger of G\textbeta y dissociation is not the electrical membrane potential per se, but rather the conformational change that occurs within the Ca\textsubscript{2} subunit during the opening of the channel, making the regulation intrinsically channel opening-dependent rather than voltage-dependent.

**What Are the Ca\textsubscript{2} Channel Molecular Determinants of G Protein Inhibition?**

Various structural determinants of the Ca\textsubscript{2} subunit have been characterized either as direct biochemical binding loci for G\textbeta y dimer or of functional importance for GPCR-mediated regulation of Ca\textsuperscript{2+} currents. These determinants are located within the I-II loop of the Ca\textsubscript{2} subunit, as well as in the amino- and carboxyl-terminal regions of the channel.

**Role of the I-II Loop of Ca\textsubscript{2} Channels.** The observation that G\textbeta y dimer is able to functionally interact with the Ca\textbeta subunit (Campbell et al., 1995) led some groups to question the role of the I-II loop of Ca\textsubscript{2} channels in G protein regulation. It is well established that G\textbeta y dimer is able to interact with the type II adenylate cyclase and the phospholipase C\textbeta via a consensus motif QXXER (Chen et al., 1995). Interestingly, this consensus site is also present within the I-II loop of Ca\textsubscript{2} channels, and is located in a proximal region of the so-called α interaction domain (AID), a 18–amino-acid
sequence (QQIERELNGY–WI–AE) initially described as the main interaction locus of the Ca_{\text{II}}b subunit (Pragnell et al., 1994), suggesting a possible interaction between the I-II loop and the Gbg dimer. Consistent with this idea, injection in human embryonic kidney (HEK)293 cells of the synthetic peptide of the Ca_{\text{II}}b subunit FLKRRQQQIERELNGYL (peptide I-II S2; Fig. 4) prevents Gbg-mediated inhibition of Cav2.2/a_{\text{II}}b_{\text{I}}b channel, suggesting that Gbg dimer is able to interact with the peptide (Zamponi et al., 1997). This interaction was then demonstrated by in vitro binding of Gbg1g2 dimer onto the glutathione S-transferase (GST)–AIDA fusion protein with an affinity of 63 nM (De Waard et al., 1997). For comparison, Cav1b1b binds to the GST-AIDA fusion protein with an apparent $K_d$ of 5 nM that is a dozen times more efficient than the binding of Gbg dimer (De Waard et al., 1995). In addition, the role of the QXXER domain in the binding of Gbg dimer was further analyzed by site-directed mutagenesis. Hence, substitution of the arginine (R) by a glutamic acid (E) is sufficient to prevent the binding of Gbg1y2 dimer to the GST-AIDA fusion protein, but also prevents GTPgS-induced inhibition of Ca_{\text{II}}b1b channels expressed in Xenopus oocyte (De Waard et al., 1997). This functional result is, however, in contradiction with another observation that the same amino acid substitution in the Ca_{\text{II}}b1b channel expressed in tsA-201 cells together with the Ca_{\text{II}}b1b subunit promotes channel inhibition by GTPgS (Herlitze et al., 1997). At this point, it is worth noting that substitution of the arginine located in the QXXER motif to a glutamic acid not only prevents the binding of the Gbg dimer, but also slows down the inactivation kinetics of the channel (Herlitze et al., 1997). Hence, the QXXER domain appears to be not only a binding locus of the Gbg dimer within the I-II loop of the Ca_{\text{II}} subunit, but also an important molecular determinant of the fast inactivation of the channel. Considering that channel inactivation is by itself an important component for back modulation of G protein inhibition, as we will see later, it is delicate to conclude on the exact functional role of the QXXER domain. It was also shown that substitution of the isoleucine (I) to a leucine (L) within the QQIER domain into the Ca_{\text{II}} subunit is intrinsically independent of the membrane potential and is rather triggered by conformational changes of the Ca_{\text{II}} subunit upon channel opening. Inactivation can occur either in the absence (E) or presence (F) of Gbg but not necessarily with the same rate.

Fig. 3. Putative kinetic model illustrating landmarks of Gbg-dependent regulation of Ca_{\text{II}} channels. This scheme relies on a single conducting state in which dissociation of the Gbg dimer has occurred, whereas the channel remains open. (A) Gbg-unbound closed channel. The transition from Gbg-bound closed channel (B) to Gbg-unbound open conducting channel (D) requires a transition through an additional transient Gbg-bound open nonconducting state of the channel (C) characterized by whole-cell current inhibition (G) (ON landmark). This transition state produces a time-dependent current recovery from Gbg-dependent inhibition, at the origin of the slowing of activation/inactivation kinetics of the whole-cell current (H) and an apparent depolarized shift of the voltage dependence of activation (I) (OFF landmark). Norm. ICa_{\text{II}}^2, normalized calcium current. Dissociation of the Gbg dimer from the Ca_{\text{II}} channel subunit is intrinsically independent of the membrane potential and is rather triggered by conformational changes of the Ca_{\text{II}} subunit upon channel opening. Inactivation can occur either in the absence (E) or presence (F) of Gbg but not necessarily with the same rate.
suggesting that, despite an important role in the biochemical coupling of Gbg dimer with the channel, the QXXER domain is not the sole molecular determinant for potent channel inhibition by G proteins. Hence, additional molecular loci of the I-II loop have been identified. For example, injection in HEK293 cells of the GVLGEFAKERERVENRRA peptide of the Ca v2.2 subunit (peptide I-II S1; Fig. 4), localized upstream the AID domain and straddling the IS6 transmembrane segment and the I-II loop is able to prevent Gbg-dependent inhibition of Ca v2.2/a2/b1b channels, suggesting a second Gbg dimer-binding locus (Zamponi et al., 1997).

Finally, the G protein interaction domain, localized 13 amino acid residues upstream the AID domain, has also been proposed as a molecular locus for Gbg binding with an affinity of 20 nM (De Waard et al., 1997), and the corresponding synthetic peptide is likewise able to prevent Gbg modulation of Ca2+ currents (Zamponi et al., 1997). Interestingly enough, protein kinase C–dependent phosphorylation of the G protein interaction domain on a threonine residue prevents binding of Gbg, suggesting a functional crosstalk between direct (i.e., mediated by Gbg dimer) and indirect (i.e., involving second messengers) GPCR modulation of VGCCs (Zamponi et al., 1997).

Altogether, it is unambiguous that the cytoplasmic I-II loop of Ca2+ channels contains various structural determinants for Gbg dimer binding. However, the functional importance of those determinants in the modulation of the Ca2+ currents remains largely discussed, and other groups rather suggested that the I-II loop was not critical for Gbg-mediated inhibition of Ca2+ channels. Hence, it has been shown that the substitution of the I-II loop of the Ca v2.2 subunit by the I-II loop of the Ca v1.2 channel (less sensitive to Gbg inhibition) or by the Ca v1.2 channel (insensitive to direct G protein modulation) does not alter somatostatine-mediated inhibition of the Ca v2.2/b1b channel (Zhang et al., 1996). Conversely, the replacement of the I-II loop of the Ca v1.2 subunit by the I-II loop of the Ca v2.2 subunit is not sufficient to restore a direct G protein modulation of the Ca v1.2/b2a channel by D2 dopaminergic receptors (Canti et al., 1999). Finally, the substitution of the I-II loop of the Ca v2.3 channel [rat rbE-II isoform (Soong et al., 1993)]—that is less sensitive to direct G protein modulation—by the I-II loop of the Ca v2.2 subunit

Fig. 4. Schematic representation of the Ca2+ molecular determinants involved in Gbg-mediated regulation of VGCCs. (A) Schematic representation of the location of the important Ca channel molecular determinants of Gbg-dependent regulation identified on the basis of functional evidence. Several Gbg determinants are present within the I-II loop, including the region S2 containing the QXXER motif and the G protein interaction domain (GID) (in red). The AID of the Ca v subunit on the I-II loop is shown in blue. Furthermore, additional molecular determinants located within the amino- (Nter) and carboxy- (Cter) terminal regions of the Ca v2.1 and Ca v2.2 subunits have been functionally documented. (B) Schematic representation of a presumable Gbg-binding pocket within the Ca v2 subunits involving the different Ca2+ molecular determinants functionally and biochemically characterized, and most likely responsible for the variable sets of Gbg-mediated modulation of the channel (ON and OFF landmarks). The carboxy-terminal region of the Ca v2 subunits also contributes in some cases to the biochemical coupling of the channel with the GPCR.
induces a slowing of activation kinetics of Ca_{2.3/3}/β_{2a} channels upon activation of G proteins by injection of GTPγS, but no net inhibition of the maximal amplitude of the Ca^{2+} current (Page et al., 1997). Hence, it was proposed that the I-II loop could be the channel molecular determinant mediating the slowing of current kinetics under direct G protein regulation, and that current inhibition per se could require other molecular determinants (Page et al., 1997). Considering that the apparent slowing of current activation kinetics is attributed to the dissociation of Gβγ dimer from the channel (OFF landmarks) (Elmslie and Jones, 1994; Stephens et al., 1998; Weiss et al., 2006), the I-II loop might be the channel molecular determinant involved in the dissociation of Gβγ dimer from the Ca_{2.2} subunit.

**Role of the Amino-Terminal Domain of Ca_{2.2} Channels.** Interestingly enough, although Ca^{2+} currents generated by the BII-2 brain rabbit isoform of the Ca_{2.3} subunit are subject to direct G protein modulation [either upon somatostatin application (Yassin et al., 1996) or by direct injection of GTPγS (Meza and Adams, 1998)], currents generated by the rat rBE-II isoform that presents a truncated amino-terminal domain remain insensitive (Page et al., 1997), suggesting an implication of the amino-terminal domain of the Ca_{2.2} channel in direct G protein modulation. Consistent with this idea, extension by polymerase chain reaction of the amino-terminal region of the rBE-II isoform, leading to a Ca_{2.3} subunit homolog to the rabbit one, is sufficient to restore quinpirole (D2 dopaminergic agonist)-mediated inhibition of Ca^{2+} currents (Page et al., 1998). Moreover, the substitution of the amino-terminal region of the Ca_{1.2} subunit by the amino-terminal domain of the Ca_{2.2} subunit makes the Ca_{1.2} channel sensitive to quinpirole-mediated inhibition (Canti et al., 1999). Further work identified a highly conserved 11-amino-acid sequence (YKQSIAQRAKT) in the amino-terminal region of Ca_{2.2} channels critical for direct G protein modulation (Canti et al., 1999) (Fig. 4A), and alanine scan of the YKQ and RAR motifs abolished quinpirole-induced inhibition of Ca_{2.2.3}/β_{2a}/α_{δ} channels (Canti et al., 1999). In addition, it was proposed that the amino-terminal region of the Ca_{2.2} subunit might constitute a G protein–gated inhibitory module acting via interaction with the cytoplasmic I-II linker of the channel (Agler et al., 2005). More recently, Ca_{2.2} amino-terminal–derived peptides have been shown to prevent noradrenaline-induced G protein inhibition of Ca^{2+} currents in superior cervical ganglion neurons, strengthening the implication of the Ca_{2.2} amino-terminal region in G protein modulation in native environment (Bucci et al., 2011).

**Role of the Carboxy-Terminal Domain of Ca_{2.2} Channels.** The carboxy-terminal region of the Ca_{2.2} subunits has also been proposed as an important determinant for direct G protein regulation, and a binding domain of Gβγ dimer was identified in the distal carboxy-terminal region of the Ca_{2.3} subunit (Qin et al., 1997) that presents some homologies with the corresponding region of the Ca_{2.1} and Ca_{2.2} subunits (Simen et al., 2001) (Fig. 4A). Hence, the substitution of the carboxy-terminal region of the Ca_{2.3} subunit by the corresponding one of the Ca_{1.2} subunit is sufficient to abolish muscarinic receptor 2 (M2)–mediated inhibition of the Ca_{2.3/3}/β_{2a} channel (Qin et al., 1997). Association of G protein β_{2a} subunit with the carboxy-terminal region of the Ca_{2.1} channel was also observed using a fluorescence resonance energy transfer approach (FRET) (Hummer et al., 2003). In addition, based on the observation that FRET signal between Ca_{2.1}/Ca_{β_{10}} FRET pairs is increased in the presence of Gβ_{2a} subunit and requires the presence of the carboxy-terminal region of the channel, it was proposed that binding of G proteins to the carboxy-terminal region may produce a conformational change of the channel that might contribute to channel inhibition (Hummer et al., 2003). However, deletion of the carboxy-terminal region of the Ca_{2.2} subunit containing the binding determinant of Gβγ dimer does not alter inhibition of Ca^{2+} currents mediated by injection of GTPγS (i.e., agonist/receptor-independent) (Meza and Adams, 1998), but only alters somatostatin-mediated inhibition (i.e., agonist/receptor-dependent) of Ca_{2.2} channels (Hamid et al., 1999), suggesting that the carboxy-terminus might rather be involved in the functional coupling of the channel with agonist-dependent activation of G proteins. Interestingly, the binding determinant of Gβγ dimer in the carboxy-terminal region of Ca_{2.2} channels is localized close to the binding domain of Gs on Ca_{2.1} and Ca_{2.2} subunits (Furukawa et al., 1998b) and Gq on the Ca_{2.2} subunit (Simen et al., 2001) (Fig. 4A), suggesting that the carboxy-terminal region could be involved in the functional and biochemical coupling of Ca_{2.2} channels with the GPCR via the Gsβγ trimer (Kitano et al., 2003; Beedle et al., 2004; Kisilevsky et al., 2008; Weiss, 2009). Paradoxically, the importance of the carboxy-terminal region of Ca_{2.2} channels in the direct regulation by G proteins was poorly investigated compared with the intensive work that was done for the I-II loop and the amino-terminal region. One possible reason is that injection of GTPγS, or overexpression of Gβγ dimer to trigger direct G protein modulation of Ca_{2.2} channels, was not suitable to highlight the functional importance of the carboxy-terminal region.

**Toward the Notion of Gβγ Protein-Binding Pocket.** As previously seen, the Ca_{2.2} subunit contains various molecular determinants for Gβγ dimer binding, and it was initially proposed that several Gβγ dimers could interact simultaneously with the Ca_{2.2} subunit in a cooperative manner (Boland and Bean, 1993). However, further analysis revealed that the kinetics of Gβγ dimer interaction with the Ca_{2.2} channel (evidenced by the functional modulation of the Ca^{2+} current) can be described by a monoexponential function with a time constant directly correlated to the free Gβγ dimer concentration, suggesting that only a single Gβγ dimer interacts with the Ca_{2.2} subunit (Zamponi and Snutch, 1998). Based on this observation, it was proposed that the various Gβγ binding sites located within the Ca_{2.2} subunit could be spatially structured to form a unique interaction domain called the Gβγ protein–binding pocket (GPBP) (De Waard et al., 2005) (Fig. 4B), in which various binding loci of the GPBP could be responsible for a particular feature of the G protein regulation. Hence, the carboxy-terminal region of the Ca_{2.2} subunit would play a critical role in the inhibition of Ca^{2+} currents by GPCRs (Qin et al., 1997; Furukawa et al., 1998a,b) through favoring the biochemical and functional coupling of the receptor with the channel via the Gαβγ trimer (Kitano et al., 2003; Beedle et al., 2004). In that context, the carboxy-terminal domain of the Ca_{2.2} subunit would not be directly involved in the inhibition of the Ca^{2+} currents, but would rather allow the rapprochement of the GPCR with the channel required for the direct G protein modulation (Forschler et al., 1986). The I-II loop of the Ca_{2.2} subunit represents an important domain of interaction with the Gβγ dimer via three motifs clearly identified. However, the binding of Gβγ dimer...
onto the I-II loop does not appear critical for the inhibition of the Ca\(^{2+}\) current (ON landmark) (Zhang et al., 1996; Qin et al., 1997), but rather seems to be involved in the relaxation of the inhibition (OFF landmarks, i.e., unbinding of G\(\beta\gamma\) dimer from the Ca\(_2\) subunit) in response, for example, to a depolarizing prepulse (Herlitze et al., 1997; Simen and Miller, 2000). Finally, the amino-terminal region of the Ca\(_2\) subunit appears as the main determinant of direct G protein inhibition of Ca\(^{2+}\) currents by G\(\beta\gamma\) dimer (Page et al., 1998; Canti et al., 1999). In that context, the GPBP emerges as a dynamic structure, composed of various loci that on one hand bring the Ca\(_{2}\) channel and the GPCR together, and on the other hand differentially mediate the ON and OFF G protein landmarks.

**How Does G\(\beta\gamma\) Dimer Inhibit Ca\(_{2}\) Channels?**

Mutagenesis studies have identified a number of amino acid residues on the surface of the G\(\beta\) subunit important for inhibition of the Ca\(^{2+}\) channel (Ford et al., 1998; Mirshahi et al., 2002; Tedford et al., 2006). Interestingly, most of these residues are located on the G\(\alpha\) surface that interacts with G\(\alpha\) and are essentially masked when G\(\alpha\) is present. However, the molecular mechanism by which binding of G\(\beta\gamma\) dimer to the channel inhibits the Ca\(^{2+}\) current remains largely unknown. It has to be mentioned that, besides the extensive work that was done to understand the molecular mechanisms of G protein regulation of VGCCs, it still remains unclear how the binding of G\(\beta\gamma\) dimer to the Ca\(_2\) subunit inhibits the Ca\(^{2+}\) current. Based on the observation that the voltage-dependence curve of the Ca\(^{2+}\) current under G protein modulation is significantly shifted to more depolarized potentials, it was initially proposed that the Ca\(^{2+}\) channel undergoes a switch from a willing mode (i.e., easily activated) to a reluctant mode (i.e., hardly activated) upon GPCR activation (Bean, 1989). The channel returns to the willing state after dissociation of the G\(\beta\gamma\) dimer from the Ca\(_2\) subunit (Bean, 1989; Elmslie et al., 1990). Consistent with this idea, single-channel recordings revealed latency in the first opening of the Ca\(_{2,2}\) channel upon M2 muscarinic receptor activation, after which the channel presents a similar behavior as the nonregulated control channel (Patil et al., 1996). Hence, it was proposed that the first opening latency could correspond to the time for G\(\beta\gamma\) dimer to dissociate from the Ca\(_{2}\) subunit in response to the membrane depolarization prior to channel opening (Patil et al., 1996). It is worth noting that low-probability openings of Ca\(_{2,2}\) channels under G protein modulation have been observed, corresponding most likely to transient dissociations of the G\(\beta\gamma\) dimer from the channel upon moderate membrane depolarizations (30 mV) (Lee and Elmslie, 2000). This simple model of regulation appears to be sufficient to support both the inhibition of the Ca\(^{2+}\) current and the depolarizing shift of the current/voltage activation curve. However, the observation that the amplitude of gating currents of Ca\(_{2,2}\) subunits is reduced upon injection of GTP\(\gamma\)S led the authors to propose that G proteins mediate inhibition of Ca\(^{2+}\) current by altering intrinsic gating properties of the channel (Jones et al., 1997). Gating currents are produced by the movement of the charged voltage-sensor domains, including positively charged S4 segments, that move into the lipid bilayer in response to electrical membrane depolarizations and lead to the opening of the channel. It is conceivable that G\(\beta\gamma\) dimer could bind either to the intracellular end of the S4 segment of the Ca\(_{2}\) subunit, or to another structural determinant of the voltage sensor exposed on the intracellular surface of the channel, preventing the proper movement of the voltage sensor and thus inhibiting the channel. Consistent with this idea, it was shown that the point mutation G177E localized in the third transmembrane segment of the first domain (IS3) of the Ca\(_{2,2}\) subunit (rat rbB-I isoform) and induces a tonic inhibition of the channel that can be reversed by a depolarizing prepulse as the classic G protein inhibition (Zhong et al., 2001). Interestingly, although this inhibition shares some of the features of the G protein modulation, activation of G proteins does not produce additional inhibition. Similarly, introduction of the G177E mutation into the Ca\(_{2,2}\) subunit (rat rbB-II isoform) produces a tonic inhibition of the channel and prevents G protein–mediated modulation of the Ca\(^{2+}\) current (Zhong et al., 2001). In contrast, the E177G mutation introduced into the Ca\(_{2,2}\) subunit reverses tonic inhibition of the channel and restores a normal G protein regulation. Hence, it was proposed that the negative charge introduced by the mutation G177E could interact with a positively charged residue of the S4 segment of the first domain of the Ca\(_2\) subunit, pushing the channel to a reluctant mode via a mechanism of voltage sensor trapping, suggesting that G\(\beta\gamma\) dimer could possibly regulate the channel in a similar way (Zhong et al., 2001; Flynn and Zamponi, 2010). Intriguingly, Gm2, which belongs to the RGK (Rad, Gem, and Kir) family of small G proteins, is also able to inhibit Ca\(_{2,2}\) channels (Chen et al., 2005). However, in contrast to the direct G protein inhibition by G\(\beta\gamma\) dimer, depolarizing prepulses do not reverse Gem2-mediated inhibition (Chen et al., 2005). Remarkably, structure analyses of Rad, a homolog of Gem, indicate the presence of a molecular motif similar to the seven repeated motifs of the G\(\beta\) subunit, suggesting a common mechanism by which G\(\beta\gamma\) dimer and RGK proteins modulate VGCCs. Another possible model of channel regulation by G proteins that does not require any alteration of gating parameters was also proposed in which willing and reluctant modes are intrinsic to the channel, whereas G proteins and other effectors simply shift the fraction of channels in these two states without modifying their intrinsic gating properties (Herlitze et al., 2001). This willing to reluctant model could also potentially explain the decrease in gating currents initially observed during G protein regulation (Jones et al., 1997). Finally, based on the observation that sodium currents recorded through Ca\(_{2,2}\) channels are less affected than calcium currents by G protein inhibition, it was proposed that binding of G\(\beta\gamma\) dimer onto the Ca\(_{2}\) subunit alters ion permeation of the channel (Kuo and Bean, 1993). Hence, consistent with the observation that a 35–amino-acid peptide (residues 271–305) of the G\(\beta\_2\) subunit is sufficient to inhibit Ca\(^{2+}\) currents (Li et al., 2005), it is also conceivable that G\(\beta\gamma\) dimer simply inhibits the channel via a pore-modifier mechanism. However, such a mechanism could only contribute partially to the overall current inhibition given the effect of G\(\beta\gamma\) dimer on single-channel gating properties (essentially latency of the first opening). Either way, the exact molecular mechanisms by which G\(\beta\gamma\) dimer inhibits VGCCs remain essentially unknown and certainly deserve further investigation. Recent structural information obtained from voltage-gated sodium channels (Payandeh et al., 2011; Zhang et al., 2012) will certainly provide key information to further
analyze the binding of G proteins to the channel subunit and find out the molecular determinant of the inhibition.

**What Is the Functional Role of the Caβ Subunit in Direct G Protein Inhibition?**

The involvement of the Caβ subunit in the direct regulation of VGCCs by GPCRs was extensively examined. Hence, the Caβ subunit and Gbg dimer share a structural binding site (QXXER located in the AID domain of the I-II loop) on the Ca2 subunit, suggesting a possible crossstalk. The initial observations that expression of the Caβ subunit in Xenopus oocytes decreases G protein inhibition of Ca2.1 and Ca2.2 channels (Roche et al., 1995), and conversely that inhibition of Ca2.1 channels by μ-opioid and M2 muscarinic receptors is increased in the absence of Caβ subunit (Bourinet et al., 1996; Roche and Treistman, 1998a), led the authors to propose that the Caβ subunit is antagonistic to the direct G protein modulation of Ca2 channels. In addition, it was shown that binding of Gbg dimer to the carboxy-terminal region of the Ca2 subunit is abolished by the guanylate kinase domain of the Caβ subunit, which also prevents M2 muscarinic receptor–dependent inhibition of the Ca2+ current, providing a possible explanation for the functional antagonism existing between the Caβ subunit and direct G protein inhibition of VGCCs (Qin et al., 1997). However, considering that the carboxy-terminal domain of the Ca2 subunit plays an important role in the functional coupling with the GPCR, it is likely that the Caβ-dependent antagonism observed relies on an altered coupling of the channel with the GPCR rather than on the direct alteration of Gbg coupling of the channel with the GPCR rather than on the direct alteration of Gbg dimer binding to the channel. In contrast to the antagonistic hypothesis, it was also proposed that the Caβ subunit could rather be essential for potent G protein modulation of the Ca2 channels (Meir et al., 2000). Consistent with this idea, it was proposed using a FRET approach that Gbg dimer induces Ca2.1 channel reluctance by displacement of the Caβ subunit from the channel (Sandoz et al., 2004). However, besides the fact that direct G protein modulation of Ca2 channels has been observed numerous times in the absence of the Caβ subunit (Roche et al., 1995; Bourinet et al., 1996; Canti et al., 2000), this observation is balanced by a study using a similar FRET approach showing that binding of Caβ1b and Gbg to the Ca2.1 subunit is not exclusive, but rather synergetic (Hummer et al., 2003). In addition, it was shown that recovery of the Ca2.2 channel from G protein inhibition is not only influenced by the presence of a Caβ subunit (Roche and Treistman, 1998b), but also depends on the Caβ isoform (β3 > β4 > β1b > β2a) (Canti et al., 2000). Considering that channel activity is an important factor of the OFF G protein landmark, these results suggest that the Caβ subunit could modulate G protein inhibition indirectly by biophysical changes induced on the channel. Consistent with this idea, the respective kinetics of channel inactivation induced by various Caβ subunits nicely correlates with the kinetic of recovery from G protein inhibition (Weiss et al., 2007a). It was thus proposed that the Caβ subunit, by controlling channel inactivation, indirectly influences the speed of G protein dissociation from the channel. Hence, fast-inactivating Caβ subunits (example Caβ3) act as a cofactor to speed the recovery from the G protein inhibition. However, the situation becomes more complicated considering the fact that fast-inactivating Caβ subunits, by accelerating the inactivation kinetics of the channel, also reduce the temporal window for G protein dissociation (Fig. 5). Hence, fast-inactivating Caβ subunits, while speeding up the rate of G protein dissociation, also reduce the maximal extent of current recovery from inhibition that leads to an apparent decrease of the prepulse facilitation. In contrast, a slow-inactivating Caβ subunit (for instance, Caβ2a) that slows down channel inactivation has minor effect on the kinetic of G protein dissociation, but prolongs the temporal window of opportunity for G protein dissociation and leads to a far more complete current recovery from inhibition, evidenced by an apparent increased prepulse facilitation (Weiss et al., 2007a). It is worth noting that the amino-terminal domain of the Ca2 subunits that has been implicated in the direct G protein inhibition of VGCCs mediates Caβ-dependent fast inactivation of Ca2.2 channel (Stephens et al., 2000). In addition, the R387E mutation in the QXXER motif of the I-II loop that alters G protein regulation (De Waard et al., 1997; Herlitze et al., 1997) also affects channel inactivation. Hence, it is possible that these molecular channel determinants contribute to G protein regulation not only by physical changes induced on the channel, but also by indirect alteration of the coupling of the channel with the GPCR.
only by providing an anchor for the Gβγ dimer, but also via their intrinsic effect on channel gating.

**How Do Synaptic Proteins Modulate Direct G Protein Inhibition?**

The two types of Ca\(^{2+}\) channels (Ca\(_{\text{2.1}}\) and Ca\(_{\text{2.2}}\)) that are most responsible for voltage-dependent release of neurotransmitter at nerve terminal endings biochemically associate with presynaptic vesicles of transmitter (Bennett et al., 1992; Yoshida et al., 1992; Leveque et al., 1994) by interacting with some of the proteins of the vesicular machinery release complex (soluble N-ethylmaleimide–sensitive fusion protein attachment protein receptors [SNAREs]), including syntaxin-1A and synaptosomal-associated protein 25 (SNAP-25). Molecular characterization of channel/SNARE interaction has identified a synprint (synaptic protein interaction) locus in Ca\(_{\text{2.1}}\) and Ca\(_{\text{2.2}}\) located within the intracellular loop between domains II and III of the channels (Sheng et al., 1994; Rettig et al., 1996). It is believed that the functional relevance of this interaction is to bring presynaptic vesicles close to the Ca\(^{2+}\) source for efficient and fast neurotransmitter release and disruption of Ca\(_{\text{2.2}}\)/SNARE complex alters Ca\(^{2+}\)-dependent release of neurotransmitter (Mochida et al., 1996; Rettig et al., 1997; Harkins et al., 2004; Keith et al., 2007). Furthermore, direct binding of syntaxin-1A and SNAP-25 to the Ca\(_{\text{2.1}}\) and Ca\(_{\text{2.2}}\) subunits also potently modulates channel gating by shifting the voltage dependence of channel inactivation toward more negative membrane potentials (Bezprozvanny et al., 1995; Wiser et al., 1996; Zhong et al., 1999; Degtiar et al., 2000; Weiss and Zamponi, 2012; Zamponi and Currie, 2013), although the physiologic relevance of this regulation is not fully understood. Interestingly, although the synprint site of Ca\(_{\text{2.1}}\) and Ca\(_{\text{2.2}}\) subunits, and in general the II-III intracellular linker, are not essential molecular determinants of Gβγ-mediated modulation of the channel activity, proteolytic cleavage of syntaxin-1A with botulinum neurotoxin C1 in primary neurons was shown to completely prevent GPCR-dependent inhibition of presynaptic Ca\(^{2+}\) channels (Stanley and Mirotznik, 1997). However, because G protein modulation of transiently expressed Ca\(_{\text{2.2}}\) channels in *Xenopus* oocytes (Bourinet et al., 1996) or HEK cells (Zamponi et al., 1997) does not require coexpression with syntaxin-1A, it is unclear why the cleavage of syntaxin-1A in native neuronal environment would lead to a total loss of G protein modulation. In contrast, Jarvis et al. (2000) reported that syntaxin-1A is not essential, but facilitates G protein modulation of the Ca\(_{\text{2.2}}\) channel. Indeed, whereas Ca\(_{\text{2.2}}\) transiently expressed in tsa201 cells in combination with Gβ1γ2 dimer is susceptible to a tonic inhibition that can be assessed (relieved) by a preceding strong membrane depolarization, coexpression of syntaxin-1A produced an even larger tonic G protein inhibition, suggesting that syntaxin-1A facilitates Gβγ-dependent inhibition of the channel. However, it remains unclear whether syntaxin-1A facilitates G protein inhibition or promotes Gβγ dimer dissociation from the channel upon prepulse depolarization. Indeed, as previously discussed in the case of the Ca\(_{\beta}\) subunit, it is possible that syntaxin-1A, by affecting channel gating, indirectly influences the kinetic of G protein dissociation from the channel, producing a larger prepulse facilitation that could be misinterpreted as the result of an increased initial G protein inhibition. In support of this idea, coexpression of SNAP-25 that reverses syntaxin-1A–dependent channel gating also reduces the magnitude of the prepulse–induced current recovery upon tonic G protein inhibition (Jarvis and Zamponi, 2001). However, this concept should also be toned down, as coexpression of a mutant syntaxin-1A that is locked permanently in an open conformation (the conformation adopted by syntaxin-1A when in interaction with SNAP-25 or synaptobrevin-2) (Dulubova et al., 1999; Brünger, 2001) has no effect on Ca\(_{\text{2.2}}\) gating, but still modulates Gβγ-dependent inhibition of the channel (Jarvis et al., 2002). Conversely, syntaxin-1B affects Ca\(_{\text{2.2}}\) channel gating, but does not support G protein modulation (Jarvis and Zamponi, 2001). Altogether, these results suggest that syntaxin-1A may present specific features responsible for G protein modulation independently of its effect on the channel gating, possibly by facilitating the colocalization of G proteins with the Ca\(^{2+}\) channel. Consistent with this idea, a syntaxin 1/GαoCa\(_{\text{2.2}}\) complex has been described at presynaptic nerve terminals of chick ciliary ganglion cells (Li et al., 2004).

Several other types of presynaptic proteins have been documented as important in the modulation of G protein inhibition of VGCCs. For example, coexpression of Rim1 significantly decreases direct [d-Ala2, N-MePhe4, Gly-ol]-enkephalin–induced G protein inhibition of Ca\(_{\text{2.2}}\) channels expressed in HEK293 cells (Weiss et al., 2011). Interestingly, careful kinetic analyses revealed that Rim1 does not prevent inhibition of the channel (ON landmark, i.e., binding of Gβγ dimer to the channel), but rather favors channel recovery from inhibition (OFF landmarks, i.e., unbinding of Gβγ dimer from the channel), most likely by slowing down channel inactivation (Kiyonaka et al., 2007), increasing the time window for functional recovery from G protein inhibition similarly to Ca\(_{\beta}\) subunits (Weiss et al., 2007a, 2011). Modulation of G protein inhibition of VGCCs has also been documented with cysteine string protein (CSP). Although CSP has been shown to stimulate Gα subunit activity by promoting the exchange of GTP for GDP (Natochin et al., 2005) that in turn is expected to reduce free Gβγ dimers, coexpression of CSP increases apparent Gβγ-dependent inhibition of Ca\(_{\text{2.2}}\) channel similarly to what was observed with syntaxin-1A (Magga et al., 2000). Although the molecular mechanism underlying CSP-dependent modulation of G protein inhibition remains to be further explored, it is possible that CSP brings G proteins together with the Ca\(^{2+}\) channel because binding of CSP has been documented with Ca\(_{\text{2.1}}\) and Ca\(_{\text{2.2}}\) subunits (Leveque et al., 1998; Magga et al., 2000), as well as with G proteins Gα subunit and Gβγ dimer (Magga et al., 2000).

**How Does the Termination of the Signal Occur?**

To be efficient, cellular events have to be localized and timely regulated. Hence, we have seen that direct G protein modulation of VGCCs requires the following: 1) the activation of the GPCR by specific extracellular ligands; 2) the release and binding of Gβγ dimer to the channel, leading to a complex regulatory phenotype; and finally 3) that this regulation can be temporally relieved, beside the continuous presence of the extracellular agonist, in response, for example, to a train of action potentials. However, how does this regulation definitively end? Although the recapture and/or degradation of the agonist terminate the activation of the GPCR, how does the channel-bound Gβγ dimer reassociate with the Gα subunit? Because channel recovery from G protein inhibition can occur at membrane potentials above the reversal potential of...
Ca\textsuperscript{2+}, ion influx is not the driving force for G\beta\gamma dimer dissociation from the channel. From a biochemical and thermodynamic point of view, it is conceivable that intrinsic hydrolysis of GTP by the G\alpha subunit and formation of GDP-bound G\alpha subunit—which presents a high affinity for G\beta\gamma dimer—could chelate free G\beta\gamma when dissociating from the channel, according to a binding/unbinding thermodynamics equilibrium. It is also possible that the GDP-bound G\alpha subunit is able to bind G\beta\gamma dimer while still on the Ca\textsubscript{v} subunit. This concept implies the molecular determinants of G\alpha and G\beta\gamma binding being accessible when the G\beta\gamma dimer is bound to the channel. Binding of GDP-G\alpha to G\beta\gamma could produce a conformational change of the channel Ca\textsubscript{v} subunit and/or G\beta\gamma dimer, unfavorable to the formation of Ca\textsubscript{v}/G\beta\gamma complex, possibly by altering an essential channel molecular determinant important for G\beta\gamma binding. Although this molecular determinant has not been identified yet, it is likely to contribute to G\beta\gamma dissociation upon channel activation, and possibly involves channel structures sensitive to electrical membrane potential, like S4 segments, for example. However, this concept remains to be investigated to be able to better understand the molecular dynamic of GPCR-induced inhibition of VGCCs.

**Are Ca\textsubscript{v2} Channels the Only Calcium Channels Susceptible to Direct G Protein Inhibition?**

It is thought that direct G protein modulation of VGCCs is mostly confined to neuronal Ca\textsubscript{v}2 channels. Indeed, structure/function studies indicate that Ca\textsubscript{v}1 channels that lack most of the believed important structural determinants involved in the binding of the G\beta\gamma dimer to the channel, such as the QXXER motif of the I-II loop (Herlitze et al., 1997), are not subject to direct G protein regulation (Roche et al., 1995; Bourinet et al., 1996; Zhang et al., 1996; Meza and Adams, 1998). However, additional studies on native channels clearly demonstrated an inhibition of L-type Ca\textsuperscript{2+} currents mediated by heterotrimeric G proteins. For example, injection of GTP\gamma S in cerebellar granule cells induces inhibition of L-type Ca\textsuperscript{2+} currents that is partly reversed upon channel activation by the dihydropyridine agonist +(-S)-202-791 (Haws et al., 1993). Moreover, inhibition of L-type Ca\textsuperscript{2+} currents has been documented upon agonist stimulation of GPCRs. Indeed, baclofen activation of GABA-B receptors in retinal bipolar neurons induces inhibition of L-type Ca\textsuperscript{2+} currents, an inhibition that is potentiated by GTP\gamma S injection (Maguire et al., 1989). In addition, activation of mGlurR2/3 receptors by application of (2S,1'S,2'S)-2-(carboxycyclopropyl) glycine on cerebellar granule cells also inhibits L-type Ca\textsuperscript{2+} currents (Chavis et al., 1994). Although these results do not provide evidence for a direct G protein inhibition, it is worth noting that GPCR-mediated inhibition of L-type Ca\textsuperscript{2+} currents in nerve cells is fully abolished by pertussis toxin treatment, indicating the implication of Gai/o proteins. However, evidence for a direct G protein modulation of Ca\textsubscript{v}1 channels comes from the observation that inhibition of L-type Ca\textsuperscript{2+} currents in pancreatic \(\beta\) cells can be reversed by application of a depolarizing prepulse (Ammala et al., 1992). Although work performed on nerve and pancreatic cells mostly involved Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 channels, G protein modulation of native Ca\textsubscript{v}1.1 channel isoform has also been described in skeletal muscle cells in which L-type Ca\textsuperscript{2+} current inhibition was observed upon \(\beta\)-adrenergic stimulation by isoproterenol or GTP\gamma S injection (Somasundaram and Tregear, 1993). In addition, it was shown that expression of G\beta\gamma dimer in vivo in adult skeletal muscle fibers specifically inhibits L-type Ca\textsuperscript{2+} currents and voltage-induced Ca\textsuperscript{2+} release (Weiss et al., 2010). Although this inhibition is not reversed by a classic depolarizing prepulse, the observation that expression of some other G\beta\gamma dimers combinations (for instance, G\beta\gamma\textsubscript{2}, G\beta\gamma\textsubscript{3}, or G\beta\gamma\textsubscript{3}) has no effect on the Ca\textsuperscript{2+} current strongly suggests that G\beta\gamma dimer specifically mediates modulation of Ca\textsubscript{v}1.1 channels. The situation is similar regarding the low-voltage–activated Ca\textsubscript{v}3 channels. Hence, application of baclofen on DRG neurons inhibits T-type Ca\textsuperscript{2+} currents, this inhibition being often counterbalanced by the concomitant presence of a current potentiation (most likely mediated by diffusible second messengers) (Scott and Dolphin, 1990). Similarly, it was shown that inhibition of T-type Ca\textsuperscript{2+} current by dopamine D1 receptor in rat adenral glomerulosa cells requires the combined action of G\beta\gamma dimer and cAMP (Drolet et al., 1997). More recently, the molecular mechanism of T-type Ca\textsuperscript{2+} current inhibition by G proteins was specified. It specifically affects Ca\textsubscript{v}3.2 channel over the Ca\textsubscript{v}3.1 isoform, and in contrast to Ca\textsubscript{v}1.1 channels specifically requires the G\beta\gamma\textsubscript{2} combination (G\beta\gamma\textsubscript{2} dimer having no significant effect on the Ca\textsuperscript{2+} current) (Wolfe et al., 2003). The inhibition relies on the direct binding of G\beta\gamma dimer to the II-III loop of the Ca\textsubscript{v}3.2 subunit, replacement of this loop by the corresponding Ca\textsubscript{v}3.1 region preventing G protein modulation. This inhibition has been attributed to a diminution of the opening probability of Ca\textsubscript{v}3.2 channels, without other alteration of channel gating or expression (DePuy et al., 2006). It is worth noting that similarly to what was observed for Ca\textsubscript{v}1 channels, G protein–mediated inhibition of the Ca\textsubscript{v}3.2 channel is not reversed by depolarizing prepulse, although mediated by the direct binding of G\beta\gamma dimer to the channel.

Taken together, these results suggest that likely most of the VGCCs are affected by G protein inhibition. However, in contrast to Ca\textsubscript{v}2 channels, it remains unclear whether inhibition of Ca\textsubscript{v}1 and Ca\textsubscript{v}3 channels is mediated by a direct binding of G\beta\gamma dimer to the channel or required activation of secondary signaling pathways. Inhibition of Ca\textsubscript{v}1 and Ca\textsubscript{v}3 channels that are usually not reversed by a depolarizing prepulse is also not characterized by a depolarizing shift of the current/voltage activation curve (DePuy et al., 2006), typical feature of the direct G\beta\gamma-dependent inhibition. However, considering the presence of numerous molecular channel determinants that contribute to the phenotype of the direct G\beta\gamma regulation of Ca\textsubscript{v}2 channels, it is possible the Ca\textsubscript{v}1 and Ca\textsubscript{v}3 channels lack important molecular determinants required to reveal OFF landmarks of the regulation. Either way, future studies will certainly uncover the molecular mechanisms by which G proteins modulate Ca\textsubscript{v}1 and Ca\textsubscript{v}3 channels.

**Voltage-Independent Inhibition of Ca\textsubscript{v}2 Channels**

Although this review is essentially focused on the so-called fast voltage-dependent regulation mediated by direct binding of G proteins onto the channel, various studies on native neurons and heterologous expression systems have identified other types of inhibition that usually take tens of seconds to develop and involve diffusible second messengers or surface remodeling of GPCR/channel complexes.
Inhibition of VGCCs by Phosphoinositides. Direct Gβγ-dependent inhibition of VGCCs is usually fast and requires the activation of Gi/o-coupled receptors. In contrast, initial recordings performed on sympathetic neurons have identified a relatively slow and voltage-independent form of inhibition that is mediated by the activation of Gq-coupled receptors (Gamper et al., 2004). Although various studies have ruled out the implication of typical Gq-dependent signaling pathways downstream of phospholipase Cβ, inositol trisphosphate, diacylglycerol, and protein kinase C, it was proposed that depletion of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) is the mediator of Gq-dependent inhibition of VGCCs (Delmas et al., 2005; Michailidis et al., 2007; Roberts-Crowley et al., 2009; Rodriguez-Menchaca et al., 2012). Consistent with this idea, loss of Ca2.2 channel activity typically observed in excised membrane patches can be either reduced by application of PIP2 or in contrast enhanced by depletion of PIP2 (Gamper et al., 2004). In addition, the time course of the slow inhibition of Ca2+ currents produced by activation of muscarinic M1 receptors in sympathetic neurons nicely correlates with the kinetics of PIP2 hydrolysis, whereas infusing of PIP2 into the cell via the patch pipette is sufficient to prevent M1 receptor-dependent inhibition of Ca2.2 channels (Gamper et al., 2004). More recently, using an exogenous voltage-sensitive 5-phosphatase that allows rapid hydrolysis of PIP2 into phosphatidylinositol 4-phosphate, it was shown that membrane depletion of PIP2 suppresses Ca1.2, Ca1.3, Ca2.1, and Ca2.2 currents, supporting the idea that depletion of PIP2 is sufficient to mimic the slow inhibition of calcium currents observed under Gq-coupled receptor activation (Suh et al., 2010). However, it is worth noting that besides the fact that voltagesensitive 5-phosphatase–induced PIP2 depletion is similar to the depletion produced by activation of muscarinic receptors, the amplitude of the Ca2+ current inhibition is significantly less, suggesting that another signaling pathway might also contribute to the slow Gq-dependent inhibition. Hence, it was proposed that production of arachidonic acid by the action of phospholipase A2 on PIP2 and other membrane phospholipids elicits modulation of Ca2.2 channels (Liu et al., 2001; Liu and Rittenhouse, 2003). Interestingly, it was also proposed that depletion of membrane PIP2 might reduce Gβγ-dependent inhibition of Ca2.1 channels expressed in Xenopus oocytes, suggesting a crosstalk between voltage-dependent and voltage-independent inhibitions (Rousset et al., 2004). Although the molecular mechanism by which PIP2 interferes with Gβγ regulation is not fully understood, it was proposed that binding of the carboxy-terminal tail of the Ca2.1 subunit to membrane phosphoinositides might stabilize a Gβγ-sensitive state of the channel (Rousset et al., 2004). However, considering that depletion of membrane PIP2 upon Gqα-dependent activation takes several minutes, it is unlikely that this mechanism accounts for the fast initial inhibition phase produced by Gβγ dimer.

Inhibition of Ca2+ Channels by Channel/GPCR Complex Internalization. Initially proposed by Forscher et al. (1986), G protein–dependent inhibition of VGCCs may require a tight GPCR-channel coupling, and evidence exists for direct biochemical interaction between the Ca2+ channel and the GPCR. Hence, physical association of Ca2.1 channels with metabotropic glutamate receptors mGluR1 has been documented in cerebellar Purkinje neurons as well as in cellular expression systems and involves the direct binding of the carboxy-terminal domain of the Ca2.1 subunit with the carboxy-terminal region of the receptor (Kitano et al., 2003). Similarly, a Ca2.2–opioid receptor like-1 (ORL-1; also known as nociception receptor) signaling complex has been documented in small DRG neurons, and supports a tonic agonist-independent G protein inhibition of the Ca2+ channel evidenced by prepulse facilitation (Beedle et al., 2004). Similar observations have been reported for μ- and δ-opioid receptors transiently expressed with Ca2.2 channel in tsA201 cells, although the existence of these protein complexes remains to be explored in native conditions (Chee et al., 2008; Evans et al., 2010). Also, a physical interaction exists between Ca2.2 channels and dopamine D1 and D2 receptors and requires other channel structural determinants, including the II-III intracellular linker (Kisilevsky et al., 2008; Kisilevsky and Zamponi, 2008; Weiss, 2009). Although the existence of Ca2.2-GPCR signaling complexes is unambiguous, the physiologic relevance of these interactions is not fully understood. It was proposed that association of the Ca2+ channels with GPCRs might control channel density at the plasma membrane, providing an additional level of control of the Ca2+ influx. Indeed, activation of ORL-1 receptors triggers an agonist-dependent cointernalization of Ca2.2–ORL-1 complexes into vesicular compartments both in tsA201 cells and DRG neurons (Altier et al., 2006; Evans et al., 2010). However, internalization of Ca2.2 channels is not accompanied by a diminution of the membrane Ca2+ current, questioning the physiologic relevance of this regulation (Murai et al., 2012). In addition, although μ-opioid receptors also physically interact with Ca2.2 channels, they do not cointernalize, indicating that biochemical coupling of the channel with the GPCR is not sufficient to mediate agonist-mediated internalization of the Ca2+ channel (Evans et al., 2010). It is also possible that the assembly of Ca2 channels and GPCRs provides a mechanism that ensures spatiotemporal regulation of the Ca2+ entering synaptic nerve terminals. In addition, tonic channel inhibition mediated by channel-GPCR complexes could also represent a mean to dynamically adjust the Ca2+ influx to the electrical input signal coming to the synaptic ending. Indeed, it was shown that the extent of current facilitation (i.e., current recovery from G protein inhibition) is dependent on both the duration (Brody et al., 1997) and the frequency of action potentials (AP) (Penington et al., 1991; Williams et al., 1997). Although low-frequency AP produces no or little recovery, increasing AP frequency significantly enhances recovery from G protein inhibition and Ca2+ influx and could contribute to short-term synaptic facilitation or depression (Bertram et al., 2003).

Contribution of G Protein Modulation to Channelopathies

As previously discussed, gating properties of the Ca2+ channel significantly affect direct G protein inhibition, essentially the OFF landmarks (i.e., the dissociation of Gβγ dimer from the Ca2 subunit). Hence, alteration of channel gating is likely to affect G protein regulation and synaptic activity. A number of congenital mutations in the gene CACNA1A encoding the Ca2.1 channel cause familial hemiplegic migraine type-1 (FHM-1), a rare and severe monogenic subtype of migraine with aura, characterized by at least some degree of hemiparesis during aura (Ophoff et al., 1996; Weiss et al., 2007b; Ducros, 2013; Pietrobon, 2013). FHM-1 mutations generally affect
structural determinants that are essential for channel gating, including the S4 transmembrane segments thought to carry the voltage sensor controlling channel activation, the S6 transmembrane segment involved in the control of channel inactivation, and the pore-forming loops. Biophysical analyses of channel gating revealed a hyperpolarizing shift of the voltage dependence of activation, as well as additional effects on channel inactivation kinetics, open probability, and unitary conductance (Kraus et al., 1998, 2000; Hans et al., 1999; Mullner et al., 2004; Tottene et al., 2005; Tonelli et al., 2006). In addition, a knock-in mouse model expressing the human pathogenic FHM-1 mutation R192Q located in the first S4 segment of the Cav2.1 subunit revealed a decreased neuronal excitability threshold, increased Ca\(^{2+}\) influx and cortical spreading depression (i.e., the mechanism underlying migraine with aura) (van den Maagdenberg et al., 2004; Pietrobon and Moskowitz, 2013), and enhanced excitatory transmission at cortical synapses (Tottene et al., 2009).

Similar alterations have also been documented for the S218L mutation (van den Maagdenberg et al., 2010). Whereas intrinsic alteration of Ca\(_{2.1}\) channel gating most likely contributes to neuronal hyperexcitability, alteration of the G protein–dependent inhibitory pathway of presynaptic Ca\(_{2.1}\) channels may also contribute to synaptic hyperexcitability. Consistent with this idea, a decreased G protein inhibition of R192Q Ca\(_{2.1}\) channels was reported (Melliti et al., 2003). Careful analysis revealed that the R192Q mutation does not affect the ON landmark, but rather favors the dissociation of G\(\beta\gamma\) dimer following channel activation (OFF landmarks), thereby decreasing the inhibitory G protein pathway (Weiss et al., 2008). Similar results were observed with various other FHM-1 mutations (Weiss et al., 2008; Garza-Lopez et al., 2012, 2013), indicating that alteration of G protein regulation of the Ca\(_{2.1}\) channel caused by FHM-1 mutations is a common underlying mechanism that certainly contributes to synaptic defects observed during the disease.

Alteration of channel gating can also be caused by change in regulatory subunits. Hence, an epileptic lethargic phenotype in mouse resulting from the loss of expression of the Ca\(_{\beta}\) subunit is accompanied by a Ca\(_{\beta}\) subunit reshuffling (Burgess et al., 1997). Considering that Ca\(_{\beta}\) subunits significantly affect G protein inhibition of Ca\(_{2}\) channels in a Ca\(_{\beta}\) isoform-dependent manner (Weiss et al., 2007a), it is likely to contribute to the altered excitatory synaptic transmission observed in those animals (Caddick et al., 1999; Hosford et al., 1999).

**Concluding Remarks and Perspectives**

Since the first description of the phenomena by Dunlap and Fischbach in the late seventies (Dunlap and Fischbach, 1978), great advances have been made in our understanding of the underlying molecular regulation of neuronal VGCCs by GPCRs and its importance in physiology. In this review, we provided an appreciation of its tremendous complexity, arising not only from the numerous molecular channel and G protein determinants involved in the regulation, but also from the channel subunit composition, GPCR subtype, interactions with synaptic proteins and other intracellular signaling pathways, and most likely many more factors that have not yet been characterized. Although numerous channel/G protein–binding determinants have been described, the molecular mechanism by which G\(\beta\gamma\) dimer mediates inhibition of the Ca\(_{2.2}\) current remains incompletely understood. It is likely that more discrete interactions have not yet been characterized support this inhibition, and the recent structural information obtained from structurally similar channels will certainly help to find out the molecular basis of G protein inhibition. In addition, the use of small molecules and peptides to selectively disrupt interaction of G protein \(\beta\gamma\) dimer with some effectors has been demonstrated in vitro and in vivo on various models of heart failure and morphine tolerance (Bonacci et al., 2006; Mathews et al., 2008; Casey et al., 2010). A deeper biochemical and functional characterization of G\(\beta\gamma\) channel interaction will certainly provide important information to identified molecules targeting G protein inhibition of VGCCs with potential therapeutic benefits.

From a more physiologic point of view, although the most evident outcome of G protein regulation of presynaptic Ca\(_{2.1}\) channel is a reduction of the Ca\(_{2.1}\) influx entering nerve terminals (ON landmark), the observation that OFF landmarks might play an important role in fine-tuning synaptic strength, possibly contributing to short-term synaptic facilitation/depression, represents an interesting concept in molecular neuroscience that certainly merits to be further investigated. In addition, the notion that G protein regulation is altered by pathologic mutations in the Ca\(_{2.1}\) channel complex not only contributes to our understanding of the pathogenesis of neuronal Ca\(_{2}\) channelopathies, but also emerges as an important signaling pathway for potential new therapeutic strategies.

Finally, merely 20 GPCRs from over nearly 1000 estimated from the sequencing of the human genome (including many orphan receptors) (Fredriksson et al., 2003; Vassilatis et al., 2003; Zhang et al., 2006) have been described for modulating VGCCs. Although investigations into this extraordinary field continue, it is likely that many new GPCRs can underlie modulation of not only neuronal VGCCs, but also channels expressed in other tissues, such as heart and skeletal muscle, and certainly represent a considerable source of potential therapeutic targets for the treatment of channelopathies in general.

**Authorship Contributions**

Wrote or contributed to the writing of the manuscript: Proft, Weiss.

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