

MINIREVIEW

# Ca<sup>2+</sup> Channels As Integrators of G Protein-Mediated Signaling in Neurons

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## ABSTRACT

The observations from Dunlap and Fischbach that transmitter-mediated shortening of the duration of action potentials could be caused by a decrease in calcium conductance led to numerous studies of the mechanisms of modulation of voltage-dependent calcium channels. Calcium channels are well known targets for inhibition by receptor-G protein pathways, and multiple forms of inhibition have been described. Inhibition of Ca<sup>2+</sup>

channels can be mediated by G protein  $\beta\gamma$ -subunits or by kinases, such as protein kinase C and tyrosine kinases. In the last few years, it has been shown that integration of G protein signaling can take place at the level of the calcium channel by regulation of the interaction of the channel pore-forming subunit with different cellular proteins.

Communication between neurons and their targets depends upon the precise timing of electrical and chemical signals. In the nervous system, short, local signals are necessary to convey appropriate timing information. Defects in such timing events produce problematic errors in the final physiological response. Voltage-dependent calcium channels are the primary triggers for electrically evoked release of chemical transmitters; therefore, understanding the molecular components and events underlying their regulation is central to the development of a mechanistic picture of key events in neuronal signaling. Inhibition of Ca<sup>2+</sup> channels can be voltage-dependent and is mediated by direct interaction of G protein  $\beta\gamma$ -subunits with the pore-forming  $\alpha_1$ -subunit of the channel (Hille, 1994; Ikeda and Dunlap, 1999). In addition, phosphorylation by kinases such as protein kinase C (Rane and Dunlap, 1986) and tyrosine kinases (Diversé-Pierluissi et al., 1997) has been shown to inhibit Ca<sup>2+</sup> channels.

## Classification and Structure of High-Voltage-Activated Calcium Channels

High voltage-activated calcium channels are classified into Ca<sub>v</sub>1.1–3 and Ca<sub>v</sub>2.1–3 types based on the gene encoding the pore subunit and their electrophysiological and pharmacological properties. Ca<sub>v</sub>1-type channels are ubiquitous (Table 1). They play an important role in excitation-contraction in cardiac and skeletal muscle (Catterall, 2000). Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 channels are involved in neurotransmission and can coexist in the same nerve terminals (Dunlap et al., 1995). Until recently, the Ca<sub>v</sub>2.3 channel was less characterized because of its lack of sensitivity to any known pharmacological blocker. The use of SNX482 as a blocker for this type of channel should facilitate the studies of the function and regulation of Ca<sub>v</sub>2.3 channels (Bourinet et al., 2001).

Voltage-dependent calcium channels are multimeric proteins composed of  $\alpha_1$ -,  $\beta$ -,  $\alpha_2\delta$ -, and  $\gamma$ -subunits (Fig. 1). The  $\alpha_1$ -subunit is the pore-forming subunit and accounts for the voltage-dependence of the channel. Channel blockers and naturally occurring toxins exert their actions by binding to this subunit. So far, four  $\beta$ -subunits, four  $\delta$ -subunits (Qin et al., 2002), and eight  $\gamma$ -subunits (Tomita et al., 2003) have been cloned. Coexpression of  $\alpha$ -subunits with different  $\beta$ -sub-

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**ABBREVIATIONS:** DRG, dorsal root ganglion; PKC, protein kinase C; BDNF, brain-derived neurotrophic factor; aa, amino acid(s); SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SH3, Src homology 3; RGS, regulator of G protein signaling; GPCR, G protein-coupled receptor; CASK, Ca<sup>2+</sup>/calmodulin-dependent kinase-like domain, SH3 domain, and a guanylate kinase-like domain containing protein; PIP2, phosphatidylinositol 4,5-bisphosphate.

the direct binding of G protein  $\beta\gamma$ -subunits ( $G\beta\gamma$ ) and results in voltage-dependent inhibition of  $Ca_v2.2$  channels (Fig. 2b) (for review, see Ikeda and Dunlap, 1999). The structural basis of this modulation will be discussed in the next section.

Phosphorylation is a common mechanism for modulation of ion channels. Early work on Ca<sub>v</sub>1 channel modulation in non-neuronal system, such as skeletal muscle and heart, has shown that the channels were a substrate for phosphorylation by cAMP-dependent protein kinase (Catterall, 2000). In neurons, receptors such as the  $\beta$ -adrenergic receptor and dopamine D1/D5 receptors modulate Ca<sub>v</sub>1 channels through cAMP-dependent pathways. Neuronal Ca<sub>v</sub>1 channels are located mainly in the cell bodies and have been shown to regulate transcription through a cAMP-dependent pathway (Catterall, 2000). Ca<sub>v</sub>2.2 channels, the focus of this review, are not main targets for modulation by cAMP. Activation of protein kinase C (PKC) results in enhancement of Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 current (Swartz et al., 1993; Zhu and Ikeda, 1994). Activators of PKC inhibit calcium current in chick DRG neurons (Rane and Dunlap, 1986); it is not known whether the difference observed in DRG neurons is a consequence of variability in the channel sequence. Some differences have been observed in the latency and time course of the effects of activation of PKC on Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 currents. One possibility is that PKC isozymes target different calcium channel subtypes and might exist in complex with the channel. This notion is supported by a recent report that shows that PKC $\epsilon$  forms a complex with the Ca<sub>v</sub>2.2 channel in rat hippocampal and cortical neurons (Maeno-Hikichi et al., 2003).

Modulation of calcium current by protein kinase C can occur through the activation of  $G_i$  or  $G_q$ . In the case of  $G_i$ , the  $\beta\gamma$ -subunits activate phospholipase C, which leads to the activation of protein kinase C (Fig. 2c). This response is prevented by pertussis toxin.  $G_q$  can inhibit  $Ca_v2.2$  channels in a pertussis toxin-insensitive manner through  $G\alpha_q$ -mediated activation of phospholipase C. This mechanism has also been reported to be involved in the modulation of  $Ca_v2.3$  channels by muscarinic receptors. Experiments in which  $Ca_v2.3$  channels and muscarinic receptors (M1, M3, and M5 receptor subtypes) receptors are expressed in human embryonic kidney 293 cells show that activation of  $G_q$  and PKC $\delta$  is required for modulation (Bannister et al., 2004).

In the last few years, it has been reported that voltage-dependent calcium channels can be modulated by tyrosine kinases (Fig. 2d). The modulation of calcium channels by tyrosine kinases can take place in either short or long time scales. Receptor tyrosine kinases can modulate channel activity by increasing the expression levels of calcium channels. This response occurs at the transcription level and is observed several hours to days after receptor activation. Long-

Name	Letter Code	$\alpha 1$ Gene	Pharmacological Blocker	Expression
Ca <sub>v</sub> 1.1	L	$\alpha 1S$	Dihydropyridines	Skeletal muscle
Ca <sub>v</sub> 1.2	L	$\alpha 1C$	Dihydropyridines	Heart, endocrine cells, neurons
Ca <sub>v</sub> 1.3	L	$\alpha 1D$	Dihydropyridines	Endocrine cells, neurons
Ca <sub>v</sub> 1.4	L	$\alpha 1F$	?	Retina
Ca <sub>v</sub> 2.1	P/Q	$\alpha 1A$	$\omega$ -Agatoxin IVA	Neurons
Ca <sub>v</sub> 2.2	N	$\alpha 1B$	$\omega$ -Conotoxin GVIA	Neurons
Ca <sub>v</sub> 2.3	R	$\alpha 1E$	SNX-482	Neurons

term exposure of rat hippocampal neurons to brain-derived neurotrophic factor (BDNF) induces the synthesis of Ca<sub>v</sub>2 channels at the soma (Baldelli et al., 2002). BDNF increases the fraction of Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 channels contributing to evoked miniature inhibitory postsynaptic currents (Baldelli et al., 2002). In a shorter time scale, Src kinase has been shown to regulate synaptic transmission (Yu and Salter, 1999). Calcium channels are potential targets for this regulation, and it could explain the effects on synaptic transmission. Src kinase forms a complex with Ca<sub>v</sub>2.2 channels in rat hippocampal and embryonic chick DRG neurons (Richman et al., 2004). Heterologous expression of Ca<sub>v</sub>2.2 channels in COS-7 cells has shown that these channels are modulated by inhibitors of Src kinase (Wijetunge et al., 2002).

A third mechanism of modulation of calcium channels may be the binding of PIP<sub>2</sub> to the channel. This lipid has been shown to interact with G protein-regulated inward-rectifier K<sup>+</sup> channels bringing the N and C termini of the channel near the inner surface of the membrane (Lei et al., 2003). PIP<sub>2</sub> prevents the rundown of Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 channels expressed in *Xenopus laevis* oocytes and plays a role in transmitter-mediated modulation of Ca<sub>v</sub>2.2 channels in bullfrog sympathetic neurons (Wu et al., 2002). Because the presence of PIP<sub>2</sub> is required for Gβγ-mediated modulation of G protein-regulated inward-rectifier K<sup>+</sup> channels (Lei et al., 2003), it will be important to test whether PIP<sub>2</sub> regulates the effects of Gβγ on Ca<sub>v</sub>2.2 current.

#### Molecular Basis for G Protein-Mediated Modulation of Ca<sub>v</sub>2.2 Channels

The molecular cloning of the channel has facilitated structure-function studies; the α<sub>1</sub>-subunit has been the focus of

many of these studies. Figure 3 summarizes the sites of interaction in the α<sub>1</sub>-subunit of Ca<sub>v</sub>2.2 channel reported thus far. The intracellular loop I–II of the α<sub>1</sub>-subunit has been found to interact with the β-subunit of the channel (Witcher et al., 1995) in the region of amino acids 387–400. The consensus sequence for this site found in both Ca<sub>v</sub>1.x and Ca<sub>v</sub>2.x is QQxE<sub>xx</sub>LxGY<sub>xx</sub>WI<sub>xxx</sub>E. In addition, in this site, the G protein βγ-subunits bind directly to the channel loop I–II (aa 359–389), inducing voltage-dependent inhibition. G protein modulation is inferred to be a result of the disruption of channel-subunit interaction. Regions of the C terminus (rat aa 2036–2074) may also be involved in this interaction (Qin et al., 1997).

The II–III loop region (aa 726–984) of the Ca<sub>v</sub>2.2 channel interacts with some synaptic proteins, such as the v-SNARE synaptotagmin and the t-SNAREs SNAP-25 and syntaxin (Mochida et al., 1996) (Fig. 3). The region of the channel at which this interaction takes place (aa 726–984) has been named the synprint region (Catterall, 1999). The synprint-SNARE interaction is disrupted by channel phosphorylation by cAMP- and calmodulin-dependent kinases (Catterall, 1999). Because SNARE proteins are involved in vesicle fusion and calcium sensitivity during exocytosis, these findings are of great interest.

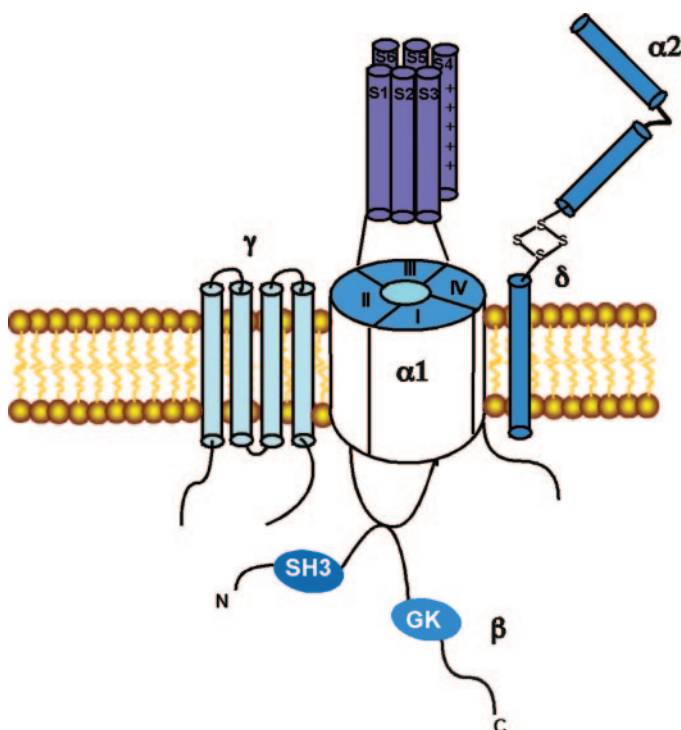
The C terminus of the α<sub>1</sub>-subunit of the Ca<sub>v</sub>2.2 calcium channel interacts with the first PDZ (Postsynaptic, Disc large, Zona occludens) domain of Munc-18 interacting protein (Mint-1) and the Src homology 3 (SH3) domain of CASK, two presynaptic modular adapter proteins (Spafford and Zamponi, 2003) (Fig. 3). In addition, calmodulin can bind to the C terminus, conferring the molecular basis for the calcium-dependent inactivation of voltage-dependent calcium channels (Spafford and Zamponi, 2003).

Although the Ca<sub>v</sub>β-subunit has been found to have a SH3-guanylate kinase module that regulates inactivation of the channel (McGee et al., 2004), there are no published reports of regulation of this module in a receptor-dependent fashion.

#### Integration in Loop I–II: Cross-Talk between G Protein βγ Subunits and Protein Kinase C

Multiple G protein-mediated pathways are known to converge to modulate calcium channels. The complex timing of events is controlled by interactions between components of several signaling pathways and a dynamic network of cytoskeletal and scaffold structures that can serve as the stratum for interaction between these components and the calcium channel. As the signaling pathways involved in calcium channel regulation have been identified and characterized, some of the questions that have arisen are: 1) Do these signaling pathways interact with each other? 2) Do they converge at the level of the channel level or are there upstream coincidence detection points for integration? 3) What are the rate-limiting steps? What molecular complexes are involved in these rate-limiting steps and thus responsible for the time course of transmitter-mediated responses? For the remainder of this review, we will discuss examples that illustrate the integration of signals at the level of the calcium channel.

The loop I–II of the α<sub>1</sub>-subunit is phosphorylated by PKC in a region near the G protein βγ binding site. Phosphorylation of threonine 422 can account for both PKC-mediated



**Fig. 1.** Subunit composition of voltage-dependent calcium channels. Schematic representation of the subunits of voltage-dependent calcium channels. Inset within the α<sub>1</sub>-subunit represents the six-transmembrane domains found in each of the four repeats of this 24 transmembrane pore-forming subunit. The Src homology domain 3 (SH3) and guanylate kinase domain (GK) are shown in the β-subunit.

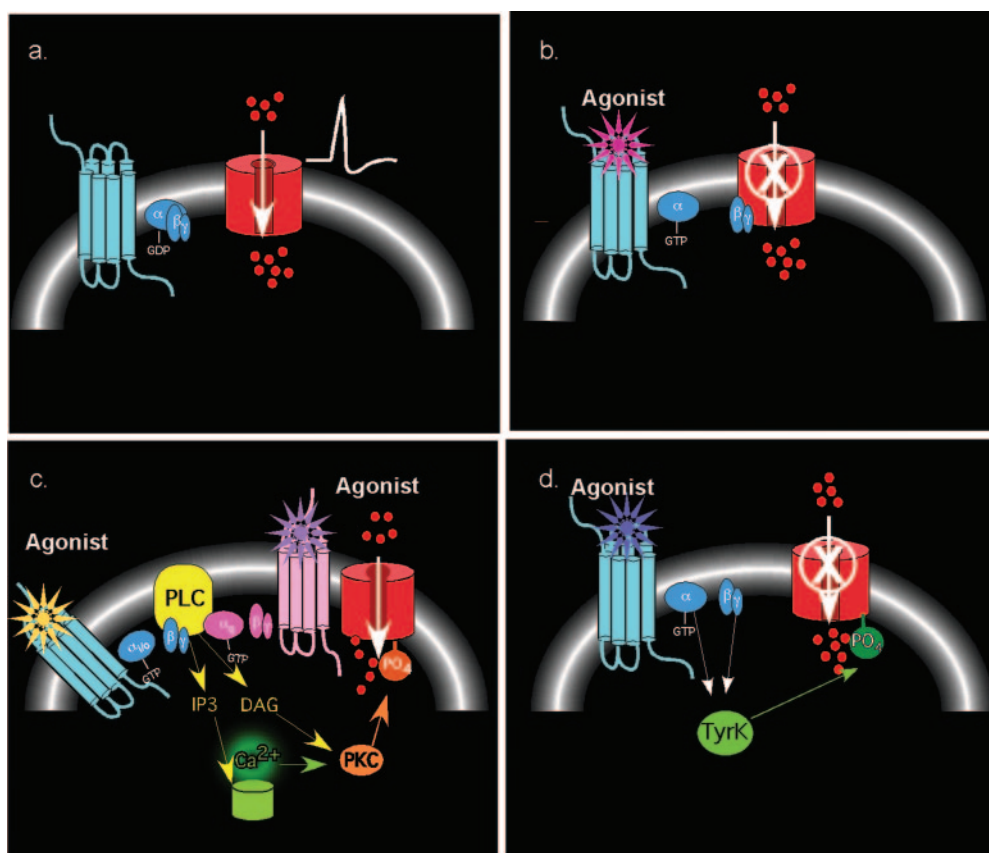


up-regulation of channel activity and antagonism of G protein-mediated inhibition (Cooper et al., 2000). Phosphorylation of threonine 422 or an amino acid substitution that mimics phosphorylation T422E prevents somatostatin- and opiate-induced modulation of  $\text{Ca}_v2.2$  channels (Cooper et al., 2000). The cross-talk between protein kinase C and the  $\text{G}\beta\gamma$ -subunits is dependent on the  $\text{G}\beta$ -subunit; phosphorylation by PKC prevents channel modulation by  $\text{G}\beta_1\gamma$  but does not prevent the effects of other  $\text{G}\beta\gamma$  combinations (Feng et al., 2001). Activation of PKC does not prevent calcium channel modulation by GTP or its hydrolysis-resistant analog guanosine 5'-O-(3-thio)triphosphate. Experiments performed in neonatal rat superior cervical ganglion neurons showed that in the absence of G protein activation, activators of PKC

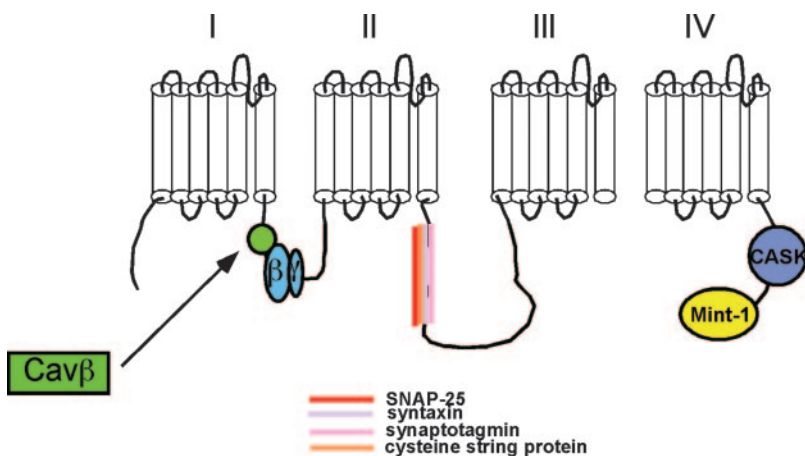
were without effect (Barrett and Rittenhouse, 2000). All these results suggest that phosphorylation by PKC prevents the calcium channel from going into a reluctant or inhibited state.

### Integration between GPCR and Tyrosine Kinase Signaling at the Level of the Calcium Channel

Src kinase can work both as an "on" and "off" signal in the modulation of  $\text{Ca}_v2.2$  channels. This kinase is preassociated with the  $\text{Ca}^{2+}$  channel such that it is rapidly activated by the receptors and regulates the magnitude of the inhibition of the  $\text{Ca}^{2+}$  channel. During the onset of channel modulation by



**Fig. 2.** Multiple G protein-mediated pathways inhibit  $\text{Ca}_v2.2$  channels. a, calcium influx through voltage-gated calcium channels during an action potential. In the absence of receptor activation, the G protein exists in its GDP-bound, heterotrimeric form. b, upon receptor activation, the G protein dissociates into  $\alpha$ -GTP and  $\beta\gamma$ .  $\text{G}\beta\gamma$ -subunits can bind to the calcium channel, resulting in the inhibition of calcium influx. c, calcium channels can be modulated by protein kinase C. GPCRs coupled to  $\text{G}_i$  or  $\text{G}_o$  activate phospholipase C (PLC). Activation of PLC results in the production of inositol 1,4,5-trisphosphate (IP3), which releases calcium from internal stores, and diacylglycerol (DAG), a lipid activator of PKC. d, activation of GPCRs can activate nonreceptor tyrosine kinases (TyrK), which can phosphorylate calcium channels.



**Fig. 3.** Interaction of the  $\alpha_1$ -subunit with cellular proteins. Schematic representation of the 24-transmembrane spanning regions of  $\text{Ca}_v2.2$   $\alpha_1$ -subunit. The calcium channel  $\beta$ -subunit ( $\text{Ca}_v\beta$ ) and G protein  $\beta\gamma$ -subunit ( $\beta\gamma$ ) bind to loop I-II. SNAP-25, syntaxin, synaptotagmin, and cysteine string protein bind to loop II-III aa 726-984; Mint-1 and CASK bind to the C terminus.

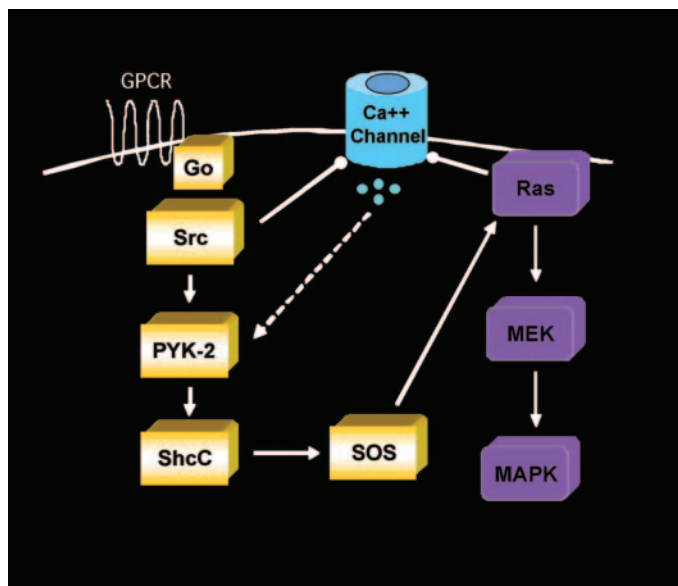
GABA<sub>B</sub> receptors, Src kinase phosphorylates the  $\alpha_1$ -subunit of the calcium channel (Schiff et al., 2000). The sites of tyrosine phosphorylation by Src kinase in the synprint region and the C terminus of the  $\alpha_1$ -subunit (Richman et al., 2004) are conserved in Ca<sub>v</sub>2.1 and Ca<sub>v</sub>2.2 channels in most species. Inhibition of Src kinase results in changes in the magnitude of the modulation of GABA-induced voltage-independent inhibition. A second effect of the activation of Src kinase is the recruitment of members of the Ras pathway (Fig. 4), which determines the rate of onset of GABA-mediated inhibition of Ca<sup>2+</sup> current (Richman et al., 2004). PYK-2, a member of the focal adhesion kinase family, is recruited downstream from Src kinase and is also involved in the determination of

the rate of onset of transmitter-mediated inhibition of calcium current. The calcium dependence of PYK-2 activity provides a point of integration of calcium signaling. The decrease in calcium influx will result in less activation of PYK-2 and a decrease in channel modulation, creating a feedback mechanism in the regulation of calcium influx. Recruitment of ShcC, the neuronal form of the Shc adaptor protein, takes place, resulting in activation and recruitment to the calcium channel complex of the Ras exchange factor Sos (Richman et al., 2004). Whereas the GABA-induced activation of MAP kinase does not alter channel activity, it provides a potential mechanism for long-term changes in the cellular environment as a result of changes in channel activity.

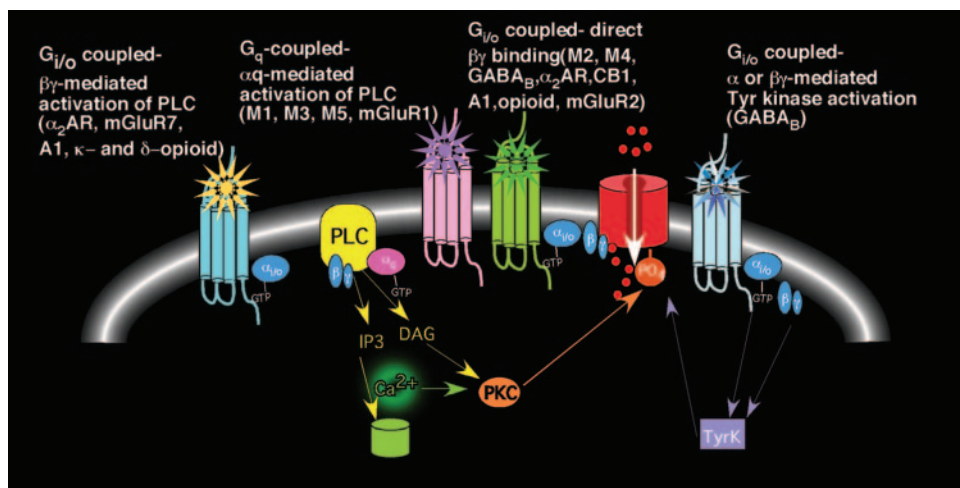
Modulation of calcium channels by G protein-coupled receptors is a transient phenomenon; neurons become unresponsive upon prolonged exposure to neurotransmitters. The inhibition of calcium current by GABA<sub>B</sub> receptors becomes desensitized within 100 s (Schiff et al., 2000). Tyrosine phosphorylation of the  $\alpha_1$ -subunit of the channel makes the channel a target for the binding of RGS12, a member of the "regulator of G protein signaling" (RGS) proteins (Schiff et al., 2000). Introduction of a recombinant protein containing the sequence of the phosphotyrosine binding domain from RGS12 slows the rate of desensitization of GABA-induced voltage-independent inhibition of Ca<sub>v</sub>2.2 calcium channels, whereas the PDZ domain is without effect. RGS12 coprecipitates with the tyrosine-phosphorylated calcium channel, whereas pretreatment with genistein, a tyrosine kinase inhibitor, decreases the degree of association (Schiff et al., 2000).

### RGS3 and Calcium-Dependence of Transmitter-Mediated Inhibition of Ca<sub>v</sub>2.2 Channels

A calcium-feedback mechanism that controls the rate of desensitization of transmitter-mediated inhibition of calcium current has recently been described by Tosetti et al. (2003). They reported that two splicing variants of RGS3 alter the rate of desensitization of GABA<sub>B</sub>-mediated inhibition of Ca<sub>v</sub>2.2 calcium channels in chick DRG neurons. A rapid effect is mediated by the EF-hand of one of the variants, whereas a slower effect on desensitization is mediated by a



**Fig. 4.** Schematic representation of the Src- and Ras-mediated pathways activated by GABA<sub>B</sub> receptors in embryonic chick DRG neurons. GPCR represents the G protein-coupled receptor, which in this case is the GABA<sub>B</sub> receptor. The lines ending in a dot represent signal molecules that interact with the calcium channel, resulting in inhibition of the current; lines with arrowheads represent the flow of the signal and activation of the signal molecule; dashed lines represent positive feedback from the calcium channel resulting in the activation of PYK2. Abbreviations: Src, p60 Src kinase; PYK2, proline-rich tyrosine kinase, a calcium-dependent member of the focal adhesion kinase family; Shc, adaptor protein; SOS, son of sevenless, a Ras guanine nucleotide exchange factor; Ras, monomeric GTP-binding protein; MEK, mitogen-activated protein kinase kinase; MAP, mitogen-activated protein.



**Fig. 5.** G protein-coupled receptors involved in the modulation of Ca<sub>v</sub>2.2 channels. Schematic representation of receptor-G protein coupling to different signaling pathways known to inhibit Ca<sub>v</sub>2.2 channels. Abbreviations: PLC, phospholipase C;  $\alpha_2$ AR,  $\alpha_2$ -adrenergic receptor; mGluR1, metabotropic glutamate receptor subtype 1; A1, adenosine receptor type 1; M, muscarinic receptor; CB1, cannabinoid receptor type 1.

variant that lacks the EF-hand and calmodulin. This kind of regulation creates a feedback loop in which the time course of transmitter-mediated inhibition of presynaptic calcium channels could be controlled by synaptic activity.

## Conclusions

A wide range of transmitters converges in the modulation of neuronal voltage-dependent calcium channels through the activation of G protein-coupled receptors (Fig. 5). A given transmitter can activate several signaling pathways mediated through different receptor subtypes (Fig. 5). Interactions between  $\text{Ca}_v2.2$  channels and signaling molecules can determine the time course of  $\text{Ca}^{2+}$  channel modulation. Over the long term, such interactions could also affect neuronal function by regulating gene expression. Thus, the integration of signals by channel-dependent scaffolding could offer the framework to produce a variety of presynaptic responses that underlie the long and short-term changes in synaptic function in neurons. Identification of the properties of systems that determine the timing and magnitude of  $\text{Ca}^{2+}$  influx is critical for understanding synaptic physiology. Characterization of the endogenous molecular components and spatiotemporal distribution of the neuronal calcium channel macromolecular complexes will be of great importance in the understanding of cellular communication in the nervous system.

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