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G Protein Modulation of Voltage-Gated Calcium Channels

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***Abstract*——Calcium influx into any cell requires fine tuning to guarantee the correct balance between activation of calcium-dependent processes, such as muscle contraction and neurotransmitter release, and calcium-induced cell damage. G protein-coupled receptors play a critical role in negative feedback to modulate the activity of the Ca_V2 subfamily of the voltage-dependent calcium channels, which are largely situated on neuronal and neuro-endocrine cells. The basis for the specificity of the relationships among membrane receptors, G proteins, and effector calcium channels will be discussed, as well as the mechanism by which G protein-mediated inhibition is thought to occur. The inhibition requires free $G\beta\gamma$ dimers, and the cytoplasmic linker between domains I and II of the Ca_V2 α1 subunits binds $G\beta\gamma$ dimers, whereas the intracellular N terminus of Ca_V2 α1 subunits provides essential determinants for G protein modulation. Evidence suggests a key role for the β subunits of calcium channels in the process of G protein modulation, and the role of a class of proteins termed "regulators of G protein signaling" will also be described.**

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I. Introduction

Voltage-gated calcium channels (VGCCs[1]) play a major role both in the normal functioning and in the pathophysiology of neurons and other excitable cells. Although they are also found at low levels in nonexcitable cells, their presence has been said to define an excitable cell (Hille, 2001). They were first identified in crustacean muscle by Fatt and Katz (1953), and subsequently extensively studied by a number of groups, including Hagiwara and Takahashi (1967). VGCCs were first classified according to their biophysical properties into low- and high-voltage-activated (LVA and HVA) channels (Carbone and Lux, 1984). Further study, with the additional aid of pharmacological tools, led to the classification of certain HVA channels as "long-lasting" or L-type channels, which were sensitive to the 1,4-dihydropyridine (DHP) class of drugs, and present in skeletal muscle, heart, smooth muscle, and neurons (Hess et al., 1984; Nowycky et al., 1985a).

In neurons it was clear that a component of the HVA calcium current was not L-type, for example, in Purkinje cells in the cerebellum (Hillman et al., 1991) and at presynaptic terminals (see Stanley and Atrakchi, 1990 for example). These additional current components were subclassified with the aid of several invaluable toxins. Two additional subtypes of calcium channel were thus identified: N-type channels, sensitive to ω-conotoxin GVIA (Nowycky et al., 1985b; McCleskey et al., 1987) and P-type channels, sensitive to ω-agatoxin IVA (Mintz et al., 1992). Another ω-agatoxin IVA-sensitive current component was subsequently identified in cerebellar granule cells and termed Q-type current (Randall and Tsien, 1995), but these two components are now combined as P/Q. There is also a residual or R-type calcium current component that is resistant to DHPs and the N and P/Q channel toxins (Randall and Tsien, 1995).

A. Molecular Subtypes of Calcium Channel

The molecular basis for the physiological subtypes of VGCCs was clarified after the identification of the subunits of voltage-gated calcium channels. This era started with the purification of skeletal muscle calcium channel complex also called the DHP receptor, which consisted of α1, α2, β, δ, and γ subunits (Flockerzi et al., 1986; Hosey et al., 1987; Takahashi et al., 1987; Chang and Hosey, 1988; Hymel et al., 1988).

After identification of individual subunits, the sequencing of peptides derived from these subunits formed the basis for the subsequent identification and cloning of the cDNA for the DHP receptor, initially from skeletal muscle (Tanabe et al., 1987) and subsequently from heart by homology with the skeletal muscle sequence (Mikami et al., 1989) (Fig. 1A). The α1 subunits have 24 putative transmembrane segments, arranged into four homologous domains, with intracellular linkers and N and C termini (Fig. 1A). Ten different α1 subunits have been cloned that have specialized functions and distributions (for review, see Ertel et al., 2000) (Fig. 1B). Those that are of most concern to this review are the Ca_V2 subfamily of HVA calcium channels that shows classical modulation by G proteins, comprising $Ca_V2.1$ or α1A, the molecular counterpart of P/Q-type calcium channels (Mori et al., 1991), $Ca_V2.2$ or α1B (Dubel et al., 1992), the molecular counterpart of N-type calcium channels, and $Ca_V2.3$ or α1E (Soong et al., 1993), thought to contribute to the molecular counterpart of the

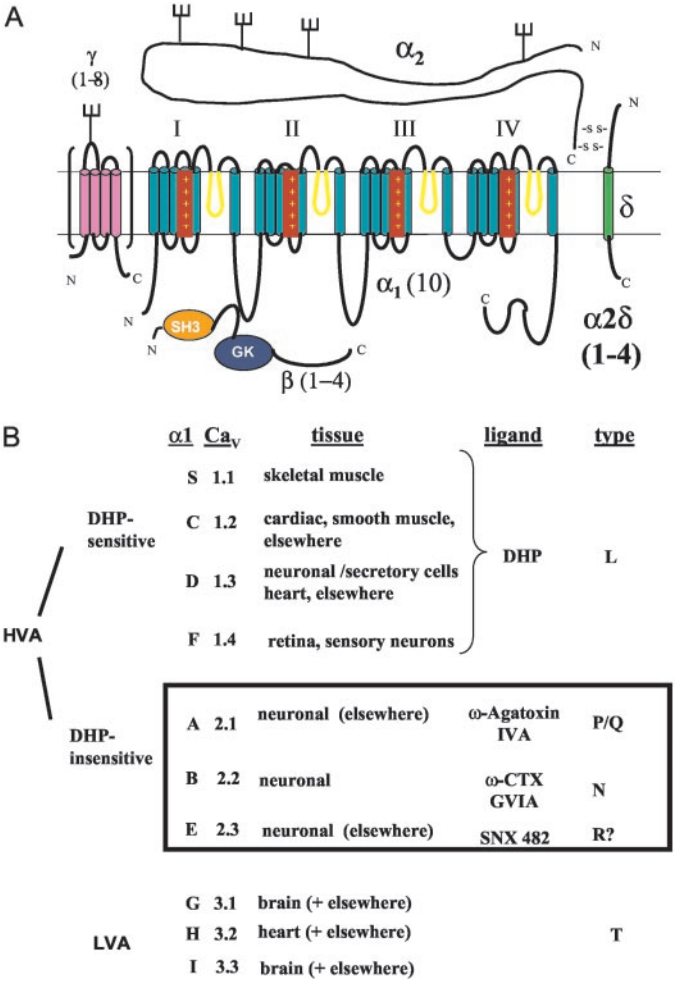

FIG. 1. Subunits making up voltage-gated calcium channels. A, diagrammatic representation of the topology of VGCCs. Cyan cylinders denote transmembrane segments of α1 subunit. Red cylinders denote the charged S4 segment, and yellow denotes the pore region. Green cylinder represents transmembrane segment of δ subunit. Two domains of β subunits [src homology (SH)-3 and guanylate kinase (GK)] were defined previously (Hanlon et al., 1999). Glycosylation is denoted by fork shapes. Redrawn with modification from Dolphin (1998). Not intended to represent exact sizes of various subunits. B, classification and nomenclature of $Ca_V\alpha$1 subunits, including nomenclature, main tissue localization, and pharmacology.

[1] Abbreviations: VGCC, voltage-gated calcium channel; HVA, high-voltage-activated (channel); DHP, 1,4-dihydropyridine; AID, α interaction domain; PKC, protein kinase C; GST, glutathione *S*-transferase; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate; GIRK, G protein-activated potassium channel; β-ARK1, β-adrenergic receptor kinase 1; RGS, regulators of G protein signaling; PIP_2, phosphatidylinositol (4,5)-bisphosphate.

R-type calcium current component (Piedras-Rentería and Tsien, 1998) (Fig. 1B, boxed).

In the case of the N- and P/Q-type as well as the L-type HVA calcium channels, the $Ca_V\alpha1$ subunit has been shown to copurify with an intracellular β subunit ($Ca_V\beta$) (Liu et al., 1996; Scott et al., 1996). Four β subunits have been cloned ($\beta1$–4), with $\beta1a$ being the skeletal muscle isoform of $\beta1$ (Ruth et al., 1989), $\beta2$ being cloned initially from cardiac muscle (Perez-Reyes et al., 1992), $\beta3$ present in cardiac and smooth muscle and neuronal tissue (Castellano et al., 1993b), and $\beta4$ cloned from brain (Castellano et al., 1993a). A number of splice variants have been identified, with one particular splice variant of $\beta2$, $\beta2a$, being N-terminally palmitoylated in certain species, giving it distinctive properties (Chien et al., 1996).

HVA calcium channels also copurify with an extracellular $Ca_V\alpha2$ subunit, which is attached by S-S bonds to a transmembrane δ subunit (Tanabe et al., 1987; Chang and Hosey, 1988; Witcher et al., 1993; Liu et al., 1996). Four $\alpha2\delta$ subunits have been cloned (Ellis et al., 1988; Klugbauer et al., 1999; Barclay et al., 2001; Qin et al., 2002).

Skeletal muscle calcium channels also copurify with a $\gamma1$ subunit (Takahashi et al., 1987). Whether any of the recently cloned novel γ-like subunits ($\gamma2$–8) (Fig. 1B) are tightly associated with other types of VGCCs remains controversial (Letts et al., 1998; Black and Lennon, 1999; Klugbauer et al., 2000; Kang et al., 2001; Moss et al., 2002; Tomita et al., 2003).

II. Role of $Ca_V\beta$ Subunits in Calcium Channel Function

The intracellular $Ca_V\beta$ subunits have marked effects on the properties of HVA $\alpha1$ subunits (Ca_V1 and Ca_V2 families), including trafficking of calcium channel complexes to the plasma membrane and modification of kinetic and voltage-dependent properties (Singer et al., 1991; De Waard et al., 1994; Chien et al., 1995; Brice et al., 1997; Bichet et al., 2000). My group has shown that the converse also applies, in that antisense-induced knockdown of $Ca_V\beta$ subunits from native neurons results in a reduction of the amplitude of endogenous calcium currents and slowed kinetics of activation (Berrow et al., 1995; Campbell et al., 1995b).

Most research indicates that all $Ca_V\beta$ subunits (except truncated splice variants described recently (Hibino et al., 2003; Hullin et al., 2003) increase the functional expression of HVA $\alpha1$ subunits (for a recent review, see Dolphin, 2003). In theory, this could be attributable to effects on a number of channel properties, an increase in the open probability of the channel, an increase in single-channel conductance, an increase in the number of functional channels inserted into the plasma membrane, or a combination of several of these processes. Initial studies did not agree whether there was an increase in number of channels at the plasma membrane, measured as charge moved in isolated gating currents, with either no increase (Neely et al., 1993) or an increase being reported (Josephson and Varadi, 1996). Much early work on the roles of $Ca_V\beta$ subunits in calcium channel expression was performed in *Xenopus* oocytes, but these cells are now known to contain an endogenous *Xenopus* β subunit that complicates the interpretation of these results (Tareilus et al., 1997; Canti et al., 2001). This endogenous $Ca_V\beta$ subunit was found to be both necessary and able to traffic at least some heterologously expressed Ca_V channels to the plasma membrane, since if endogenous β subunit expression was reduced or eliminated by injection of $\beta3$ antisense oligonucleotides, Ca_V expression was largely lost (Tareilus et al., 1997; Canti et al., 2001).

In COS-7 cells, small currents were observed when $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$ were expressed alone, but exogenous β subunits all increased $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$ maximum conductance about 10-fold (Berrow et al., 1997; Stephens et al., 1997; Meir and Dolphin, 1998; Stephens et al., 2000). COS-7 cells do contain mRNA for endogenous β subunits (Meir et al., 2000), but the protein for corresponding β subunits was not detectable by immunocytochemistry (Meir et al., 2000), although a low level might be found if higher sensitivity detection methods were used. Thus, at the moment there are no expression systems that definitively contain no $Ca_V\beta$ subunits that can be used conclusively to answer the question as to whether HVA calcium channels can be trafficked to the plasma membrane without a β subunit.

The increase in current density brought about by $Ca_V\beta$ subunits can be attributed to a number of effects on biophysical properties as well as the important influence on trafficking. All $Ca_V\beta$ subunits hyperpolarize the voltage dependence of activation of all HVA VGCCs (Fig. 2), whereas all, except the $\beta2a$ splice variant that is N-terminally palmitoylated, hyperpolarize the voltage dependence of steady-state inactivation (Birnbaumer et al., 1998). Where it has been studied, the β subunits all produce an increase in mean open time, which is at least in part due to a hyperpolarizing shift in the voltage dependence of the mean open time (Wakamori et al., 1999; Meir et al., 2000). Although $Ca_V\alpha1$ subunits contain inherent determinants of voltage-dependent inactivation (Zhang et al., 1994; Herlitze et al., 1997; Cens et al., 1999; Spaetgens and Zamponi, 1999), association with different $Ca_V\beta$ subunit isoforms dictates their overall kinetics of inactivation (Olcese et al., 1994; Meir and Dolphin, 2002). At the whole-cell level, the inactivation rate was affected in the following order (highest first) $\beta3 > \beta1b > \beta4 > \beta2$ subunits. Retardation of inactivation has been shown to be particularly dramatic for the palmitoylated $Ca_V\beta2a$ subunit expressed with $Ca_V1.2$ (Chien and Hosey, 1998), $Ca_V2.2$ (Bogdanov et al., 2000; Stephens et al., 2000), or $Ca_V2.3$ (Qin et al., 1998).

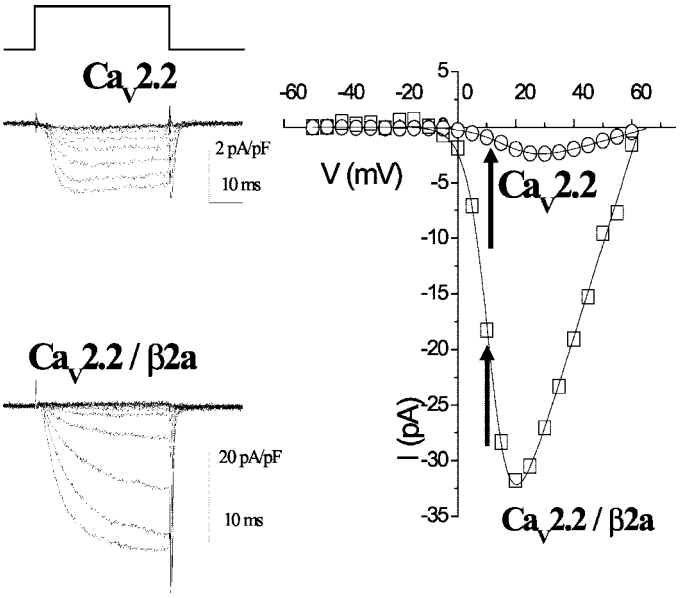

FIG. 2. Properties of $Ca_V2.2$ voltage-gated calcium channels expressed in the absence and presence of a $Ca_V\beta$ subunit. Left, recordings of $Ca_V2.2$ channels transiently transfected in COS-7 cells in the absence of a coexpressed β subunit (upper traces) or the presence of $Ca_V\beta2a$ (lower traces). The currents are activated by 40-ms voltage steps in 5-mV increments from a holding potential of −100 mV, and only those on the rising phase of the I-V relationship are shown for clarity. Right, I-V curve showing the increase in $Ca_V2.2$ current amplitude in the presence of $Ca_V\beta2a$ (open squares) compared with its absence (open circles). Recordings were in 10 mM Ba^{2+} extracellular solution. These I-V data were fitted with the equation: current = $G_{max} \cdot (V - V_{rev})/\{1 + \exp[(V - V_{1/2})/k]\}$, where G_{max} is maximum slope conductance, $V_{1/2}$ is the voltage at which 50% of the current is activated, V_{rev} is the null potential, and k is the slope factor. Coexpression of $\beta2a$ increased G_{max} and induced a hyperpolarizing shift in $V_{1/2}$ and a reduction in k. In these examples, G_{max} = 0.08 and 0.83 nS/pF in the absence and presence of $Ca_V\beta2a$. The arrows indicate the voltage for 50% activation for the two curves, showing the hyperpolarization induced by $\beta2a$. Data replotted from Stephens et al. (2000).

A. Binding of $Ca_V\beta$ to the $\alpha1$ I-II Linker

$Ca_V\beta$ subunits have been found to bind with very high affinity to the cytoplasmic intracellular linker between domains I and II of all HVA calcium channels, via an 18-amino acid motif called the α interaction domain (AID) on the I-II linker (Pragnell et al., 1994). The AID sequence of rabbit $Ca_V2.2$ is QQIERELNGYLEWIFKAE, and the consensus sequence present in both $Ca_V1.x$ and $Ca_V2.x$ subfamilies is QQxExxLxGYxxWIxxxE.

A 41-amino acid sequence (BID) on the $Ca_V\beta$ subunit was identified as the minimal motif required to influence $\alpha1$ subunit expression and to bind to the $\alpha1$ subunit (De Waard et al., 1994, 1996). The consensus sequence of BID is K—E—PYDVV**P**SMR**P**—LVG**P**SLKG**Y**EVTDMMKQALFDF; the underlined serines are consensus protein kinase C (PKC) phosphorylation sites. The residues in bold have been identified as particularly important for binding to $Ca_V\alpha1$ subunits (Walker and De Waard, 1998). This small BID sequence alone can produce an increase in calcium current density, albeit not to the same extent as the full-length protein (De Waard et al., 1994). The affinity between $Ca_V\beta$ subunits and a I-II linker fusion protein has been measured to be between 5 and 60 nM (De Waard et al., 1994), but has been proposed to be state-dependent (De Waard et al., 1995; Canti et al., 2001). In one study (De Waard et al., 1995), no dissociation was seen for $\beta1b$ from the $Ca_V2.1$ I-II linker fusion protein after 10 h, but this may reflect a technical difficulty of overlay assays, because the bound protein may become directly anchored to the membrane. In our own binding studies using surface plasmon resonance, the affinity of $\beta3$ for the GST fusion protein of the I-II linker of $Ca_V2.2$ was about 20 nM, and the k_{off} was 5.2×10^{-3} s^{-1} (Canti et al., 2001). We found similar data (Fig. 3A) for $\beta1b$ binding to the I-II linker of both $Ca_V2.2$ and $Ca_V1.3$ (Bell et al., 2001).

We have studied the in vivo concentration dependence of the effects of $Ca_V\beta$ subunits. Our evidence supports the hypothesis that there are two distinct binding processes for β subunits on $Ca_V2.2$ (Canti et al., 2001). One is a high-affinity process related to the effect of $Ca_V\beta$ on

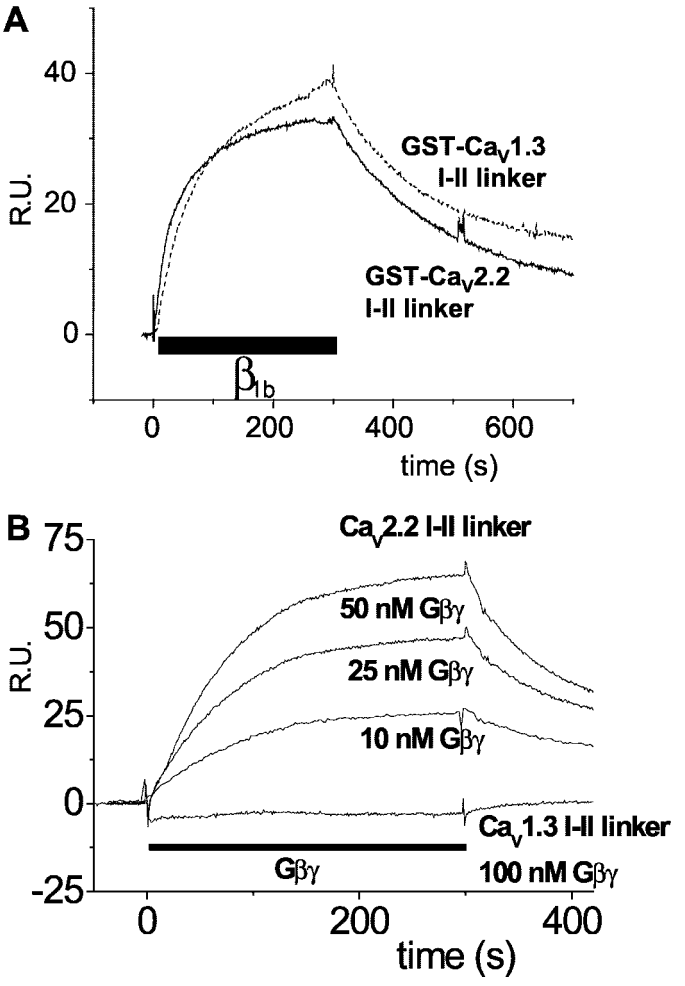

FIG. 3. Binding of $\beta1b$ and $G\beta\gamma$ dimers to I-II linker of $Ca_V2.2$ and $Ca_V1.3$. A, GST fusion proteins of the I-II linker of $Ca_V2.2$ and $Ca_V1.3$ were bound to a Biacore 2000 sensor chip (Biacore International SA, Stevenage, Hertfordshire, UK) via a GST antibody, and the reversible binding of purified recombinant $\beta1b$ (10 nM) was then examined. y-axis, R.U. (arbitrary units of mass bound to the sensor chip). B, the binding of increasing concentrations of purified bovine brain $G\beta\gamma$ was determined to the same fusion proteins. Data replotted from Bell et al. (2001).

the maximum conductance of $Ca_V2.2$, presumably involving its trafficking to the plasma membrane, whose affinity corresponds closely to the in vitro affinity for the I-II linker (~17 nM), which is coincidentally the concentration of endogenous β3 estimated to exist in *Xenopus* oocytes (Canti et al., 2001). The second process is of lower affinity (K_D ~120 nM), associated with the voltage-dependent effects of the β subunit, for example steady-state inactivation. One explanation for the discrepancy in these two calculated affinities is that a single binding site undergoes a marked reduction in affinity for $Ca_V\beta$ subunits once the $Ca_V\alpha1$ subunits have been trafficked from the endoplasmic reticulum and are inserted in the polarized plasma membrane. Alternatively, one might postulate the coexistence of two separate $Ca_V\beta$ subunit binding sites on each $Ca_V2.2$ molecule, but the binding of two $Ca_V\beta$ subunits has not been demonstrated directly (Canti et al., 2001). Whichever hypothesis is correct, it is highly likely that the $Ca_V\beta$ subunit interacts with other domains on the $Ca_V\alpha1$ subunit as well as the I-II linker.

B. Binding of $Ca_V\beta$ Subunits to the N and C Termini of $Ca_V\alpha1$ Subunits

Two other β subunit interaction sites have been identified on various α1 subunits on the C terminus (Qin et al., 1997; Walker et al., 1998) and the N terminus (Walker et al., 1999; Stephens et al., 2000). These appear to be of lower affinity and may be selective for certain $Ca_V\beta$ subunits. Whether they represent part of a single complex β subunit binding pocket made up of the I-II linker and the N and C termini remains to be established. However, the binding site found for β2a on the C terminus of $Ca_V2.3$ appeared to involve binding to the BID domain of β2a, the same as that which binds to the $Ca_V\alpha1$ I-II linker, making it an alternative, rather than an additional site, for an individual $Ca_V\beta$ subunit (Qin et al., 1997). Walker et al. (1999) showed that the N terminus of $Ca_V2.1$ interacted with $Ca_V\beta4$ and β2a but not β1b or β3. The region of β4 involved was within its C terminus (amino acids 446–482). The C terminus of $Ca_V\beta4$ also bound to the C terminus of $Ca_V2.1$. The N and C termini of $Ca_V2.1$ were found to occupy overlapping binding sites that were mutually exclusive, but either could bind in combination with binding to the I-II linker (Walker et al., 1999). This group also showed that $Ca_V\beta4$ produced a smaller hyperpolarizing shift of $Ca_V2.1$ currents than did $Ca_V\beta3$, and that this differential was due to the $Ca_V2.1$ N terminus. Stephens et al. (2000) showed that $Ca_V2.2$ N-terminal residues in the same region as the essential site for G protein modulation (see *Section IV.D.*) were involved in retardation of inactivation kinetics by β2a. Palmitoylated β2a has been suggested to retard inactivation by tethering the I-II linker so that it cannot mediate inactivation (Restituito et al., 2000; Stotz et al., 2000), but our data show an additional role for the N terminus of $Ca_V2.2$.

III. Modulation of Calcium Channels

There are several means by which VGCCs may be both up- and down-regulated by second messenger pathways, for example by phosphorylation (Nunoki et al., 1989; Dolphin, 1999; Catterall, 2000). These include regulation by kinases, for example, up-regulation of cardiac L-type channels by cyclic AMP-dependent protein kinase (Reuter, 1987) and regulation by protein kinase C (Stea et al., 1995). In this review, however, I shall concentrate on the classical G protein pathway and describe first how this was examined in native neurons.

For the neuronal channels, particularly N- and P/Q-types, a major mechanism of inhibitory modulation occurs via the activation of heterotrimeric G proteins by seven transmembrane G protein-coupled receptors (GPCRs). GPCR activation was first found to reduce action potential duration in dorsal root ganglion neurons in the 1970s (Dunlap and Fischbach, 1978). Subsequently, this effect was found to result from inhibition of voltage-gated calcium channels (Dunlap and Fischbach, 1981). Such modulation has since been observed in many types of neuron, including superior cervical ganglion neurons (Ikeda and Schofield, 1989) and submucosal neurons (Surprenant et al., 1990).

The GPCRs typically involved in this type of modulation include α2-adrenoceptors, μ and δ opioid receptors, GABA-B receptors (Fig. 4, A and B), and adenosine A1 receptors (Dunlap and Fischbach, 1978; Dolphin et al., 1986; Scott and Dolphin, 1986). The key features that typify this inhibition are a slowing of the current activation kinetics, which is thought to be due to a time- and depolarization-dependent recovery from voltage-dependent inhibition (Bean, 1989). The voltage dependence is manifested by a shift to more depolarized potentials of the current activation-voltage relationship and the loss of inhibition at large depolarizations, because of a shift from "reluctant" to "willing" channels (Bean, 1989). Removal of inhibition can also be induced by a depolarizing prepulse applied immediately before the test pulse (Ikeda, 1991). Additional mechanisms that are not voltage-dependent have also been described in various cell types manifested by a scaled reduction in the current and an inability of a depolarizing prepulse to reverse this component of the inhibition (for example, see Diversé-Pierluissi and Dunlap, 1993).

N- and P/Q-type calcium channels support synaptic transmission and are concentrated at nerve terminals. P/Q-type channels are most important for transmitter release at central terminals (Takahashi and Momiyama, 1993), although N-type channels are also present and particularly contribute earlier in development. In contrast, N-type channels are more prevalent in peripheral nerve terminals and are largely responsible for synaptic transmission in autonomic and sensory terminals (Mochida et al., 1996; Koh and Hille, 1997). Modulation of these channels by activation of GPCRs has been shown

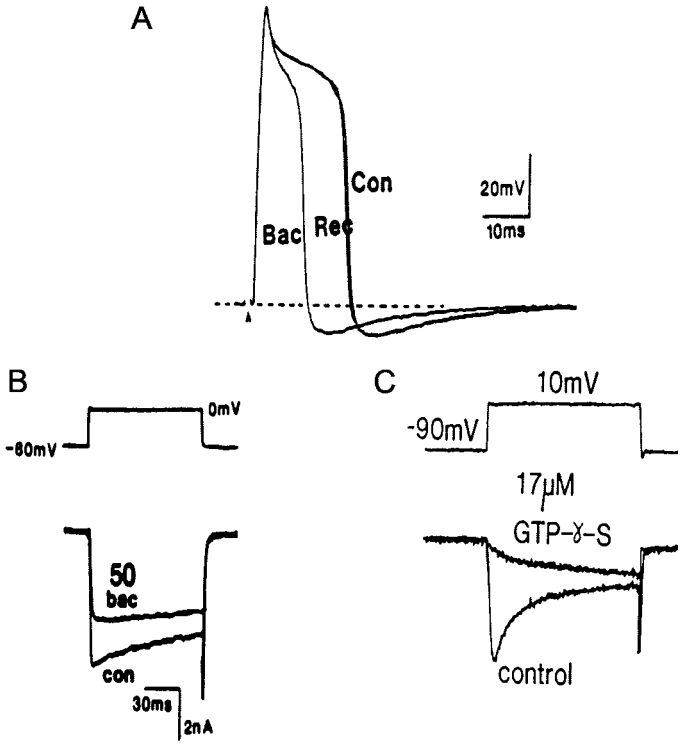

FIG. 4. Effect of the GABA-B agonist (−)-baclofen on action potential duration and calcium channel currents recorded from dorsal root ganglion neurons: involvement of G proteins. A, action potentials, whose duration was lengthened by blockade of potassium channels by extracellular tetraethylammonium (10 mM), were recorded in current clamp, induced by a brief depolarizing pulse. (−)-Baclofen (50 μM, Bac) was applied by pressure ejection and shortened the action potential duration, compared with the control (Con), before agonist application. The effect was readily reversible after terminating agonist application (Rec). B, calcium channel currents were recorded from these cells in voltage clamp, and application of (−)-baclofen (50 μM) reduced the current amplitude. C, when GTPγS (17 μM) was included in the patch pipette, the current recorded was small in amplitude and more slowly activating than control. Data redrawn from Dolphin and Scott (1987) and Dolphin and Scott (1990). The Ba^{2+} concentration was 2.5 mM.

to occur both in cell bodies (Holz et al., 1986; Scott and Dolphin, 1986; Dolphin and Scott, 1987; Ikeda, 1991) and at presynaptic terminals (Takahashi et al., 1998). This mechanism may be responsible for at least some of the presynaptic inhibition of synaptic transmission mediated by a wide variety of GPCRs in many areas of the nervous system (Dolphin and Prestwich, 1985; Man-Son-Hing et al., 1989; Toth et al., 1993). Activation of GPCRs such as the GABA-B receptor will reduce calcium entry into presynaptic terminals via VGCCs by the same mechanism that is observed in cell bodies (Fig. 4A), and the effect should also be frequency- and potential-dependent. Inhibition will be reduced during a high-frequency train as a result of the voltage dependence of the inhibitory modulation. Relief of inhibition of calcium currents, evoked by action potential-like voltage waveforms, has been reported during high-frequency trains (Williams et al., 1997; Brody and Yue, 2000) and may contribute to the modulation of presynaptic inhibition according to input frequency.

IV. Inhibitory Coupling between G Proteins and Voltage-Gated Calcium Channels in Native Tissue

The role of G proteins in the inhibition of calcium currents by GPCR activation was first demonstrated some years later (Holz et al., 1986; Scott and Dolphin, 1986). It was also identified that there was a direct membrane-delimited link between G protein activation and N- or P/Q-type calcium channel inhibition. A key experiment enabling this idea to be accepted was the finding that inhibition of calcium channels recorded in the cell-attached patch mode only occurs when the receptor agonist is present in the patch pipette, and not when it bathes the remainder of the cell membrane (Forscher et al., 1986). This indicates that the inhibitory process is very localized and that a soluble second messenger is not involved. In most studies, it is found that this direct linkage only applies to the Ca_V2 family of calcium channels, but additional non-voltage-dependent pathways, which may be direct or via down-stream soluble intracellular messengers also occur in certain cell types and may additionally apply to L-type channels (Hille, 1992; Dunlap and Ikeda, 1998) and also to T-type channels (Wolfe et al., 2003).

A. The G Protein Subunits Involved in the Direct Inhibitory Modulation of Native and Heterologously Expressed Calcium Channels

The modulation of neuronal VGCCs in most native neurons is usually mediated by receptors coupled to pertussis toxin-sensitive G proteins (G_i and G_o subtypes) (Holz et al., 1986; Scott and Dolphin, 1986). The response to an agonist can be mimicked by nonhydrolyzable analogs of GTP such as guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (Fig. 4C) (Dolphin and Scott, 1987) and by photoactivation of a caged GTP analog (Dolphin et al., 1988). The effect of GTP analogs is, as expected, more extensive than that of receptor agonists and is irreversible because G proteins are permanently activated. To provide evidence that the effect of agonists is mediated by G proteins, initial experiments showed that the effect of agonists is enhanced and made irreversible by a low concentration of GTPγS (Scott and Dolphin, 1986) and prevented by a GDP analog such as guanosine 5′-*O*-(2-thiodiphosphate) (Holz et al., 1986; Dolphin and Scott, 1987).

To identify which G proteins are involved in receptor-mediated inhibition of calcium channels in native systems (such as dorsal root ganglion neurons and sympathetic neurons), a number of studies were performed with blocking antibodies and antisense oligonucleotides complementary to G protein α subunits, which showed that $G\alpha_o$ was primarily responsible for the response (McFadzean et al., 1989; Baertschi et al., 1992; Campbell et al., 1993; Menon-Johansson et al., 1993). However, others found that both $G\alpha_i$ and $G\alpha_o$ were involved

(Ewald et al., 1989), and in several studies, G_s- or G_q-coupled receptors produced similar modulation (Shapiro and Hille, 1993; Golard et al., 1994; Zhu and Ikeda, 1994). This led to the hypothesis that the species involved was the moiety common to all these G proteins, $G\beta\gamma$ rather than any particular $G\alpha$, and this was subsequently found to be the case (Herlitze et al., 1996; Ikeda, 1996), although previously other groups had directly investigated the involvement of $G\beta\gamma$ in calcium channel modulation and not found any effect of its infusion (Hescheler et al., 1987). However, there was a clear precedent for an effector role for $G\beta\gamma$ in the G protein-activated potassium channels (GIRKs). Although for many years a controversy reigned concerning which G protein subunit was responsible for modulation of the native GIRKs (e.g., Yatani et al., 1987), they were eventually shown conclusively to be activated by $G\beta\gamma$s (Logothetis et al., 1987; Kurachi et al., 1989; Clapham and Neer, 1993). Furthermore, most $G\beta\gamma$ combinations tested except transducin ($G\beta_1\gamma_1$) are similarly effective (Wickman et al., 1994; Yamada et al., 1997). From the work of two groups (Herlitze et al., 1996; Ikeda, 1996), it became clear that transfection of either primary neurons or cell lines with $G\beta\gamma$ subunits mimicked agonist effects and led to tonic inhibition of the calcium current, which could be transiently reversed by a depolarizing prepulse, applied just before the test pulse, a hallmark of voltage-dependent inhibition of these channels.

Taking examples for illustration from our own work (Meir et al., 2000), the effect of coexpression of $G\beta\gamma$ with N-type calcium channels can be seen both at the whole-cell and at the single-channel level (Figs. 5A, and 6A). When $Ca_V2.2$ was coexpressed in COS-7 cells with β2a and $G\beta_1\gamma_2$, the whole-cell currents were very small and slowly activating but were markedly enhanced by a depolarizing prepulse (Fig. 5A, left panel). The time constant of activation of the peak current at 0 mV in the presence of $G\beta\gamma$ was about 27 ms, compared with less than 5 ms in the presence of the $G\beta\gamma$ binding domain of β-adrenergic receptor kinase 1 (β-ARK1) to bind any free endogenous $G\beta\gamma$. At the single-channel level, the slow activation of $Ca_V2.2/\beta$2a channels was seen as a marked prolongation of the latency to first opening (Fig. 6A, compare traces in right panel with $G\beta\gamma$ with those in the

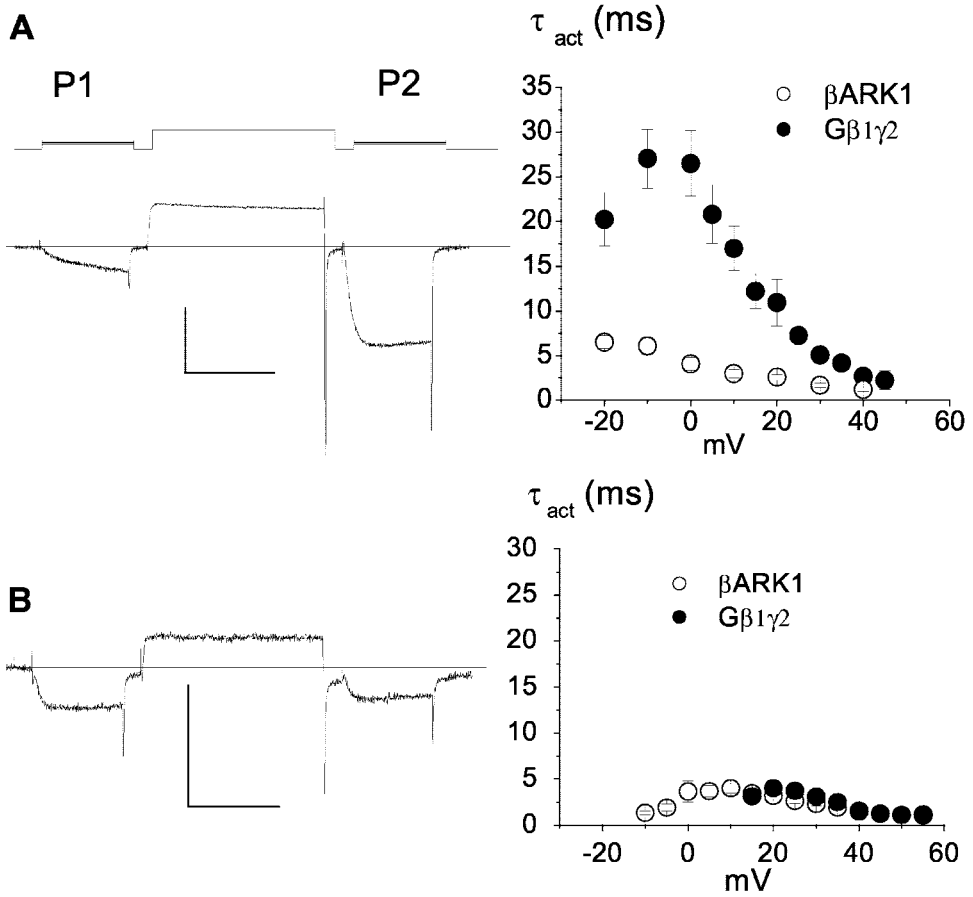

FIG. 5. Requirement for a β subunit for the modulation of whole-cell $Ca_V2.2$ currents by $G\beta\gamma$ in COS-7 cells. A, slowed activation of $Ca_V2.2/\beta$2a currents by $G\beta_1\gamma_2$ coexpression. Left top panel, voltage protocol, holding potential −80 mV, P1 and P2 test pulses to between −40 and +60 mV, with intervening prepulse to +100 mV; lower panel, example current traces for P1 and P2 to 0 mV for $Ca_V2.2/\beta$2a/$G\beta_1\gamma_2$. Right, plot of time constant for activation (τ_{act}) against voltage for P1 currents in the presence of $G\beta\gamma$ (●), compared with coexpression of β-ARK1 minigene (○). B, lack of slowed activation of $Ca_V2.2$ currents by $G\beta\gamma$ in the absence of $Ca_V\beta$ subunits. Left, example current traces for P1 and P2 to 0 mV for $Ca_V2.2/G\beta_1\gamma_2$. Right, plot and symbols as above. Note that traces in A are recorded in 1 mM Ba^{2+} and those in B are recorded in 10 mM Ba^{2+}. Scale bars represent 10 pA/pF and 50 ms for both. Data replotted from Meir et al. (2000).

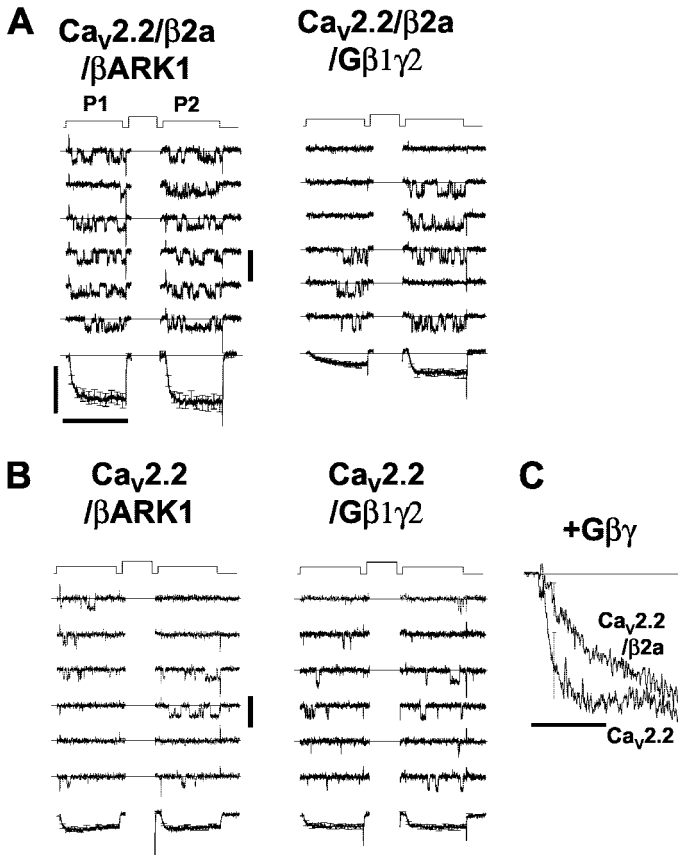

FIG. 6. Requirement for a $Ca_V\beta$ subunit for the modulation of $Ca_V2.2$ single-channel currents by $G\beta\gamma$ in COS-7 cells. A, effect of $G\beta_1\gamma_2$ (right) compared with β-ARK1 (left) coexpression on $Ca_V2.2$ single-channel currents in the presence of $Ca_V\beta2a$ subunits, recorded from cell-attached patches in COS-7 cells. Upper voltage trace, holding potential −100 mV, test potential both in P1 and P2, +30 mV, separated by a depolarizing prepulse to +120 mV. Top, representative single-channel records from a single channel-containing patch. Scale bar 1 pA for all single-channel records. Bottom panel, single-channel ensemble average currents, with error bars only every 5 ms for clarity (n = 13). The slowed activation of $Ca_V2.2/\beta2a$ currents by $G\beta_1\gamma_2$ and reduced amplitude compared with coexpression of β-ARK1 are evident, as well as its partial reversal by a prepulse. B, lack of effect of $G\beta_1\gamma_2$ (right) compared with β-ARK1 (left) on $Ca_V2.2$ single-channel currents in the absence of $Ca_V\beta$ subunits. Top, representative single-channel records, 100-ms long; bottom, ensemble average currents (n = 13), showing no effect of $G\beta\gamma$ on current activation. Scale bar represents 0.1 pA and 100 ms and refers to all ensemble currents. C, comparison of the scaled ensemble averages in the presence of $G\beta\gamma$ and in the presence and absence of $\beta2a$. Scale bar represents 20 ms. Data replotted from Meir et al. (2000).

left panel with β-ARK1). Indeed, there were many instances where no openings were observed to a test pulse. The latency to first opening was significantly reduced by a large depolarization (Fig. 6A, right panel). $G\beta\gamma$ overexpression also occluded modulation by agonist (Ikeda, 1996). The $G\beta\gamma$-mediated inhibition presents a picture that is very similar to that of agonist-mediated inhibition in terms of slowed activation and reversal by a large depolarizing prepulse. This is illustrated in an example from work by my own group, showing the effect of quinpirole-mediated activation of a coexpressed D2-dopamine receptor on the same channel combination (Fig. 7A) (Meir et al., 2000).

The involvement of $G\beta\gamma$ dimers as mediators of the G protein-signaling pathway does not call into question the finding by many groups that $G\alpha_o$ is involved in receptor-mediated inhibition in many native systems (Ewald et al., 1989; McFadzean et al., 1989; Campbell et al., 1993; Menon-Johansson et al., 1993; Degtiar et al., 1996), because G_o is present in very high concentrations, particularly in neurons. Thus, in the absence of the $G\alpha_o$ subunit, GPCR-mediated signaling will be markedly attenuated since it depends on the G protein heterotrimer.

Nevertheless, a number of studies have further concluded that there is a specific role for $G\alpha$ subunits. In

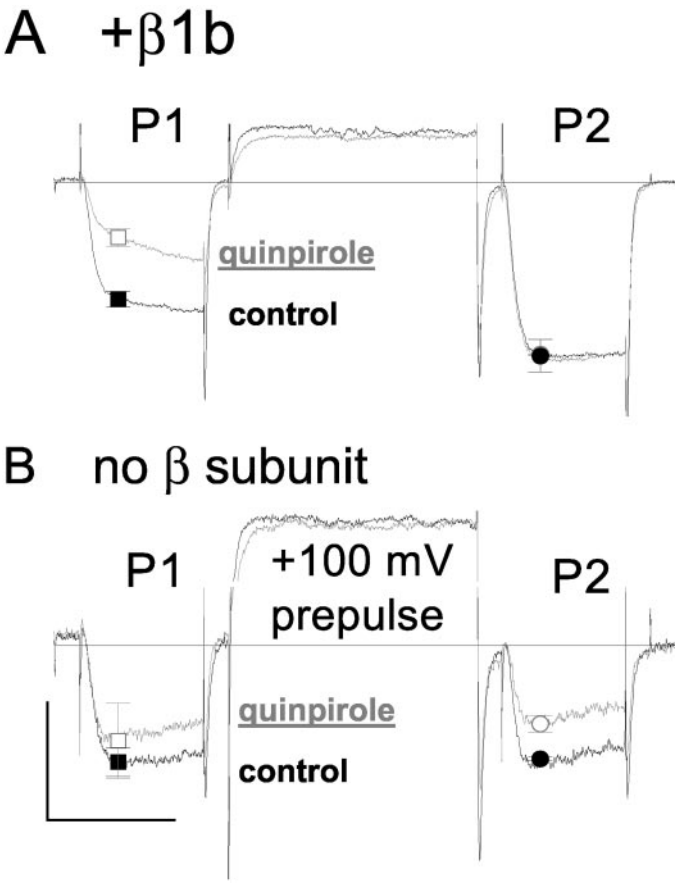

FIG. 7. Requirement for a β subunit for the modulation of whole-cell $Ca_V2.2$ currents by activation of the dopamine D2 receptor in COS-7 cells. Whole-cell $Ca_V2.2$ currents recorded from transfected COS-7 cells. The voltage protocol consists of two 50-ms test pulses (P1, P2) to +10 mV from a holding potential of −80 mV, with an intervening 100-ms prepulse to +120 mV. The D2 dopamine receptor was activated with 100 nM quinpirole (during control and wash, the bath was perfused with extracellular solution). The traces shown are mean normalized currents before [P1 (■) and P2 (●)] and during [P1 (□) and P2 (○)] application of quinpirole. Currents were normalized to the current at 20 ms after the onset of the test pulse in P1 before quinpirole application and then averaged. The scale bars apply to A and B and represent one normalized current unit and 50 ms. Mean ± S.E.M. are shown in the same symbols. A, effect of the dopamine D2 receptor agonist quinpirole (gray traces) on $Ca_V2.2$ currents in the presence of $Ca_V\beta1b$ subunits. The slowed activation of $Ca_V2.2/\beta2a$ currents by quinpirole and reversal by a depolarizing prepulse are evident. B, lack of voltage-dependent effect of the D2 receptor agonist quinpirole (gray traces) on $Ca_V2.2$ current activation kinetics in the absence of $Ca_V\beta$ subunits. A small degree of inhibition is observed, which is not reversed by a prepulse (compare P2 traces to those in P1). Data replotted from Meir et al. (2000).

some cell types, a marked specificity of different $G\alpha\beta\gamma$ combinations for signaling pathways between different receptors and calcium channels has been demonstrated (Kleuss et al., 1991; Degtiar et al., 1996). These findings might also be reconciled with the evidence that most $G\beta\gamma$ subunits are able to transduce the signal to calcium channels (Ikeda, 1996; Garcia et al., 1998; Ruiz-Velasco and Ikeda, 2000) by interpreting that a specific G protein heterotrimer combination may selectively couple to a particular receptor in intact cells, and the selectivity is therefore largely at the receptor-G protein interaction step or due to segregation into different compartments, rather than due to $G\beta\gamma$ specificity. In one study, however, it was concluded that there was an effector role for $G\alpha_{i3}$ subunits (Furukawa et al., 1998b). In this study, either $G\alpha$ or $G\beta\gamma$ moieties were coexpressed in *Xenopus* oocytes with $Ca_V2.2$ or $Ca_V2.1$ channels and the μ-opioid receptor. Receptor-mediated inhibition was found to be enhanced by coexpression of $G\alpha$, which was therefore said to mediate this inhibition; but a more likely explanation of this finding is that expression of exogenous $G\alpha$ increases the amount of $G\alpha\beta\gamma$ available for coupling to the receptor (Jeong and Ikeda, 1999; Canti and Dolphin, 2003) rather than that a specific $G\alpha$ mediates the response. In another study in chick sensory neurons, it was originally suggested that following activation of α2-adrenoceptors, $G\beta\gamma$ dimers were responsible for the voltage-independent inhibition via activation of PKC and activated $G\alpha$ for voltage-dependent inhibition via an unknown second messenger (Diversé-Pierluissi and Dunlap, 1993; Diversé-Pierluissi et al., 1995). It is unknown whether different pathways might be activated in avian neurons. Interpretation of the role of phosphorylation in mediating a pathway must always bear the proviso that phosphorylation might also occlude receptor-mediated effects by receptor down-regulation.

I have further examined whether $G\alpha$ subunits play any role in mediating calcium channel inhibition, by the use of receptor-$G\alpha$ fusion proteins. I found that there was no difference between α_2 adrenoreceptor-$G\alpha_o$ and -$G\alpha_i$ tandems and the wild-type α_2 adrenoreceptor in their ability to support G protein-mediated inhibition of N-type calcium channels in an expression system, and also no difference in the voltage dependence of the inhibition (Fig. 8), despite the fact that no $G\alpha$ amplification would occur in the case of the tandems (Bertaso et al., 2003). This is in contrast to the selective inhibition by G_o rather than G_i in sympathetic neurons (Delmas et al., 1999), which may depend on localization within discrete membrane compartments in native cells (Delmas et al., 2000). Thus, coexpression studies in heterologous systems provide information on what is possible, but studies in native cells, using different means of interfering with the signal transduction pathway or its intermediates, provide information on what actually happens in any given cell. Both types of study are essential to place the correct interpretation on data from native cells.

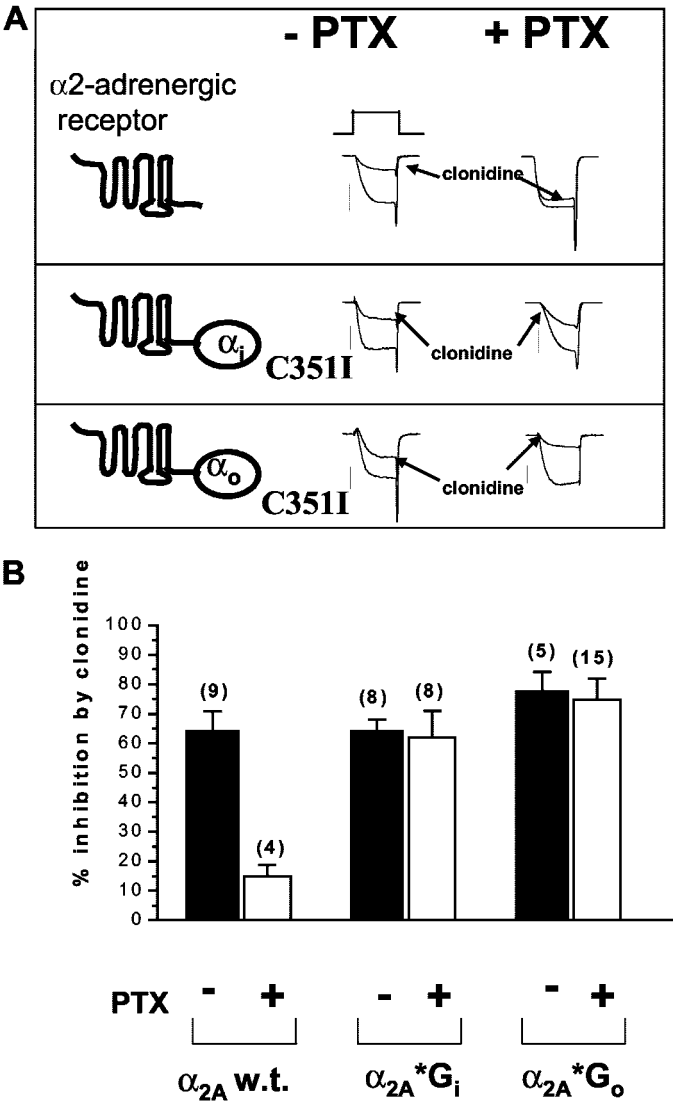

FIG. 8. G protein modulation via receptor-G protein tandems. A, inhibition of $Ca_V2.2/\beta1b$ currents by activation of heterologously expressed α2A-adrenoceptors, either alone (top) or as tandem constructs with $G\alpha_i$ (middle) or $G\alpha_o$ (bottom). The receptor-G protein tandems have a mutation at the C terminus of the $G\alpha$ to prevent ADP-ribosylation and inactivation by pertussis toxin (C351I) (left panel). The charge carrier was 10 mM Ba^{2+}. Clonidine (10 μM) was the agonist and the inhibition is shown either without (middle panel) or with (right panel) pertussis toxin pretreatment. The holding potential was −100 mV, and currents were evoked by 30-ms steps to 0 mV. B, mean data for inhibition by clonidine for the three conditions described above, given as a histogram. Solid bars, data obtained in the absence of pertussis toxin; open bars, data obtained in the presence of pertussis toxin. Data replotted from Bertaso et al. (2003).

One $G\beta$ subunit, $G\beta_5$, when overexpressed in sympathetic neurons was less effective than other $G\beta$ subunit combinations at producing G protein modulation of VGCCs (Ruiz-Velasco and Ikeda, 2000). This may be because $G\beta_5$ preferentially interacts with certain regulators of G protein signaling (RGS) proteins with G protein γ-like domains, rather than with $G\gamma$ subunits themselves (Snow et al., 1998), and also couples selectively to the G_q family of $G\alpha$ subunits (Fletcher et al., 1998).

B. Voltage Dependence of G Protein Modulation of Calcium Channels

It is commonly accepted that the relief of G protein-mediated inhibition by depolarization is a result of rapid dissociation of G$\beta\gamma$ dimers from the channel at depolarized potentials. This process is thought to be strongly voltage- and time-dependent (see Figs. 5A and 6A for examples) and, as suggested above, is also believed to cause the slow relaxation observed in response to a test pulse (Jones and Elmslie, 1997). Furthermore, re-establishment of inhibition after a prepulse, during a period at the holding potential, is likely to result from rebinding of G$\beta\gamma$ dimers. Whether these processes actually result in physical dissociation and reassociation between the G protein subunits and channel remains formally to be established. However, the finding that the rate of reblock is dependent on the concentration of activated G protein is consistent with this view (Elmslie and Jones, 1994; Stephens et al., 1998a; Zamponi and Snutch, 1998). The interpretation of these results is that the process involves binding from the pool of free G$\beta\gamma$ dimers.

C. The Role of the $Ca_V\alpha1$ I-II Linker in G Protein Modulation of Ca_V2 Calcium Channels

The combination of two findings, 1) that G$\beta\gamma$ dimers are the mediators of inhibitory modulation, and 2) that there is a functional interaction between $Ca_V\beta$ subunits and G$\beta\gamma$ (Campbell et al., 1995), led a number of groups to examine the intracellular I-II linker in detail. G$\beta\gamma$ dimers have been found previously to bind to sites on type 2 adenylyl cyclase and phospholipase C $\beta2$ (Chen et al., 1995), which have a characteristic central motif consisting of QXXER. Whereas this motif is not necessarily indicative of a functional G$\beta\gamma$ binding site, it is also found to occur in the I-II loop of $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$, intriguingly within the binding site described for the VGCC β subunit (**QQIER**ELNGY–WI-KAE) (Pragnell et al., 1994). Furthermore, it is modified in the cognate region in L-type channels (**QQLEE**DL-GY–WITQ-E).

It is clear that G$\beta\gamma$ binds to the I-II linker of G protein-modulated calcium channels. This has been shown by several groups using overlay assays (De Waard et al., 1997; Zamponi et al., 1997) and also by the use of a surface plasmon resonance-based system to measure reversible binding, where the on- and off-rates can be measured, showing an affinity of G$\beta\gamma$ for the I-II linker of $Ca_V2.2$ of 62 nM (Fig. 3B) (Bell et al., 2001). Furthermore, the I-II linker of the L-type channel $Ca_V1.3$ does not bind G$\beta\gamma$, although it will bind $Ca_V\beta$ subunits (Bell et al., 2001). The residues in the I-II linker critical for G$\beta\gamma$ binding have been mapped (De Waard et al., 1997). As predicted, the AID part of the linker is one important domain, and some residues of the QQIER sequence were shown to be essential for G$\beta\gamma$ binding (De Waard et al., 1997).

The role of the I-II linker G$\beta\gamma$ binding site in the process of G protein modulation remains controversial. Initial electrophysiological studies that supported a major role for the I-II linker used peptides derived from the I-II linker region in the patch pipette and found that they blocked G protein modulation (Herlitze et al., 1997; Zamponi et al., 1997). However, peptides alone do not prove that the $Ca_V\alpha1$ I-II loop is the site of modulation but rather indicate whether the peptides bind to G$\beta\gamma$ and can therefore effectively compete for this mediator. Chimeric and mutant channels have also been made between those channels that showed the greatest G protein modulation, such as $Ca_V2.2$, and those that exhibited no or less modulation, in an attempt to define the regions involved in this process. Three groups found that the I-II linker was key to G$\beta\gamma$ modulation (De Waard et al., 1997; Herlitze et al., 1997; Zamponi et al., 1997). Zamponi et al. (1997) made chimeras between $Ca_V2.1$ and $Ca_V2.2$ by putting the I-II linker of $Ca_V2.2$ into $Ca_V2.1$, which resulted in an increased modulation, with $Ca_V\beta1b$ as the coexpressed β subunit. However, both the channels used in this study were G protein modulatable. De Waard et al. (1997) found that a mutation that prevented G$\beta\gamma$ binding (R $\rightarrow$ E in QQIER) also prevented inhibitory modulation of $Ca_V2.1/\beta4$ by GTPγS injection into oocytes, although in this study only a small amount of inhibition was observed with GTPγS even in the wild-type $Ca_V2.1$. However, Herlitze et al. (1997) mutated the QQIER sequence in $Ca_V2.1$ to that in $Ca_V1.2$, which is QQLEE, and found a reduction of modulation of the channel coexpressed with $\beta1b$ by GTPγS, but not an abolition. Interestingly, in this study, they observed that $Ca_V2.1$ with the sequence QQIEE showed increased, rather than decreased, modulation by GTPγS, in contrast to the results of De Waard et al. (1997).

Two other groups found that the presence of an I-II linker from a G protein-modulatable channel was either not essential for G protein modulation or not the most critical region (Zhang et al., 1996; Page et al., 1997, 1998; Canti et al., 1999). The results of Zhang et al. (1996) showed that neither the I-II linker from $Ca_V2.1$ nor that from $Ca_V1.2$ reduced modulation in a $Ca_V2.2$ backbone when coexpressed with $\beta1$. In agreement, the results of Page et al. (1997) showed that insertion of the I-II linker of $Ca_V2.2$ into a $Ca_V2.3$ construct (RbEII) (Soong et al., 1993), which had a truncated N terminus, did not restore the G protein modulation shown by $Ca_V2.2/\beta1b$. Furthermore, Canti et al. (1999) showed that the I-II linker of $Ca_V2.2$ inserted into a $Ca_V1.2$ backbone and coexpressed with $\beta2a$ did not allow any G protein modulation (Fig. 9). Both groups found that domain I of G protein-modulated channels was a key region in the process of G protein modulation (Zhang et al., 1996; Stephens et al., 1998b). The discrepancies do not appear to be due to the different G$\beta\gamma$ dimers or $Ca_V\beta$

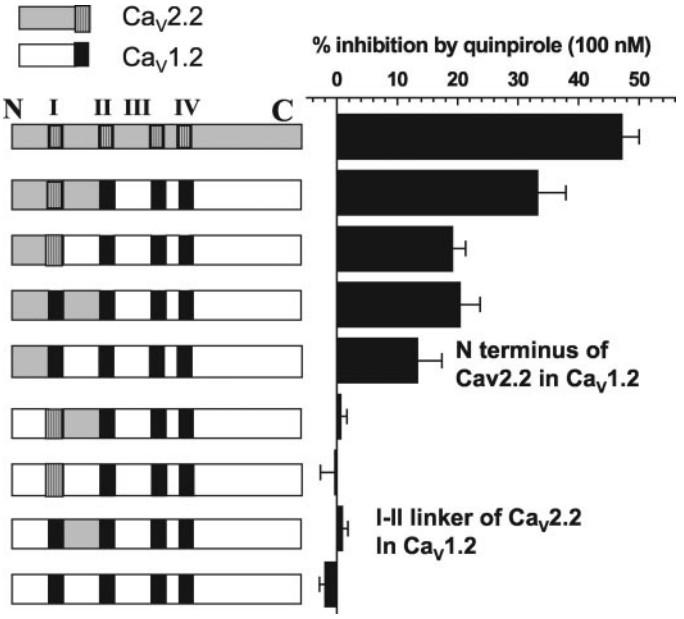

FIG. 9. Modulation of $Ca_V1.2$-$Ca_V2.2$ chimeras by activation of a dopamine D2 receptor. Left, diagrammatic representation of the chimeras made between $Ca_V2.2$ (rabbit BIII clone, represented by gray bars, top) and $Ca_V1.2$ (rat rbC-II clone, white bars, bottom). The four transmembrane domains are shown in black/gray stripes for $Ca_V2.2$ and solid black for $Ca_V1.2$. Right, the constructs were expressed in *Xenopus* oocytes together with auxiliary subunits and dopamine D2 receptors. Percentage of inhibition of peak calcium current at 0 mV by the agonist quinpirole (100 nM). Results taken from Canti et al. (1999).

subunits used, because the different groups have coexpressed channels with a variety of different $Ca_V\beta$ subunits, and the $G\beta\gamma$ combination, where used, was $G\beta_1\gamma_2$ or $G\beta_2\gamma_3$.

Thus, the I-II linker of G protein-modulated channels has a clear ability to bind $G\beta\gamma$ (Fig. 3B), although there appear to be no motifs in the I-II linker whose presence, despite being essential for high-affinity binding to the I-II linker, is absolutely essential for the process of modulation of $Ca_V2.x$ channels by these $G\beta\gamma$ dimers.

D. The Essential Role of the $Ca_V\alpha1$ N Terminus in G Protein Modulation

In reconstituted systems, consisting minimally of a VGCC $\alpha1/\beta$ combination, with or without an $\alpha2\delta$ and either an endogenous or an expressed G protein-coupled receptor, classical G protein modulation could be demonstrated for $Ca_V2.2$ and, to a lesser extent, for $Ca_V2.1$, but modulation of $Ca_V2.3$ was only observed by some groups (Yassin et al., 1996; Meza and Adams, 1998) but not by my group using a particular rat $Ca_V2.3$ clone (Page et al., 1997). My group subsequently identified the reason for this discrepancy; one of the initial clones from rat brain, rbEII (Soong et al., 1993), had a truncated 5′ coding region, commencing at the second methionine, and this showed no G protein modulation (Page et al., 1998). PCR extension of the N terminus formed a full-length rat $Ca_V2.3$ clone homologous to the rabbit and human clones, and this full-length $Ca_V2.3$ is strongly G protein-modulated (Page et al., 1998). To examine whether the requirement for an intact N terminus was a general conclusion, not limited to $Ca_V2.3$, my group further showed that partial truncation of the highly homologous N terminus of $Ca_V2.2$ abolished its ability to be G protein modulated (Page et al., 1998). Furthermore, a chimeric channel consisting of only the cytoplasmic N terminus of $Ca_V2.2$ in a rat $Ca_V1.2$ backbone showed all the elements of classical G protein modulation, whereas $Ca_V1.2$ did not (Canti et al., 1999) (Fig. 9). We then extended this study to show that an 11-amino acid motif YKQSIAQRART in $Ca_V2.2$ N terminus that is also highly conserved in $Ca_V2.1$ and $Ca_V2.3$ was essential for G protein modulation. Within this sequence, mutation of either YKQ or RAR to AAA abolished G protein modulation (Canti et al., 1999). Elements of this 11-amino acid motif were also involved in interaction with $Ca_V\beta$ subunits, because deletion of this motif in $Ca_V2.2$ or mutation of certain residues countered the $Ca_V\beta2a$-mediated retardation of inactivation (Stephens et al., 2000).

E. Basis for the Selectivity of Calcium Current Inhibition by Transmembrane G Protein-Coupled Receptors

Activation of the G_q family of G proteins does not produce typical voltage-dependent inhibition of N-type calcium channels despite the production of $G\beta\gamma$ dimers, but instead produces a non-voltage-dependent inhibition (Kammermeier et al., 2000; Bertaso et al., 2003). The reason for this is unclear, but a number of hypotheses have been put forward. One possibility is that, as stated above, $G\alpha_q$ frequently interacts with $G\beta_5$ in native tissues, and $G\beta_5$ is unique among the G proteins in that it does not interact with most $G\gamma$ subunits (Zhou et al., 2000). A second possibility is that the inhibition is via $G\alpha_q$ itself or a downstream effector. Indeed, the voltage-dependent inhibitory modulation of calcium currents resulting from activation of the pertussis toxin-sensitive G protein pathway or expression of $G\beta\gamma$ dimers can be reversed by coactivation of $G\alpha_q$ (Zamponi et al., 1997; Simen et al., 2001; Bertaso et al., 2003). $G\alpha_q$ activates phospholipase C and downstream signal transduction events including PKC. There is a threonine, Thr422, in a PKC consensus phosphorylation site just C-terminal to the AID motif in the sequence KRAA**T**KKSR within the I-II linker of rat $Ca_V2.2$. It has been proposed that phosphorylation by PKC of this threonine residue counteracts $G\beta\gamma$ binding to the I-II linker and thus counters inhibitory modulation (Zamponi et al., 1997; Hamid et al., 1999). However, this was subsequently found only to hold true for $G\beta_1$ and not other $G\beta$ subunits (Cooper et al., 2000). Furthermore, the sequence is not completely conserved in rabbit $Ca_V2.2$, which has alanine in place of threonine at the equivalent residue 422 (KRAA**A**KKSR in rabbit $Ca_V2.2$), although the same phenomenon of cross talk between G protein modulation and PKC activation occurs (Bertaso et al., 2003). Thus, the site(s) of

phosphorylation by PKC responsible for the reversal of G protein modulation is not yet certain (Bertaso et al., 2003). It has been shown recently that $G\alpha_q$ binds to the C terminus of N-type calcium channels, to which PKC was also found to bind, and this colocalization may facilitate the phosphorylation of the N-type calcium channel (Simen et al., 2001). It is also possible that, if there is a reduction in membrane levels of phosphatidylinositol (4,5)-bisphosphate (PIP_2) resulting from phospholipase C activation, this may play a role in the reversal or attenuation of G protein modulation, since PIP_2 has been shown to regulate calcium channels (Wu et al., 2002). However, it remains controversial whether PIP_2 levels are reduced substantially after activation of G protein-coupled receptors linked to phospholipase C, because no global change was observed in the heart (Nasuhoglu et al., 2002), but the reduction may be localized and also linked to increased synthesis of PIP_2 (Zhao et al., 2001).

F. Is There a Role for the C Terminus in Calcium Current Inhibition by G Protein-Coupled Receptors?

A number of studies have suggested that the C terminus of certain Ca_V2 calcium channels is essential for their G protein modulation and indeed binds either $G\beta\gamma$ or $G\alpha$ subunits (Qin et al., 1997; Furukawa et al., 1998a,b; Kinoshita et al., 2001). However, my group has found that the chimeric constructs containing domain I of $Ca_V2.2$ and the entire final three domains and C terminus of $Ca_V1.2$ is strongly G protein-modulated (Stephens et al., 1998b). Furthermore, truncation of the C terminus of $Ca_V2.2$ including the region homologous with that found to bind $G\beta\gamma$ in $Ca_V2.3$ (Qin et al., 1997) did not affect G protein modulation by GTPγS (Meza and Adams, 1998), and a similar truncation of $Ca_V2.2$ was found by another group to reduce, but not to abolish modulation by somatostatin receptor activation (Hamid et al., 1999). Thus, any direct role for the C terminus is probably a minor one.

V. Essential Role of $Ca_V\beta$ Subunits in G Protein Modulation of Calcium Channels

The identification of a QQIER motif, known to bind $G\beta\gamma$ dimers in other proteins, in the region of the $\alpha1$ subunit I-II linker where the $Ca_V\beta$ subunit binds, suggested that the $Ca_V\beta$ subunit might be involved in G protein modulation.

A. Initial Evidence for the Role of $Ca_V\beta$ Subunits in G Protein Modulation in Native Neurons

To investigate the involvement of $Ca_V\beta$ subunits in G protein modulation, I first developed an antisense strategy to deplete dorsal root ganglion neurons of their $Ca_V\beta$ subunits by microinjection of an antisense oligonucleotide complementary to a region common to all β subunits (Berrow et al., 1995). Antisense knockdown of the $Ca_V\beta$ subunit by about 90% resulted in an enhancement of the ability of the GABA-B receptor agonist (−)-baclofen to inhibit the residual currents (Campbell et al., 1995b). We hypothesized from these results that there might be a functional interaction between activated G protein and VGCC β subunit for interaction with the relevant channels (Campbell et al., 1995b).

B. The Involvement of $Ca_V\beta$ Subunits in G Protein Inhibition of Heterologously Expressed Calcium Channels

The role of $Ca_V\beta$ subunits in G protein inhibition of expressed calcium channels has now been extensively examined (Bourinet et al., 1996; Qin et al., 1997; Roche and Treistman, 1998; Canti et al., 2000, 2001; Meir et al., 2000). In initial studies in *Xenopus* oocytes, there was reported to be less or even a complete loss of G protein inhibition following coexpression of a β subunit (Bourinet et al., 1996; Qin et al., 1997), although these studies only examined inhibition at a single potential and need to be interpreted with caution because of the presence of an endogenous oocyte $Ca_V\beta$ subunit. The result was interpreted in terms of a competition or displacement of $Ca_V\beta$ by $G\beta\gamma$ at an overlapping binding site (Bourinet et al., 1996). However, since β subunits shift the calcium current activation to more hyperpolarized potentials, it is inappropriate to measure G protein-mediated inhibition at a single potential. By studying the voltage dependence of receptor-mediated inhibition in *Xenopus* oocytes, my group has shown that this is a bell-shaped curve, peaking at about the voltage for 50% current activation (Canti et al., 2000). In the absence of coexpressed β subunits, the maximum amount of inhibition induced by activation of the coexpressed dopamine D2 receptor was about 70% at −10 mV. This curve is hyperpolarized in the presence of coexpressed β subunits, and the peak inhibition observed with $\beta1$b, $\beta3$, and $\beta4$ was little changed at 70, 62, and 59%, although it occurred at −20 mV, whereas with $\beta2$a coexpression, maximal inhibition was modestly reduced, being 51% at −10 mV (Canti et al., 2000). Thus, it is likely that this cannot represent a simple competition between $Ca_V\beta$ subunits and $G\beta\gamma$ dimers, but the interaction is dynamic and depends on the membrane voltage.

C. Does $G\beta\gamma$ Displace $Ca_V\beta$ Subunits?

In this section, the evidence will be assessed for two different views concerning the mechanism of inhibition of calcium channels by G proteins: 1) that $Ca_V\beta$ subunits do not dissociate during this process, which involves an allosteric rearrangement of the $\alpha1$-β interaction associated with the voltage-dependent binding and unbinding of $G\beta\gamma$: $Ca_V\alpha1$-$Ca_V\beta + G\beta\gamma \rightleftharpoons Ca_V\alpha1$-$\beta$-$G\beta\gamma$ as proposed in Meir et al. (2000); or 2) that G protein modulation is favored in the absence of $Ca\beta$ subunits and opposed by the presence of β subunits, indicating that $Ca_V\beta$ and $G\beta\gamma$ compete for a single binding site on the $\alpha1$ subunit,

as in the reaction: $Ca_V\alpha1$-$Ca_V\beta$ + $G\beta\gamma \rightleftharpoons Ca_V\alpha1$-$G\beta\gamma$ + $Ca_V\beta$ as proposed by Bourinet et al. (1996) and Qin et al. (1997). Such a reaction would either require transient formation of an intermediate ternary $Ca_V\alpha1$-β-$G\beta\gamma$ complex or require that $Ca_V\beta$ dissociates before $G\beta\gamma$ binds, if they bind to the same site.

If $G\beta\gamma$ binding either displaced $Ca_V\beta$ or allosterically resulted in the physical dissociation of $Ca_V\beta$, then the effects of the two species should oppose one another. Indeed, in many respects $G\beta\gamma$ dimers do appear to have the opposite effect from $Ca_V\beta$ subunits on calcium channel properties. All $Ca_V\beta$ subunits shift calcium channel activation to more hyperpolarized potentials (for review, see Birnbaumer et al., 1998) and $G\beta\gamma$ has the opposite effect (Bean, 1989). However, all $Ca_V\beta$ subunits except palmitoylated β2a hyperpolarize the steady-state inactivation by about 30 mV (Canti et al., 2000). In contrast, where it has been studied, little or no effect of G protein activation or $G\beta\gamma$ dimers has been observed on steady-state inactivation (Bean, 1989; Meir and Dolphin, 2002). This suggests that $G\beta\gamma$ does not simply displace $Ca_V\beta$ subunits and prevent their interaction with the channel.

For the Ca_V2 subfamily of channels, prepulse facilitation of G protein-modulated channels is thought to involve $G\beta\gamma$ unbinding from the channel, induced by depolarization, as described above. This finding can be used to test the hypothesis that there is a competition between $G\beta\gamma$ and $Ca_V\beta$ subunits, since if $G\beta\gamma$ unbinds during a prepulse, then $Ca_V\beta$ might be expected to bind in its place. If so, the rate of facilitation would be directly dependent on $Ca_V\beta$ concentration in the cytosol. Indeed, the rate of facilitation during a prepulse was markedly increased by the heterologous expression of all $Ca_V\beta$ subunits (Roche and Treistman, 1998; Canti et al., 2000), which might be construed as supporting this view.

My group therefore developed a means of testing this hypothesis by expressing increasing amounts of $Ca_V\beta3$ cDNA with a constant amount of $Ca_V2.2$ cDNA in *Xenopus* oocytes. We first showed that there was a linear relationship between $\beta3$ cDNA injected and $\beta3$ protein expressed (Canti et al., 2001). The $\beta3$ subunit was used in these experiments because it is almost identical to the endogenous *Xenopus* $\beta3$ present in oocytes (Tareilus et al., 1997). We then performed an intracellular dose-response curve for $Ca_V\beta$ subunits to examine the concentration dependence of the effect of β subunits to increase the facilitation rate (Canti et al., 2001). This experiment is illustrated in Fig. 10. At high $Ca_V\beta$ concentrations in oocytes (between about 20 and 100 ng of $\beta3$/oocyte), the facilitation rate during the depolarizing prepulse can be fit by a single fast exponential (Fig. 10, A and B), which we have interpreted as corresponding to the $G\beta\gamma$ off-rate from the channel that has $Ca_V\beta$ bound. The reason for this interpretation is that with these concentrations of $\beta3$ coexpressed, the steady-state inactivation of the $Ca_V2.2$ channels is fit by a single Boltzmann function, which is hyperpolarized compared with that for $Ca_V2.2$ expressed without a β subunit (Fig. 10C).

At intermediate $Ca_V\beta$ concentrations, the facilitation rate is not well fit by a single exponential (Canti et al., 2001), but can be fit by the sum of the same invariant fast exponential plus a slow exponential (Fig. 10, A and B). The value of the fast time constant, interpreted above as the $G\beta\gamma$ off-rate, is invariant over 100-fold change of $\beta3$ concentration and is therefore highly unlikely to involve any process requiring actual binding of $\beta3$ from the bulk solution. However, one aspect of the process does show a dependence on $Ca_V\beta$ concentration. The proportion of current showing the fast facilitation rate (Fig. 10D) shows exactly the same dependence on $Ca_V\beta$ protein concentration as the proportion of current with a hyperpolarized steady-state inactivation (see the biphasic steady-state inactivation curves at intermediate $Ca_V\beta$ concentrations in Fig. 10C). This agrees with our interpretation that the fast time constant of facilitation represents the behavior of a population of channels that has $Ca_V\beta$ bound. Reciprocally, the proportion of current showing a slow time constant of facilitation decreases as $Ca_V\beta$ concentration is increased, as does the proportion of current with a depolarized steady-state inactivation (Fig. 10C). We interpret this component as representing Ca_V channels without a bound $Ca_V\beta$. Unlike the component with the fast time constant of facilitation, this slow component of facilitation has a time constant that does vary with $Ca_V\beta$ concentration (Fig. 10D). We have interpreted this finding as representing $Ca_V\beta$ subunit binding to the population of free channels during the depolarizing prepulse, after which $G\beta\gamma$ then unbinds rapidly with the invariant fast time constant (Canti et al., 2001). From this study we conclude that there is not a simple competition between $G\beta\gamma$ and $Ca_V\beta$ subunits, but rather that under normal circumstances, $G\beta\gamma$ dissociates at depolarized potentials from and rebinds at hyperpolarized potentials to channels that have $Ca_V\beta$ bound. Only under circumstances when $Ca_V\beta$ is limiting, which might rarely occur in native tissues, does $Ca_V\beta$ bind with higher affinity during depolarization and thence induces $G\beta\gamma$ unbinding.

Thus, $Ca_V\beta$ must have a higher affinity for the channels in their depolarized state. This phenomenon of depolarization-dependent displacement of the $\alpha1$-β equilibrium toward increased $Ca_V\beta$ binding would only be observed under conditions where $Ca_V\beta$ is limiting, and it remains unknown whether this is ever the case in native tissues. In agreement with this interpretation, we could still observe significant tonic facilitation under conditions where $G\beta\gamma$ was minimized for $Ca_V2.2$ channels expressed in oocytes without a $Ca_V\beta$ subunit, in sharp contrast to the lack of tonic facilitation in the additional presence of exogenous $\beta1$b (Fig. 11) (Canti et al., 2000).

It was found that the reinhibition rate following a prepulse (for the $Ca_V2.2$/$\beta1$b/$\alpha2\delta$-1 combination) was in-

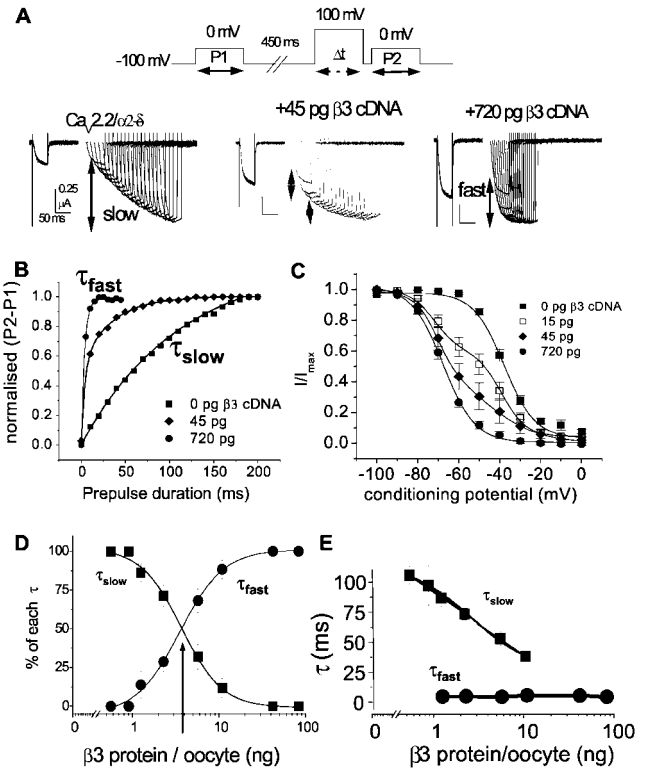

FIG. 10. Concentration dependence of the facilitation rate of $Ca_V2.2$ currents in *Xenopus* oocytes. $Ca_V2.2$ was expressed either in the absence of exogenous β subunit or in the presence of increasing concentrations of coinjected $\beta 3$ cDNA. The amount of $\beta 3$ cDNA injected is linearly related to the amount of $\beta 3$ protein expressed (Canti et al., 2001). A, top panel, voltage protocol used: P1 and P2 are identical test pulses to 0 mV, with P2 being preceded by a depolarizing prepulse to +100 mV, of varying duration. Lower panel, examples of three families of currents generated by increasing the duration of the depolarizing prepulse. Left, $Ca_V2.2$ in the absence of exogenous $Ca_V\beta$; middle, $Ca_V2.2$ in the presence of an intermediate concentration of exogenously expressed $Ca_V\beta 3$ (45 pg of $\beta 3$ cDNA); right, $Ca_V2.2$ in the presence of a maximal concentration of $Ca_V\beta 3$ (720 pg of $\beta 3$ cDNA). Five millimolars Ba^{2+} is the charge carrier. B, prepulse potentiation was measured by subtracting the current amplitude in P2 from that in P1 and normalizing this to the asymptotic value. The graph shows the slow exponential time course of facilitation for $Ca_V2.2$ in the absence of exogenous $Ca_V\beta 3$ (closed squares), the time course of facilitation for $Ca_V2.2$ in the presence of a representative intermediate concentration of exogenous $Ca_V\beta 3$ (45 pg of $\beta 3$ cDNA, closed diamonds), which can only be fit by two exponentials to generate a slow and fast time constant of facilitation (see Canti et al., 2001, for details), and the fast exponential time course for $Ca_V2.2$ in the presence of a maximal concentration of $Ca_V\beta 3$ (720 pg of $\beta 3$ cDNA, closed circles). C, steady-state inactivation was measured by delivering a 15-s conditioning pulse to the potential shown, followed by a test pulse to 0 mV. The proportion of current available is given by I/I_{max}, where I_{max} is the current in the absence of a conditioning step, and the curves are fit by either a single or a double Boltzmann function. The graph shows the steady-state inactivation for $Ca_V2.2$ in the absence of exogenous $Ca_V\beta 3$, which is fit by a single Boltzmann function with a depolarized midpoint of about −40 mV (closed squares). It also shows the steady-state inactivation for $Ca_V2.2$ in the presence of a maximal concentration of exogenous $Ca_V\beta 3$ which is fit by a single Boltzmann function with a hyperpolarized midpoint of about −70 mV (720 pg of $\beta 3$ cDNA, closed circles). The steady-state inactivation curves time for $Ca_V2.2$ in the presence of two representative intermediate concentration of exogenous $Ca_V\beta 3$ (45 pg of $\beta 3$ cDNA, closed diamonds, and 15 pg of $\beta 3$ cDNA, open squares) can only be fit by varying proportions of a double Boltzmann function with midpoints at −40 and −70 mV (see Canti et al., 2001, for details). D, variation of the proportion of the slow and fast time constants, determined as in B, τ_{slow} (closed squares) and τ_{fast} (closed circles) with the amount of $Ca_V\beta 3$ subunit protein expressed per oocyte. The $\beta 3$ protein expression level was determined by Western blotting (see Canti et al., 2001, for details). The plots are fit by sigmoid curves, the midpoint of which (arrow) is calculated to represent a concentration of 120 nM $\beta 3$ (see Canti et al., 2001, for details). E, variation of the values of τ_{slow} (closed squares) and τ_{fast} (closed circles) with $Ca_V\beta$ subunit concentration. Results taken from experiments described in detail in Canti et al. (2001).

creased as the concentration of $G\beta\gamma$ protein in the patch pipette was increased (Zamponi and Snutch, 1998), but in my group, we have observed that this rate is also increased slightly by overexpression of any $Ca_V\beta$ subunit together with $Ca_V2.2$, compared with the rate in the presence only of endogenous oocyte $Ca_V\beta$ (Canti et al., 2000). If there were direct competition between $G\beta\gamma$ and $Ca_V\beta$ for an overlapping binding site, then $Ca_V\beta$ should unbind during this process, before $G\beta\gamma$ rebinds. During the process of reinhibition following a prepulse, the depolarizing shift in activation and consequently the inhibition observed would result from $Ca_V\beta$ unbinding, rather than $G\beta\gamma$ binding, and therefore should not be dependent on $G\beta\gamma$ concentration. Elevation of $Ca_V\beta$ would be expected to slow the overall reinhibition rate, which is the opposite of what is observed. The conclusion

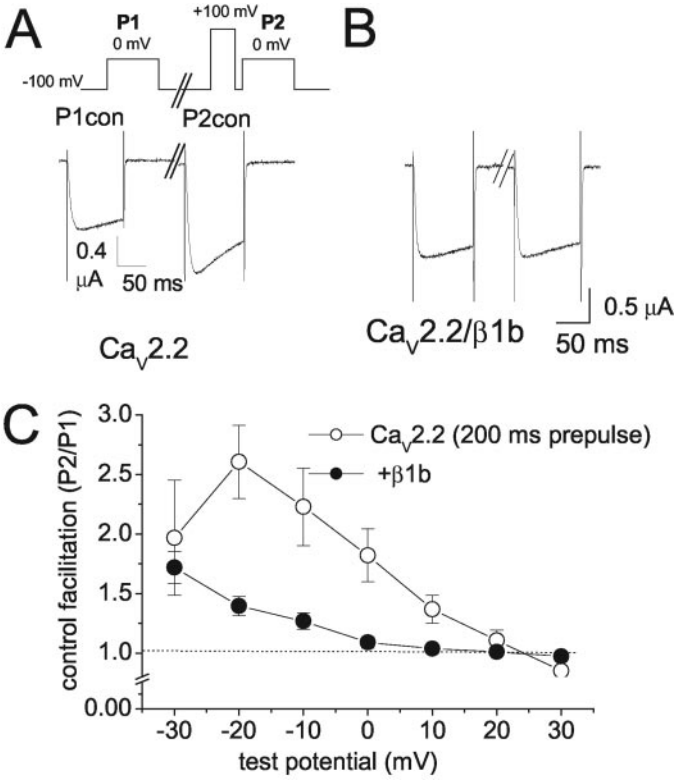

FIG. 11. Facilitation of $Ca_V2.2$ currents in the absence and presence of $Ca_V\beta$ subunits expressed in *Xenopus* oocytes. The protocol shown at the top was used to generate control P1 and P2 currents. Tonic G protein modulation has been removed by a previous application of quinpirole followed by recovery, as described previously (Canti et al., 2000). Constructs are expressed in the absence (A) or presence (B) of coexpressed $\beta 1b$ subunit. Ba^{2+} (5 mM) is the charge carrier. The prepulse was 200 ms in A and 50 ms in B. Lengthening the prepulse to 200 ms had no additional effect on facilitation in B. Expression was in *Xenopus* oocytes, which contain an endogenous low level of β subunit. Note the presence of control facilitation (P2 con/P1 con) when β subunits are not coexpressed, interpreted as resulting from enhanced binding of endogenous β subunits during the depolarizing prepulse. In B, where $\beta 1b$ is coexpressed, the lack of control facilitation can be interpreted as suggesting that most channels are already associated with coexpressed $\beta 1b$ subunits at the holding potential of −100 mV. C, control facilitation is quantified, for the two conditions, no coexpressed β (n = 4, open circles) where facilitation reaches 2.6, and with coexpressed $\beta 3$ (n = 5, closed circles) where facilitation is 1.4 at the same potential. Results taken from experiments described in Canti et al. (2000).

must be that Gβγ preferentially rebinds to the $Ca_V\alpha1$-β combination, and this species predominates when $Ca_V\beta$ is overexpressed.

Our model for the functional interplay between Gβγ dimers and $Ca_V\beta$ subunits does not support the idea that there is a simple competition between $Ca_V\beta$ and Gβγ for binding to the channel, or that $Ca_V\beta$ dissociates from the channel during G protein modulation, but rather that under normal conditions where the channels all have a $Ca_V\beta$ bound, Gβγ allosterically disrupts the effect of $Ca_V\beta$ on $Ca_V\alpha1$ channels. Conversely, depolarization, such as occurs during a prepulse, results in a state-dependent conformational change between $Ca_V\alpha1$ and $Ca_V\beta$, which decreases the stability of Gβγ binding.

The observation that at some potentials G protein modulation is enhanced in oocytes in the absence of overexpressed $Ca_V\beta$ in *Xenopus* oocytes (Bourinet et al., 1996), or following antisense depletion of $Ca_V\beta$ subunits in sensory neurons (Campbell et al., 1995b), may be explained as follows. The slowed current activation in the presence of Gβγ is one of the main components of G protein-mediated inhibition, and is a reflection of the fact that Gβγ-bound channels either do not open upon depolarization until Gβγ dissociates, or show a very brief reluctant opening (Patil et al., 1996; Lee and Elmslie, 2000). Since a reduction of $Ca_V\beta$ produces slowing of overall facilitation rate of G protein-modulated channels during the prepulse, then the same will be true for the smaller degree of Gβγ unbinding that occurs during the test pulse. This is likely to be the reason that a reduction in $Ca_V\beta$ levels results in the observation of enhanced inhibition during a test pulse, or at least a shift in the voltage dependence of inhibition to more depolarized potentials (Canti et al., 2000). Conversely, an elevation of $Ca_V\beta$ reduces the inhibition observed. It should be noted that the direct effects of $Ca_V\beta$ subunits on inactivation during the test pulse, while not directly influencing the process of G protein modulation, will also influence the net amount of modulation exhibited (Meir et al., 2000; Meir and Dolphin, 2002).

In an expression system (COS-7 cells) in which (unlike *Xenopus* oocytes) no endogenous $Ca_V\beta$ subunit protein was detected by immunocytochemistry (Meir et al., 2000), coexpression of Gβγ with $Ca_V2.2$ in the absence of $Ca_V\beta$ resulted in calcium channel currents that were rapidly activating and not facilitated by a prepulse. This was observed both at the whole-cell level (Fig. 5B), and at the single-channel level (Fig. 6B). However, Gβγ did produce a small reduction in the current amplitude, compared with currents recorded in the absence of Gβγ. For all these sets of currents, their depolarized activation clearly showed that no $Ca_V\beta$ was associated. The additional presence of heterologously expressed $Ca_V\beta$ subunits was required for the relief of Gβγ-mediated inhibition by a depolarizing prepulse (Figs. 5A, 6A, and 7A) (Meir et al., 2000).

At the single-channel level, in the cell-attached patch mode, when only one channel is present, the effect of Gβγ and $Ca_V\beta$ can be compared in the absence of the confounding effect of $Ca_V\beta$ on the number of channels expressed. The main effect of coexpression $Ca_V\beta2a$ on $Ca_V2.2$ channel properties is an increase in the mean open time and a hyperpolarizing shift in the latency to first opening (Meir et al., 2000). For the model in which $Ca_V\beta$ is displaced by Gβγ to be correct, the currents in the presence of Gβγ should display the same properties as those in the absence of $Ca_V\beta$, that is, the combination $Ca_V2.2\alpha1$/Gβγ. This is not the case as the main effect of Gβγ in the presence of $Ca_V\beta$ is an increased latency to first opening. For the competition hypothesis to be true, if it is assumed that the N-type channels do not open before Gβγ unbinds, then before the first opening when the $Ca_V2.2\alpha1/\beta$/Gβγ combination is coexpressed, only Gβγ should be bound; however, the channels do not show the same properties as the $Ca_V2.2\alpha1$/Gβγ combination (Meir et al., 2000).

We concluded from that study that $Ca_V\beta$ subunits were essential for the process of facilitation or Gβγ dissociation. In the same system, receptor-mediated inhibition via activation of the D2 dopamine receptor was also examined. It was much reduced in the absence of coexpressed $Ca_V\beta$ subunits (Fig. 7B), and reversal of this inhibition by a 100-ms prepulse was lost, implying that in the absence of $Ca_V\beta$ subunits, Gβγ dimers are able to bind and produce a small non-voltage-dependent inhibition of the $Ca_V2.2$ current, but their unbinding is not influenced by voltage (Meir et al., 2000).

D. Potential Overlap of Determinants for $Ca_V\beta$ Subunit and Gβγ Subunit Function

There is overlap in the determinants for G protein modulation and $Ca_V\beta$ binding or function for all three of the sites discussed above: the I-II linker, the C terminus, and the N terminus of Ca_V2 calcium channels. The Gβγ binding site on the $Ca_V\alpha1$ subunit intracellular I-II loop (De Waard et al., 1997; Zamponi et al., 1997) partially coincides with binding sites for auxiliary $Ca_V\beta$ subunits (Pragnell et al., 1994). However, the main amino acids that are critical for $Ca_V\beta$ subunit interaction are not within but adjacent to the QxxER consensus sequence implicated in Gβγ binding (Herlitze et al., 1996; De Waard et al., 1997).

Within the N terminus of $Ca_V2.2$ between amino acids 45 and 55, four individual point mutations (S48A, I49A, R52A, and R54A) were isolated, which significantly compromised modulation of $Ca_V2.2$ by G proteins (Canti et al., 1999). My group has subsequently shown that both the $Ca_V2.2$-R52,54A and $Ca_V2.2$-R52A constructs also exhibited compromised β2a-mediated retardation of inactivation as did $Ca_V2.2$-Q47A, which was shown previously to undergo normal Gβγ modulation (Stephens et al., 2000). Taken together with our initial study that identified this site (Canti et al., 1999), the results indi-

cate that the $Ca_V2.2$ amino terminus contributes determinants for both $Ca_V\beta$ subunit and $G\beta\gamma$ dimer function. However, the differentiating effect of $Ca_V2.2$-Q47A suggests that although the overall region involved may partially coincide, the determinants are not identical.

A partial overlap in $Ca_V\beta$ subunit and $G\beta\gamma$ binding sites has also been proposed for the $Ca_V2.3$ carboxyl-terminal site (Qin et al., 1997). However, whereas deletion of the majority of this $Ca_V2.3$ site affected $G\beta\gamma$ modulation, it allowed retention of full sensitivity to β2a (Qin et al., 1997), suggesting that this is not the prime mediator of the β subunit response (see also Jones et al., 1998).

VI. Molecular Mechanism of G Protein-Mediated Inhibition

It is still not understood how $G\beta\gamma$ binding to the $Ca_V\alpha1$ subunit results in inhibition of the calcium current. It is first necessary to establish the nature of the inhibitory effect at the level of a single calcium channel. It was initially suggested either that the G protein-bound channels opened with different gating properties (Kasai and Aosaki, 1989) or that there was a modal shift in gating (Delcour et al., 1993; Delcour and Tsien, 1993). However, the view that now prevails is that G protein-bound channels are reluctant to open, and that dissociation of bound G protein from the channel is required to convert them into willing channels (Bean, 1989; Elmslie et al., 1990). The simplest model would involve opening only the free and not the G protein-bound channel (for review, see Dolphin, 1991). At the single-channel level there is a prolonged latency to first opening in the presence of an agonist (Patil et al., 1996). However, once a channel had opened, no difference was observed on subsequent open probability or gating pattern compared with nonmodulated channels (Patil et al., 1996). This result is in agreement with the hypothesis that the delay to first opening is due to dissociation of the $G\beta\gamma$ dimers from the channel, allowing it to open, and that the G protein-bound channel does not open even with large depolarizations. This indicates either that the $G\beta\gamma$ binding is itself strongly voltage- or state-dependent or that $G\beta\gamma$ binds to a site on the channel that produces a voltage-dependent inhibition. More recently, it has been suggested that reluctant or $G\beta\gamma$-bound channels can open, albeit with a low probability (Lee and Elmslie, 2000).

It is still unclear how many $G\beta\gamma$ dimers are required to bind to each Ca_V2 channel to produce inhibition. Models have suggested that more than one activated G protein may be bound per channel in a cooperative manner (Boland and Bean, 1993), but more recently it was suggested that a single $G\beta\gamma$ was bound per channel (Zamponi and Snutch, 1998).

The mechanism by which bound $G\beta\gamma$ prevents the channel from opening is also unknown. In one study, the effect of G protein activation on voltage sensor movement in $Ca_V2.2$ was examined. This revealed that GTPγS produced a depolarizing shift in the voltage dependence of charge movement that could be reversed by a large depolarizing prepulse and also induced the appearance of a slow component of "on" gating charge (Jones et al., 1997). The greatest effect was the large separation on the voltage axis between gating charge movement and channel opening. Thus, $G\beta\gamma$ is acting both to slow voltage sensor movement and to inhibit the subsequent transduction of this movement into channel opening (Jones et al., 1997). In partial agreement with this, from a study exploiting a spontaneous mutation in $Ca_V2.2$ channels, G177E in domain IS3, which converts channels into a form that behaves as if it is tonically G protein-modulated in the absence of $G\beta\gamma$ dimers, it was suggested that the normal role of $G\beta\gamma$ dimers is voltage sensor trapping (Zhong et al., 2001).

There are a number of potential mechanisms whereby the N terminus might exert its essential role in G protein modulation. Three possibilities will be considered here. The first possibility is that, bearing in mind the effect of the N terminus of $Ca_V2.x$ channels on the actions of $Ca_V\beta$ subunits, it might form part of a complex $Ca_V\beta$ subunit binding pocket, into which $G\beta\gamma$ dimers could intercalate. However, the interaction of the N terminus with β subunits is unlikely to be of high affinity; as in a yeast two-hybrid assay, the N terminus did not interact with $Ca_V\beta$ subunits or with the I-II linker of $Ca_V2.2$ (Canti et al., 2001), although as described above (*Section II.B.*), the N terminus of $Ca_V2.1$ has been shown to bind to β4 subunits in overlay assays (Walker et al., 1999).

The second possible mechanism is that the N-terminal motif identified in $Ca_V2.x$ channels might also form the effector of G protein modulation, as suggested in Canti et al. (1999). It might, for example, create a blocking particle, in a manner somewhat analogous to the ball and chain model of potassium channel block by the N terminus (MacKinnon et al., 1993). However, the N-terminal motif is not at the extreme N terminus, because it represents amino acids 44 to 55 in $Ca_V2.2$, and amino acids N-terminal to this motif (1–44 in $Ca_V2.2$) are not required for G protein modulation (Canti et al., 1999). The sequence of the N-terminal ball peptide in $K_V1.1$ (Shaker B) is MAAVAGLYGLGEDRQHRKKQ, and there are no similarities with the N-terminal motif of $Ca_V2.2$ (YKQSIAQRART), apart from the presence of a number of essential positively charged residues and a KQ motif. In the case of K_V channels, the ball peptide inserts into the inner vestibule of the pore of the open channel and produces inactivation by open-channel block. If this type of action is involved in G protein modulation of Ca_V2 channels, the inhibition is not an open-channel block mechanism; rather it is both retarding voltage sensor movement and preventing channel opening in response to the voltage sensor movement,

and the effect is relieved by a large depolarization. One might envisage that the N-terminal motif is held in place, for example, to anchor the voltage sensor(s) by the binding of a G$\beta\gamma$ dimer, and its association is weakened by an altered interaction between the $Ca_V\alpha1$ and $Ca_V\beta$ subunits induced by depolarization.

A third possibility is that the relevant part of the N terminus may form a PIP_2 binding site, since RAR is similar to motifs containing positively charged residues in GIRK channels involved in binding the negatively charged head groups of PIP_2, resulting in membrane association. In GIRK channels, PIP_2 binding is thought to lead to association of residues on the N and C termini with the inner surface of the plasma membrane, producing a channel conformation that favors activation. The interaction of these regions of GIRK channels with PIP_2 is found to be stabilized by G$\beta\gamma$, and the presence of PIP_2 in the membrane is a prerequisite for G$\beta\gamma$ modulation (Huang et al., 1998; Logothetis and Zhang, 1999; Zhang et al., 1999). If such a mechanism were to occur for calcium channel modulation, PIP_2 might be expected to coregulate the channel together with G$\beta\gamma$ dimers. Indeed, this has been studied (Wu et al., 2002), and PIP_2 was found to have a dual effect, both preventing calcium channel rundown in patches and producing an inhibitory modulation. Whether the inhibitory effect of PIP_2 directly interacts with G$\beta\gamma$ modulation remains unclear.

VII. Recovery from G Protein-Mediated Inhibition

The speed of termination of GPCR-mediated inhibition of calcium currents depends on a number of factors. The rates of onset and offset of an agonist-mediated response are slower than the dissociation and reassociation rates of the activated G protein-channel complex, obtained by the prepulse protocol (Zhou et al., 1997). The onset of agonist-mediated inhibition using 10 μM noradrenaline was found to have a time constant of 0.7 s, whereas for reinhibition following a prepulse, the time constant was about 0.2 s. This difference is accounted for by the time taken for agonist binding to the receptor and for G protein activation. The rate of recovery from the agonist-mediated response (time constant of approximately 6 s for 10 μM noradrenaline) is very much slower than the facilitation rate, representing the dissociation of G$\beta\gamma$ measured during the depolarizing prepulse (Zhou et al., 1997). This discrepancy may be explained both by the off-rate of the agonist, which for some drugs may be very slow, the slower dissociation of G$\beta\gamma$ from the calcium channel at polarized rather than depolarized potentials (the basis for the voltage dependence of inhibition), and by the slow decay of the free G$\beta\gamma$ concentration, which determines the rebinding rate. This will depend on lateral diffusion in the membrane and reassociation of G$\beta\gamma$ and Gα-GDP, which will in turn be dependent on the rate of hydrolysis of activated Gα-GTP to Gα-GDP. The intrinsic hydrolysis rates of most heterotrimeric G proteins are much slower than the measured off-rate of this response. A family of RGS proteins has been identified that stimulates the GTPase activity of the Gα moiety of specific heterotrimeric G proteins (Watson et al., 1996; Dohlman and Thorner, 1997; García-Palomero et al., 2001). Overexpression of certain RGS proteins accelerated the off-rate of the response (Jeong and Ikeda, 2000). They also slowed the rate of recovery of inhibition after prepulse facilitation, indicating that they had reduced the level of free G$\beta\gamma$ by increasing the Gα-GDP available to rebind G$\beta\gamma$ dimers. Endogenous RGS proteins are likely to be involved in recovery from inhibition as expression of an RGS-insensitive $G\alpha_o$ in sympathetic neurons resulted in a dramatic slowing of the rate of recovery of calcium currents after inhibition by noradrenaline (Jeong and Ikeda, 2000). Of interest in this regard, my group has shown that the GTPase activity of $G\alpha_o$ in neuronal membranes is blocked by an antibody against $Ca_V\beta$ subunits (Campbell et al., 1995a). It remains to be determined whether there is an association between RGS proteins or G protein subunits and $Ca_V\beta$ subunits. It will also be fascinating to examine whether GPCRs are included in such macromolecular signaling complexes.

VIII. Conclusion

A number of experiments indicate that the Ca_V2 calcium channel $\alpha1$ I-II linker is involved in the modulation of the Ca_V2 family of calcium channels by G$\beta\gamma$ dimers. However, several pieces of evidence suggest that this is not the main site involved in mediating the effects of G$\beta\gamma$, since the N terminus is essential in this regard (Page et al., 1998; Canti et al., 1999). Goals for the future include elucidation of the molecular mechanism of modulation by G$\beta\gamma$ dimers since there is still little understanding of the way in which G protein binding is converted into an effect on latency of channel opening (Patil et al., 1996). It will also be of interest to evaluate whether the G protein α subunit plays a role in terminating the signal transduction process, which may be the case for GIRKs (Schreibmayer et al., 1996), and to examine the exact mechanism of the functional interaction between $Ca_V\beta$ subunits and G$\beta\gamma$ dimers in the inhibition of the Ca_V2 family of calcium channels.

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