Calcium Channel β Subunits Differentially Regulate the Inhibition of N-type Channels by Individual Gβ Isoforms*

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The direct inhibition of N- and P/Q-type calcium channels by G protein β subunits is considered a key mechanism for regulating presynaptic calcium levels. We have recently reported that a number of features associated with this G protein inhibition are dependent on the G protein β subunit isoform (Arnot, M. I., Stotz, S. C., Jarvis, S. E., Zamponi, G. W. (2000) J. Physiol. (Lond.) 527, 203–212; Cooper, C. B., Arnot, M. I., Feng, Z.-P., Jarvis, S. E., Hamid, J., Zamponi, G. W. (2000) J. Biol. Chem. 275, 40777–40781). Here, we have examined the abilities of different types of ancillary calcium channel β subunits to modulate the inhibition of α1B N-type calcium channels by the five known different Gβ subunit subtypes. Our data reveal that the degree of inhibition by a particular Gβ β subunit is strongly dependent on the specific calcium channel β subunit, with N-type channels containing the β2 subunit being less susceptible to Gβγ-induced inhibition. The calcium channel β2a subunit uniquely slows the kinetics of recovery from G protein inhibition, in addition to mediating a dramatic enhancement of the G protein-induced kinetic slowing. For Gββγ-mediated inhibition, the latter effect is reduced following site-directed mutagenesis of two palmitoylation sites in the β2a N-terminal region, suggesting that the unique membrane tethering of this subunit serves to modulate G protein inhibition of N-type calcium channels. Taken together, our data suggest that the nature of the calcium channel β subunit present is an important determinant of G protein inhibition of N-type channels, thereby providing a possible mechanism by which the cellular/subcellular expression pattern of the four calcium channel β subunits may regulate the G protein sensitivity of N-type channels expressed at different loci throughout the brain and possibly within a neuron.

The inhibition of presynaptic calcium channels by the activation of seven-helix transmembrane receptors is an important mechanism for modulating calcium influx into presynaptic nerve termini. It is now well established that this inhibitory mechanism occurs via a direct interaction between the G protein βγ dimer and the calcium channel α1 subunit (Refs. 1 and 2; for review, see Ref. 3). Upon Gβγ binding, the channels experience an increase in first latency to opening (4), which is seen as a decrease in peak current amplitude, a slowing of the time course of activation, and a slowed macroscopic time course of inactivation at the whole cell level. The inhibition can be relieved by application of a strong depolarizing voltage pre-pulse (5), which causes a transient dissociation of the G protein complex from the channel (6) and, consequently, a temporary restoration of normal current kinetics. The overall degree of inhibition is strongly dependent not only on the nature of the calcium channel α1 subunit (7–10), but also on the Gβ/γ subunit subtype (10–12). The notion of a Gβγ isoform dependence of this G protein effect is also supported by the observation that protein kinase C is able to antagonize the inhibition of N-type calcium channels by Gβγ subunits, but not that mediated by other Gββγ subunit isoforms (13).

It is now widely accepted that Gβγ physically interacts with the domain I–II linker (14, 15) as well as the carboxyl-terminal region of the channel (8, 16) and that both regions also bind the ancillary calcium channel β subunit (17, 18). In view of this target site overlap, it may not be surprising to note that the presence of the calcium channel β subunit has been shown to antagonize G protein action (7, 19–21). Four different types of calcium channel β subunits (β1a, β2a, β3, and β4) (22) are expressed in rat brain and have been shown to differentially affect dopamine receptor-mediated inhibition of cloned N-type calcium channels in Xenopus oocytes (20). However, use of receptor-coupled inhibition of the channels does not permit access to the detailed effects of the calcium channel β subunit on the inhibition profile of the five known types of G protein β subunits. Here, we have utilized transient coexpression of α1H calcium channels with specific combinations of calcium channel β subunits and Gβ isoforms to address this issue. Our data show that G protein modulation of N-type calcium channels is dependent on the β subunit and varies with the Gβ subunit isoform present. Moreover, the β2a subunit modulates G protein action in a unique manner by slowing the interaction rates between the channel and the G proteins as well as by mediating a dramatic enhancement of the abilities of Gββγ subunits to stabilize the closed conformation of the channel. Thus, the nature of the calcium channel β subunit is an important determinant of G protein efficacy and may give rise to substantial heterogeneity in the susceptibility of native N-type channels to G protein inhibition.
MATERIALS AND METHODS

Molecular Biology—Wild-type calcium channel cDNA constructs were supplied by Dr. Terry Snutch. The wild-type G protein cDNA constructs used were the same as those described by us previously (10).

Creation of Enhanced Green Fluorescent Protein-Gβ Fusion Constructs—Fluorescently tagged Gβ proteins were created using the Living Colors® C-terminal enhanced green fluorescent protein (EGFP®) vector from CLONTech, Palo Alto, CA, which attaches a GFP tag to the N terminus of the protein of interest. Each Gβ subunit was excised from the pMT2 vector using previously engineered (10) XhoI and KpnI restriction sites flanking the inserts, subcloned in frame into the EGFP vector, and sequenced.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out on the full-length β2a construct contained in the pMT2 expression vector using the QuikChange mutagenesis kit (Stratagene) as described by us previously (23). The mutagenesis primers included a diagnostic silent restriction enzyme site (SacI) that was used to confirm the presence of the mutation. A 2.9-kilobase ApaI-EcoRI fragment was excised from the original, non-mutated, full-length construct and substituted into the construct that had undergone mutagenesis. The remaining ~600 base pairs of coding sequence of the "mutated" construct were entirely sequenced to rule out mutagenesis errors and to confirm the presence of the mutation.

Tissue Culture and Transient Transfection of tSA-201 Cells—Human embryonic kidney tSA-201 cells were grown to 80% confluence in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum and penicillin/streptomycin) as described previously (23). After splitting with trypsin/EDTA, the cells were plated on glass coverslips at 5 × 10^4 cells per coverslip in medium (supplemented with 10% fetal bovine serum). The cells were washed, allowed to recover for 12 h, and then moved to 28 °C and maintained for 24–72 h before electrophysiological characterization or fluorescence confocal microscopy.

Imaging—Imaging was carried out at the Seaman Family MR Research Center Confocal Microscopy and Imaging Facility. Coverslips containing cells of interest were placed in a glass-bottom Petri dish and visualized with an inverted Olympus IX70 microscope (magnification ×40). Confocal images were created using an Olympus Fluoview confocal laser scanning microscope (confocal aperture 2). Cells were stimulated with an argon 488-nm laser, and settings for laser power, photo multiplier tube gain, and offset were chosen such that pixel densities were just below saturation levels. Visualization of the transmitted images was done with differential interference contrast/Nomarski interference contrast.

Patch Clamp Recordings—Whole cell recorders were carried out as described in detail by Arnot et al. (10). In brief, cells were bathed in a recording solution consisting of 20 mM BaCl₂, 1 mM MgCl₂, 100 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 65 mM CsCl (pH 7.2 with tetraethylammonium hydroxide). Whole cell patch clamp recordings utilized an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) and pCLAMP Version 7.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15) were pulled using a Sutter P-87 puller and fire-polished using a Narashige Microforge, with series resistance and capacitance being compensated, but no leak subtraction was used. Currents were evoked by stepping from -100 mV to a test potential of +20 mV. The degree of voltage-dependent G protein inhibition was assessed by the degree of current facilitation that occurred after application of a 50-ms depolarizing prepulse to +150 mV 5 ms prior to the test depolarization. For Fig. 1, the peak 1% prepulse inhibition was determined by integrating the area under the current records obtained before and after the prepulse up to an arbitrary time point of 5 ms, thus reflecting the effect of G proteins on net charge entry (24). To determine the kinetics of development of facilitation, R(1-PP)/R(PP) peak current ratios as a function of prepulse duration were fitted monoexponentially as described (10), where R(PP) and R(1-PP) are the peak current amplitudes before and after the prepulse (see Fig. 2 for pulse paradigms). The time constant for recovery from facilitation after the prepulse was determined by monoexponential fits to R(1-PP)/R (PP) peak current ratios obtained at various time points after the termination of the prepulse (10). The time constants for activation (before and after the prepulse) were estimated from monoexponential fits to the late rising phase of the whole cell currents. The raw data were analyzed using Clampfit and Sigmaplot (Jandel Scientific) software. All figures were generated using Sigmaplot Version 4.0.

Numbers in parentheses reflect individual experiments that typically were obtained in multiple transfections. Error bars given reflect standard errors; p values reflect either Student’s t tests or one-way ANOVAs followed by a Tukey post-ANOVA test.

Western Blot Analysis—Western blots on cell lysates were generated from transfected tSA-201 cells as we have described previously (13, 25). The monoclonal antibody to the bovine G protein β3 subunit was purchased from Transduction Laboratories (catalog no. G29629). Immunoblots were subjected to chemiluminescence analysis using ECL Plus (Amersham Pharmacia Biotech) and detected on Eastman Kodak BioMax film.

RESULTS

Gβ5y Modulation of N-type Channels Is Dependent on the Nature of the Calcium Channel β Subunit—Upon coexpression of N-type calcium channels with Gβ5y subunits, the channels undergo a tonic G protein inhibition and enter a reluctant gating mode that is seen at the whole cell level as a slowed time course of current activation (termed kinetic slowing) and a decrease in peak current amplitude (10). Both the kinetic slowing and the reduction in peak current amplitude can be conveniently removed by application of a strong depolarizing prepulse to reveal the control (i.e. non-inhibited) current waveform. The increase in peak current amplitude following the prepulse (termed facilitation) is commonly used an indication of the degree of voltage-dependent inhibition mediated by the G proteins.

Fig. 1A displays a family of current records obtained with α₁b (α₁b + αδβ) calcium channels coexpressed with Gβδ5γ2 and one of the four individual types of calcium channel β subunits. As shown in Fig. 1A, the degree of facilitation seen with Gβδ5 was dependent on the nature of the calcium channel β subunit in at least two ways. First, in the presence of the β3 subunit, Gβδ5 induced a dramatic slowing of the activation kinetics such that activation remained incomplete 150 ms into the test depolarization (note the expanded time scale in Fig. 1A). Second, the degree of prepulse facilitation seen with Gβδ3 was less pronounced in the presence of the calcium channel β3 subunit than 2a subunit. Thus, the calcium channel β3 subunit appears to be an important determinant in regulating Gβδ5 modulation of N-type calcium channel activity.

To obtain a quantitative measure of the degree of facilitation, we integrated the areas under the first 5 ms of the current waveforms obtained before and after the prepulse for various calcium channel β subunit/Gβ5y combinations (Fig. 1B–D). As shown in Fig. 1B, in the absence of exogenously expressed G proteins, little if any facilitation was seen in the presence of the calcium channel β1 subunit, whereas slightly higher background levels were observed with β2a. In the presence of exogenously expressed Gβ3, Gβδ3, or Gβ4, significant facilitation was observed for all calcium channel β subunits examined, but the total degree of facilitation appeared to vary with both the Gβ subtype and the nature of the calcium channel β subunit. For example, with the calcium channel β1 subunit, a significantly larger degree of facilitation (p < 0.05) was seen with Gβ3 compared with the other Gβ isoforms. By contrast, for the other three types of calcium channel β subunits, the degree of facilitation did not differ significantly among the three Gβ isoforms.

To provide a better indication of the effects of the four calcium channel β subunits on the modulation by a particular Gβ subunit, the data shown in Fig. 1B were plotted in a three-dimensional format in Fig. 1C. As shown for Gβ3, there was no
significant difference among the facilitation ratios obtained following coexpression of $G_\beta_1$, $G_\beta_2$, and $G_\beta_3$, whereas facilitation was significantly smaller ($p < 0.05$) in the presence of the calcium channel $G_\beta_4$ subunit. For $G_\beta_1$, a slightly different pattern became apparent, with facilitation being significantly greater in the presence of the calcium channel $G_\beta_2$ subunit compared with $G_\beta_1$ ($p < 0.05$).

The responses observed with $G_\beta_2$ and $G_\beta_3$ are illustrated in Fig. 1D on an expanded scale. $G_\beta_2$, which we had previously found to mediate only weak effects on $G_\beta_1$-containing $\alpha_1$ channels (10), did not appear to affect $\alpha_1$ calcium channels coexpressed with any of the other three calcium channel $G_\beta$ subunits. Perhaps more interestingly, $G_\beta_3$, which we had shown previously not to affect $G_\beta_1$-containing $\alpha_1$ calcium channels (10), induced a significant inhibition of N-type calcium channels containing the $G_\beta_3$ subunit (Fig. 1C), indicating that this structurally unique $G_\beta$ subunit is capable of interacting with the N-type calcium channel under certain circumstances. Overall, the data in Fig. 1 show that the inhibitory effects of various $G_\beta$ isoforms are differentially modulated by the four different calcium channel $G_\beta$ subunits.

$G_\beta_1$ protein-induced calcium channel inhibition is differentially modulated by calcium channel $G_\beta$ subunits. A, whole cell Ba$^{2+}$ currents from N-type channels ($\alpha_1$ and various $\beta$ subunits) coexpressed with the $G_\beta_1$ isoform (+$G_\gamma_1$). The currents were evoked by a +20-mV test depolarization with (+pp) or without (-pp) prior application of a 50-ms prepulse of +150 mV. The dashed lines indicate the baseline. B and C, effect of coexpression of the four types of calcium channel $G_\beta$ subunits on the facilitation of $\alpha_1$ calcium channels seen with $G_\beta_1$, $G_\beta_2$, and $G_\beta_4$. The data shown in C are the same as in B, but are presented in a three-dimensional format to facilitate comparison (error bars have been omitted here for clarity). In C, asterisks indicate statistical significance relative to $G_\beta_1$ ($p < 0.05$, ANOVA). D, effect of $G_\beta_2$ and $G_\beta_5$ on N-type calcium channel activity. Note that $G_\beta_2$ and $G_\beta_5$ are capable of inducing a significant degree of inhibition only when coexpressed with the calcium channel $G_\beta_2$ and $G_\beta_3$ subunits, respectively. Also note the different scaling of the ordinate compared with B, indicating a much smaller degree of facilitation seen with these two $G_\beta$ protein $G_\beta$ subunit isoforms. Error bars indicate S.E. values; numbers in parentheses reflect numbers of experiments; and asterisks indicate significance relative to control.

G Protein $G_\beta$ Subunits Are Effectively Expressed and Targeted in tsA-201 Cells—To unequivocally interpret the experiments shown in Fig. 1, it is important to ensure appropriate expression levels of the five $G_\beta$ subunits to rule out the possibility of a mixed population of $G_\beta$-bound/unbound channels. We have previously shown via Western blot analysis that $G_\beta_1$, $G_\beta_2$, $G_\beta_3$, and $G_\beta_4$ can be effectively exogenously expressed in tsA-201 cells and at levels that are well beyond endogenous background $G_\beta$ subunits (13). Moreover, we obtained similar results with $G_\beta_5$ subunits. To determine whether the $G$ proteins are also appropriately targeted to the plasma membrane, we generated EGFP-conjugated versions of the five $G_\beta$ subunit subtypes in which a GFP tag was fused to the $G_\beta$ N terminus. These were then expressed in tsA-201 cells to confirm their appropriate expression and targeting. As $G_\beta$ protein-induced calcium channel inhibition is differentially modulated by calcium channel $G_\beta$ subunits. A, whole cell Ba$^{2+}$ currents from N-type channels ($\alpha_1$ and various $\beta$ subunits) coexpressed with the $G_\beta_1$ isoform (+$G_\gamma_1$). The currents were evoked by a +20-mV test depolarization with (+pp) or without (-pp) prior application of a 50-ms prepulse of +150 mV. The dashed lines indicate the baseline. B and C, effect of coexpression of the four types of calcium channel $G_\beta$ subunits on the facilitation of $\alpha_1$ calcium channels seen with $G_\beta_1$, $G_\beta_2$, and $G_\beta_4$. The data shown in C are the same as in B, but are presented in a three-dimensional format to facilitate comparison (error bars have been omitted here for clarity). In C, asterisks indicate statistical significance relative to $G_\beta_1$ ($p < 0.05$, ANOVA). D, effect of $G_\beta_2$ and $G_\beta_5$ on N-type calcium channel activity. Note that $G_\beta_2$ and $G_\beta_5$ are capable of inducing a significant degree of inhibition only when coexpressed with the calcium channel $G_\beta_2$ and $G_\beta_3$ subunits, respectively. Also note the different scaling of the ordinate compared with B, indicating a much smaller degree of facilitation seen with these two $G_\beta$ protein $G_\beta$ subunit isoforms. Error bars indicate S.E. values; numbers in parentheses reflect numbers of experiments; and asterisks indicate significance relative to control.

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shown in Fig. 2A, all of the five G protein β subunit isoforms were effectively targeted to the plasma membrane and appeared at comparable levels. To rule out the possibility that one of the four calcium channel β subunits might negatively affect Gβ expression, we carried out Western blot analysis on tsA-201 cells transfected with Gβ1, α1B, α2δ, and each of the four calcium channel β subunits. As shown in Fig. 2B, the exogenous expression of Gβ3 resulted in a dramatic increase in Gβ protein that did not appear to be affected by the nature of the calcium channel β subunit. Together with our previous findings (13), these results indicate that all five G protein β subunits are effectively expressed and targeted in tsA-201 cells and that the expression occurs independently of the type of calcium channel β subunit that is present.

**Calcium Channel β Subunits Modulate the Kinetics of G Protein Action**—To examine the dependence of the G protein kinetics (Fig. 3) for various calcium channel β subunit/Gβ isoform combinations, we used the paradigms described in detail by Arnott et al. (10). Varying the duration of the prepulse yields the time constant for development of facilitation, which reflects the kinetics of the dissociation of the G protein-channel complex. In contrast, varying the interval between the prepulse and the test depolarization yields the time constant for recovery from facilitation, which is a reflection of the kinetics of reassociation between the G protein and the channel (6). Assuming a 1:1 interaction between the G protein and the channel (6, 26), the former will be independent of G protein concentration and thus independent of the absolute levels of Gβ expression. However, the recovery from facilitation is dependent on the absolute levels of Gβ, which are not known in a given cell and must therefore be cautiously interpreted.

For these experiments, we focused on Gβ1, Gβ2, and Gβ4 because the inhibitory effects of Gβ2 and Gβ4 could be observed only with a single calcium channel β subunit. Fig. 3 illustrates the effects of the calcium channel β subunit on the time constant for recovery from facilitation (Fig. 3A) and on the time constant for development of facilitation (Fig. 3B) by the three active Gβ subunit isoforms. For Gβ1, the time constant for recovery from facilitation was similar for all calcium channel β subunits, whereas that seen with Gβ2 and Gβ4 was dramatically slowed in channels containing the β2a subunit. A similar effect of the calcium channel β2a subunit could be observed with the time constant for development of facilitation; however, here, primarily responses by Gβ4 and Gβ3 were affected. Interpreted mechanistically, these data suggest that the calcium channel β2a subunit induces a channel conformation in which G proteins might encounter a reduced access to their site of action on the channel, but once associated with the channel, are more tightly bound compared with channels containing other types of calcium channel β subunits. We reiterate that the kinetics of recovery from facilitation are expected to be dependent on the free concentration of Gβγ. However, we note that for each of the three Gβ isoforms, the only variable is the type of calcium channel β subunit; and hence, for a particular Gβ subunit isoform, the effects observed with β2a are likely due to a direct effect of this subunit on G protein access to its binding site on the channel, rather than due to a diffuse concentration effect. Overall, these data indicate that the nature of the calcium channel β subunit is important in determining the rate of unbinding (and possibly binding) between certain G protein β subunit isoforms and the channel, consistent with a partial overlap of the binding sites for the calcium channel β subunit and Gβγ on the α1B subunit.

**G Protein-induced Kinetic Slowing Is Selectively Enhanced by the Calcium Channel β2a Subunit**—Fig. 4 examines the effects of different Gβ subunit isoforms on the activation kinetics of α1B calcium channels, coexpressed with different ancillary calcium channel β subunits, in the form of ratios of activation time constants obtained before and after application of the prepulse. Although a slowing of the activation kinetics was seen with every calcium channel β subunit/Gβ isoform combination, the kinetic slowing induced by the three Gβ isoforms was dramatically enhanced in channels coexpressed with the β2a subunit such that G proteins were able to slow the activation kinetics of the α1Bβ2aαΔ heterotrimer by as much as 25-fold (see also Fig. 1A). A subset of these experiments was repeated using the EGFP-coupled Gβ1 subunit to ensure high G protein expression levels in cells chosen for patch recordings. Compared with wild-type Gβ1, the GFP-fused constructs resulted in slower kinetics for development of and recovery from facilitation (data not shown), which is perhaps not surprising given the relative sizes of the GFP tags and the G protein subunit (23 and 35 kDa, respectively). And yet, with regard to the ability of β2a to enhance the G protein-induced kinetic slowing, the pattern seen with the GFP-tagged Gβ1 subunit closely paralleled that observed with the wild-type protein (Fig. 4C), suggesting that the unique effects of β2a are not skewed by cells expressing low amounts of Gβ. Perhaps more importantly, the activation time constants obtained before and after the prepulse could in every case (i.e. for both wild-type and GFP-fused Gβ subunits) be fitted monexponentially (Fig. 4A). The absence of biphasic (i.e. mixed fast and slow) activation components prior to application of the prepulse indicates that all of the channels are tonically in a reluctant mode and consequently must be associated with a G protein. This lends additional support to our contention that we are not dealing with a heterogeneous population of G protein-bound and -unbound channels in our experiments.

Finally, we note that there was no significant difference in the activation time constants obtained immediately after ap-
plication of the prepulse for all of the calcium channel β subunit/Gβ subunit combinations (Table I), indicating that the mere presence of the G protein subunits did not per se affect the activation time course and, more importantly, that the pre-pulses were sufficient to effectively remove tonic voltage-dependent G protein inhibition in all cases. Overall, our data indicate that the β2a subunit helps to stabilize the G protein-induced reluctant mode of channel opening; and when extrapolated to the situation in an intact neuron, this would imply that a single action potential may be insufficient to allow significant calcium entry through such a channel.

Palmitoylation Sites in the β2a Subunit Contribute to the Gβ-induced Slowing of Activation—It is well established that coexpression of the rat β2a subunit exerts a dramatic slowing of the macroscopic time course of calcium channel inactivation (27). This is nicely shown in Fig. 1 when comparing the time course of inactivation seen with the four calcium channel β subunits following the prepulse. It has been shown that this effect is critically dependent on the N-terminal region of β2a (28), which contains a pair of cysteine residues at positions 3 and 4 that are not present in other types of calcium channel β subunits and which forms a palmitoylation site (29–31). Replacement of the two cysteines by serines ablates the slowing of inactivation (31), and replacement of the N-terminal region with the transmembrane domain of the CD8 receptor restores the slowing of inactivation, suggesting that palmitoylation of the cysteines and subsequent membrane insertion underlie this inactivation phenomenon (32).

To determine whether this palmitoylation site is also responsible for the effects of β2a on G protein-induced kinetic slowing, we mutated the two cysteines to serines and examined the effects of this mutant β2a subunit on G protein inhibition of N-type calcium channels. As shown in Fig. 5, the serine mutations significantly (p < 0.05) reduced the degree of kinetic slowing observed with Gβ3, although the remaining degree of kinetic slowing was still substantially larger than that seen with the other types of calcium channel β subunits. Hence, the ability of β2a to interact with the plasma membrane appears to be an important (albeit not exclusive) determinant of the unique effects of this subunit on Gβ3 regulation. Since both the calcium channel β subunit (17) and Gβγ (14, 15) physically interact with the N-type calcium channel I–II linker region, the selective palmitoylation and membrane tethering of β2a may perhaps result in a conformational change in the Gβγ target

**Fig. 3.** Time constants for recovery from facilitation (A) and development of facilitation (B) obtained with Gβ1, Gβ3, and Gβ4 in the presence of different calcium channel β subunits. The kinetics were determined as described under “Materials and Methods” using the pulse paradigms shown in A and B. In A, asterisks indicate significant differences within each Gβ group (ANOVA). In B, asterisks indicate statistical significance within each Gβ group, and the number symbols indicate statistical significance relative to both β1b and β2a (ANOVA). Note that the β2a subunit slowed both the kinetics of development of and recovery from facilitation. PP, prepulse.
FIG. 4. A, current records illustrating the effect of prepulses on the activation phase of whole cell currents. The thick lines are monoexponential fits to the raw data (the time constants obtained from the fits are included). The Gβ1-induced kinetic slowing (i.e., the ratio of the activation time constants obtained in the presence and absence of a depolarizing prepulse) was dramatically enhanced in the presence of the β2a subunit (note the expanded time scale in the right panel).

B, degree of kinetic slowing induced by various Gβ isoforms as a function of the calcium channel β subunit subtype. The bars reflect the activation time constants obtained via monoexponential fits to the time course of current activation (see A) before (−pp) and after (+pp) application of the prepulse. Error bars indicate S.E. values; numbers in parentheses reflect number of experiments; and asterisks denote statistical significance within the data set (ANOVA). Note that the β2a subunit dramatically enhanced the kinetic slowing induced by all three Gβ subunits examined.

C, comparison of kinetic slowing seen with wild-type and EGFP-tagged Gβ1 subunits for the four types of calcium channel β subunits. Note that when cells were chosen based on robust EGFP-Gβ1 fluorescence, the unique ability of the β2a subunit to augment kinetic slowing was retained.

Role of Ca2+ Channel β Subunit in G Protein Modulation

DISCUSSION—Expression Levels of G Proteins and Calcium Channel Subunits—Our experiments rely on three key players (the calcium channel α1B subunit, the calcium channel β subunit, and Gβ), and unambiguous interpretation of our experiments requires the presence of a homogeneous population of G protein-bound channels. The expression of the calcium channel α1B subunit is not an issue since its presence is required for current activity (note that robust current activity was observed for each of the calcium channel β subunits; see Table I). In tsA-201 cells, we observed little (if any) N-type channel activity when the calcium channel β subunit was omitted. Hence, under our conditions, the transiently expressed channels are likely to form a homogeneous population; or in other words, all of the channels that we recorded from must be in a complex with a β subunit. This is supported by examination of the time course of inactivation seen with β2a subunits (Fig. 1A). After removal of voltage-dependent G protein inhibition via the prepulse, channels coexpressed with the β2a subunit uniformly exhibit slowed inactivation. If a mixed population of β2a-bound/β2a-free channels had been present, the time course of inactivation would have been composed of both rapid and slow components. Hence, the calcium channel β subunit expression levels are not a variable that will affect our experiments.

By contrast, the presence of saturating Gβ expression levels is critical, and several lines of experiments suggest that this is indeed the case. For the three Gβ subtypes that mediated significant voltage-dependent inhibition, the time course for activation seen in the absence of the prepulse was slowed and was well described by a single exponential in every case, indicating a single population of reluctant and thus G protein-bound channels. In addition, the imaging of the GFP-tagged Gβ subunits indicates that all five Gβ isoforms can be effectively expressed in tsA-201 cells and are appropriately targeted to the plasma membrane. Finally, the results from Western blot analysis shown here and reported previously (13) indicate a massive up-regulation of Gβ levels following transient transfection that occurred independently of the type of calcium channel β subunit that was coexpressed. Since even the low endogenous amounts of Gβ present in these cells are sufficient to maximally inhibit N-type channels via activation of G protein-coupled receptors (13), the massive up-regulation of Gβ2a levels in transfected cells implies a large molar excess of Gβ2a subunits over N-type channels when they are exogenously expressed. Hence, we conclude that we are dealing with a homogeneous population of G protein-bound N-type calcium channels under our conditions. Under these circumstances, most of the key parameters examined here (degree of facilitation, time constant for development of facilitation, degree of kinetic slowing) are predicted to be independent of the absolute concentration of Gβ subunits (6, 26); and hence, our data likely reflect a true calcium channel β subunit dependence of G protein action. On the other hand, the time constant for recovery from facilitation is inversely proportional to the Gβ concentration, which is unknown in a given cell (6). Hence, although the calcium channel β2a subunit appears to uniquely slow the time course for recovery from facilitation, it is difficult to rule out a contribution from variability in Gβ levels. However, these data were included here mainly for completeness and should not detract from the unique ability of the β2a subunit to modulate other, more precisely controlled parameters of G protein inhibition of N-type calcium channels. Taken together, these considerations strongly suggest that the interpretations of our experiments are not skewed by variability of G protein or calcium channel β subunit expression.

Comparison with Previous Work—We have recently reported
Domain of Ca\(^{2+}\) Channel \(\beta\) Subunit in G Protein Modulation

There was no significant difference in the activation time constants within each group of calcium channel \(\beta\) subunits (ANOVA), indicating that the prepulses (pp) completely remove the effects of the G proteins on current activation. There was also no significant difference in the current densities obtained with the four different types of calcium channel \(\beta\) subunits, pF, picofarads.

### Table I

| Current density, no \(\mu A/\mu F\) | \(t_{activation}\) \(ms\) | \(n\) | \(G_\beta_1\) | \(G_\beta_3\) | \(G_\beta_4\) | \(G_\beta_5\) |
|-----------------------------------|-----------------|------|-------------|-------------|-------------|-------------|
| \(\beta_{1b}\)                   |                 |      |             |             |             |             |
| 60.26 ± 12.2                     | 1.53 ± 0.18     | (n = 14) | 1.75 ± 0.37 | 1.79 ± 0.13 | 1.57 ± 0.10 | 1.25 ± 0.09 |
| (n = 14)                         |                 |      |             |             |             |             |
| \(\beta_{2a}\)                   |                 |      |             |             |             |             |
| 72.62 ± 15.4                     | 1.22 ± 0.12     | (n = 14) | 1.49 ± 0.94 | 1.56 ± 0.13 | 1.92 ± 0.29 | 1.55 ± 0.10 |
| (n = 14)                         |                 |      |             |             |             |             |
| \(\beta_3\)                      |                 |      |             |             |             |             |
| 48.93 ± 7.9                      | 1.32 ± 0.09     | (n = 14) | 1.56 ± 0.11 | 1.88 ± 0.36 | 1.53 ± 0.12 | 1.28 ± 0.09 |
| (n = 14)                         |                 |      |             |             |             |             |
| \(\beta_4\)                      |                 |      |             |             |             |             |
| 63.25 ± 12.11                    | 1.43 ± 0.08     | (n = 10) | 1.69 ± 0.13 | 1.99 ± 0.19 | 1.45 ± 0.09 | 1.55 ± 0.10 |
| (n = 10)                         |                 |      |             |             |             |             |

**Fig. 5.** A, time course of current activation seen in the presence of \(G_\beta_3\) and in the absence of a prepulse for N-type channels coexpressed with either wild-type (wt) or mutant (mut) \(\beta_{2a}\) subunits in which the cysteines at positions 3 and 4 were replaced with serines. Note that the kinetic slowing induced by \(G_\beta_3\) was reduced in the presence of the mutant \(\beta_{2a}\) subunit. B, effect of the \(\beta_{2a}\) mutations on the degree of kinetic slowing, expressed as activation time constants obtained before (−pp) and after (+pp) application of a prepulse (as described in the legend to Fig. 4) in cells expressing \(G_\beta_3\) or \(G_\beta_4\). Note that removal of the cysteine residues in the \(\beta_{2a}\) subunit significantly \((p < 0.05)\) reduced \(G_\beta_{3/4}\)-mediated kinetic slowing, but did not affect that induced by \(G_\beta_2\). The data for the wild-type \(\beta_{2a}\) subunits are the same as those shown in Fig. 4B. Error bars indicate S.E. values; numbers in parentheses reflect number of experiments; and the asterisk denotes statistical significance between the wild-type and mutant \(\beta_{2a}\) subunits.

A strong dependence of the inhibition of \(\beta_{2a}\) subunit-containing N- and P/Q-type calcium channels on the G protein \(\beta\) subunit isoform (10). Here, we have extended our work to elucidate the role of the calcium channel \(\beta\) subunit in regulating the inhibitory profile of the individual \(G_\beta\) subunit isoforms. Recently, Canti et al. (20) reported that dopamine receptor-mediated inhibition of N-type calcium channels in *Xenopus* oocytes was dependent on the type of calcium channel \(\beta\) subunit that was coexpressed. Although the authors did not observe a significant calcium channel \(\beta\) subunit dependence of the time constant for recovery from facilitation, they showed that the time constant for recovery from inhibition was fastest in the presence of the calcium channel \(\beta_4\) and \(\beta_2\) subunits and slowest in the presence of \(\beta_{2a}\). Because it is not known which specific G protein \(\beta_\gamma\) subunit isoforms are involved in receptor-mediated inhibition of the channels, it is difficult to make firm comparisons between our work and this prior study. Nonetheless, the data shown in Fig. 2 reveal two striking parallels to the work of Canti et al. (20). First, we observed a similar trend for the time constant for development of facilitation: the \(\beta_{2a}\) subunit slowed the development of facilitation the most, whereas the \(\beta_3\) and \(\beta_4\) demonstrated the least slowing. Second, for \(G_\beta_{3/4}\)-mediated responses, we found no significant calcium channel \(\beta\) subunit dependence of the recovery kinetics. In contrast, for \(G_\beta_2\)-mediated responses, our data reveal a dramatic slowing of the time constant for recovery from facilitation in the presence of the \(\beta_{2a}\) subunit. Hence, the calcium channel \(\beta\) subunit dependence of the dopamine receptor-mediated inhibition reported by Canti et al. (20) most closely resembles that seen with \(G_\beta_1\) in our experiments. However, although it is tempting to speculate that the dopamine D2 receptor might preferentially couple to \(G_\beta_1\), such a possibility will need to be substantiated experimentally.

The general absence of facilitation seen with the \(G_\beta_1\) subunit appears to be at odds with previous reports (11, 12). We have shown previously via Western blot analysis that \(G_\beta_2\) expresses well in tsA-201 cells. Moreover, the GFP-tagged \(G_\beta_{2a}\) subunits were effectively targeted to the plasma membrane. Hence, the lack of \(G_\beta_{2a}\) modulation in our experiments is unlikely to arise from ineffective expression of this subunit. There are two major differences between our experiments and those reported by Ikeda and Ruiz-Velasco (11) and Hille and co-workers (12). First, both groups carried out their experiments in intact neurons and hence may not have dealt with a homogeneous channel population (for example, the presence of different \(\alpha_{1B}\) splice isoforms could affect the degree of inhibition). Second, as we reported previously (10), our rat \(G_\beta_2\) construct (GenBank\textsuperscript{TM}/EBI accession number AF277892) contains 11 amino acid differences relative to previously published sequence that we confirmed in multiple independent polymerase chain reactions and that may underlie some of the differences with previous reports. In summary, our experiments provide the first detailed approach to determining the role of the calcium channel \(\beta\) subunit in modulating the inhibition by each of the five known G protein \(\beta\) subunit under well controlled and identical conditions.
Possible Physiological Implications—Our results have important implications for our understanding of the G protein modulation seen with native calcium channels. Depending on the experimental preparation, native N-type calcium channels can undergo a wide range of responses to G protein–coupled receptor activation with regard to the magnitude of inhibition as well as the degree of kinetic slowing (33–37). This is not surprising in view of an increasing number of reports showing that G protein inhibition of N-type calcium channels is strongly dependent on the G protein β subunit isoform (10–12, 38, 39). However, our observation that different calcium channel β subunits are able to differentially modulate the inhibitory properties of individual types of Gβ subunit isoforms provides an additional mechanism by which the activation of a seven-helix transmembrane receptor can produce diverse effects on native N-type channels. Considering reports showing that calcium channel β subunits vary in their temporal expression patterns and can be differentially distributed across brain regions/loci within a cell could be differentially modulated by the same neurotransmitter. Together with the observation that G protein inhibition of these channels can be modulated by protein kinase C (13, 14, 37, 42) and by the presence of SNARE proteins (24, 25, 43–45), this provides further intricacy in the molecular mechanisms that govern the entry of calcium through presynaptic calcium channels and thus the release of the neurotransmitter.

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