Ca^{2+} Channel β_3 Subunit Enhances Voltage-Dependent Relief of G-Protein Inhibition Induced by Muscarinic Receptor Activation and $G_{\beta\gamma}$

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The Ca^{2+} channel β subunit has been shown to reduce the magnitude of G-protein inhibition of Ca^{2+} channels. However, neither the specificity of this action to different forms of G-protein inhibition nor the mechanism underlying this reduction in response is known. We have reported previously that coexpression of the Ca^{2+} channel β_3 subunit causes M_2 muscarinic receptor-mediated inhibition of α_{1B} Ca^{2+} currents to become more voltage-dependent. We report here that the β_3 subunit increases the rate of relief of inhibition produced by a depolarizing prepulse and also shifts the voltage dependency of this relief to more hyperpolarized voltages; these effects are likely to be responsible for the reduction of inhibitory response of α_{1B} channels to G-protein-mediated inhibition seen after coexpression of the Ca^{2+} channel β_3 subunit. Additionally, the β_3 subunit alters the rate and voltage dependency of relief of the inhibition produced by coexpressed $G_{\beta1\gamma1}$, in a manner similar to the changes it produces in relief of M_2 receptor-induced inhibition. We conclude that the Ca^{2+} channel β_3 subunit reduces the magnitude of G-protein inhibition of α_{1B} Ca^{2+} channels by enhancing the rate of dissociation of the G-protein $\beta\gamma$ subunit from the Ca^{2+} channel α_{1B} subunit.

Key words: Ca^{2+} channels; G-proteins; α_{1A}; α_{1B}; Ca^{2+} channel β subunit; G-protein α subunit; G-protein $\beta\gamma$ subunit; voltage-dependent inhibition; Xenopus *oocyte; muscarinic M_2 receptor; NEM*

G-protein-mediated inhibition of voltage-gated Ca^{2+} channels provides an important mechanism for regulating synaptic strength (Holz et al., 1986; Wheeler et al., 1994; Dittman and Regehr, 1996; Takahashi et al., 1996). Although many types of Ca^{2+} channels can undergo this class of inhibition, N-type Ca^{2+} current is the most frequently studied target of this modulation (Schultz et al., 1990; Anwyl, 1991; Dolphin, 1991; Hille, 1994). Various members of the seven membrane-spanning family of receptors, after binding neurotransmitter, transduce their signal via activation of a variety of heterotrimeric G-proteins. The activated G-proteins then either directly interact with the channel to cause inhibition, in a process known as membrane-delimited inhibition (Bean, 1989; Brown and Birnbaumer, 1990), or subsequently activate a second messenger cascade that ultimately acts on the channel to cause inhibition. N-type Ca^{2+} channels are inhibited via both a membrane-delimited pathway (Schultz et al., 1990; Anwyl, 1991; Dolphin, 1991; Hille, 1994) and a pathway requiring diffusible intracellular second messengers (Beech et al., 1992; Shapiro et al., 1994a).

Membrane-delimited G-protein inhibition encompasses both voltage-dependent and voltage-independent inhibition. Voltage-dependent inhibition exhibits two main characteristics in voltage-clamp studies: (1) slowed activation kinetics and (2) diminished inhibition at more depolarized voltages (Marchetti et al., 1986; Wanke et al., 1987; Bean, 1989; Kasai and Aosaki, 1989). The diminished inhibition at more depolarized voltages gives rise to a third characteristic of voltage-dependent inhibition, prepulse current facilitation (Elmslie et al., 1990; Ikeda, 1991; Lopez and Brown, 1991). Strongly depolarizing voltages are thought to cause a temporary dissociation of the G-protein from the Ca^{2+} channel (Bean, 1989; Lopez and Brown, 1991; Golard and Siegelbaum, 1993); thus, a current elicited during this period of G-protein dissociation will be facilitated compared with current elicited by the same test voltage step without a depolarizing prepulse.

Voltage-independent inhibition is characterized by equivalent current inhibition at all voltages, with no change in current kinetics during the inhibition. Frequently, voltage-independent G-protein inhibition requires intracellular signaling cascades and thus is not membrane-delimited (Beech et al., 1991, 1992; Bernheim et al., 1991; Shapiro et al., 1994a). However, there are instances of membrane-delimited voltage-independent inhibition (Shapiro and Hille, 1993; Diverse-Pierluissi et al., 1995; Wollmuth et al., 1995).

Voltage-dependent inhibition of N-type Ca^{2+} currents in rat superior cervical ganglion (SCG) sympathetic neurons (Herlitze et al., 1996; Ikeda, 1996), as well as α_{1A} Ca^{2+} channel currents expressed in tsA-201 cells (Herlitze et al., 1996), is mediated by the G-protein $\beta\gamma$ subunit. $G_{\beta\gamma}$, however, seems not to be responsible for voltage-dependent inhibition of N-type currents in embryonic chick dorsal root sympathetic ganglion neurons (Diverse-Pierluissi et al., 1995). The G-protein $\beta\gamma$ subunit is capable of binding to at least two regions of the intracellular loop between transmembrane regions I and II of α_{1A} and α_{1B} Ca^{2+} channels (De Waard et al., 1997; Zamponi et al., 1997); the same intracel-

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lular loop contains the binding region for the Ca^{2+} channel β subunit (Pragnell et al., 1994). Mutations that reduce *in vitro* G-protein $\beta\gamma$ subunit binding to this region of the Ca^{2+} channel also block some characteristics of voltage-dependent G-protein inhibition of α_{1A} current (De Waard et al., 1997), although similar mutations do not affect somatostatin-induced inhibition of α_{1B} currents (Zhang et al., 1996). The critical amino acids within α_{1A} responsible for $G_{\beta\gamma}$ binding are not the same as those critical for Ca^{2+} channel β subunit binding (De Waard et al., 1997), suggesting that direct competition for a binding site on the α_1 subunit is unlikely.

The Ca^{2+} channel β subunit reduces the magnitude of G-protein inhibition of both α_{1A} and α_{1B} Ca^{2+} channels expressed in *Xenopus* oocytes (Roche et al., 1995), as well as Ca^{2+} currents in rat dorsal root ganglion neurons (Campbell et al., 1995). Speculation on the mechanism underlying this reduction in sensitivity to G-protein inhibition includes: (1) direct competition between the Ca^{2+} channel β_3 subunit and the G-protein for the same site on the Ca^{2+} channel α_1 subunit (McAllister-Williams and Kelly, 1995; Roche et al., 1995; Bourinet et al., 1996; Clapham, 1996), (2) steric blockade of the G-protein binding site (Roche et al., 1995; Bourinet et al., 1996), and (3) a Ca^{2+} channel β subunit-induced increase in the GTPase activity of the G-protein (Campbell et al., 1995). Examination of M_2 muscarinic receptor-induced inhibition of α_{1B} currents in *Xenopus* oocytes revealed that not only is the magnitude of the G-protein inhibition reduced after coexpression of the Ca^{2+} channel β_3 subunit but the portion of inhibited current that is voltage-dependent is increased as well (Roche and Treistman, 1998). Here, we examine possible mechanisms that underlie the increase in voltage-dependence and discuss whether this mechanism can explain the reduction in the magnitude of M_2 receptor-induced inhibition of α_{1B} currents after coexpression of the Ca^{2+} channel β_3 subunit. We address these questions using α_{1B} Ca^{2+} channels coexpressed with muscarinic M_2 receptors in *Xenopus* oocytes. The coexpressed M_2 receptor couples to the endogenous pertussis toxin-sensitive G-proteins of the *Xenopus* oocyte (Lechleiter et al., 1991). We also coexpress G-protein α and $\beta\gamma$ subunits individually to determine the G-protein subunit mediating inhibition of both α_{1B} and $\alpha_{1B}\beta_3$ Ca^{2+} channel currents and to assess the influence of the Ca^{2+} channel β_3 subunit on the direct actions of these G-protein subunits on α_{1B} Ca^{2+} channels.

MATERIALS AND METHODS

Expression plasmids and oocyte preparation. Capped RNA transcripts encoding full-length α_{1A} (*Xba*I-linearized/SP6 RNA polymerase; gift of Dr. Y. Mori, University of Cincinnati Medical Center), α_{1B} (*Sal*I/SP6; gift of Dr. Y. Fujita, Kyoto University), and β_3 (*Not*I/T7; gift of Dr. Edward Perez-Reyes, Loyola University Medical Center) calcium channel subunits as well as the muscarinic M_2 receptor (*Eco*RI/*Bgl*II; gift of Dr. Wolfgang Sadee, University of California San Francisco) and G-protein αi_2 (gift of Dr. Randall Reed, HHMI, Baltimore, MD) and $\beta_1\gamma_1$ (gift of Drs. Melvin Simon and Anna Aragay, California Institute of Technology, Pasadena, CA) subunits were synthesized using the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Austin, Texas). *Xenopus laevis* stage V–VI oocytes were removed and treated with collagenase (Sigma type IV; Sigma, St. Louis, MO) to remove the follicular layer. The oocytes were then injected with cRNA encoding α_{1B} in combination with M_2 in a ratio of 2:1 or in combination with both M_2 and β_3 (2:2:1). The concentration of all individual RNAs before injection was 0.1 μg/μl, with the exception of the G-protein α and $\beta\gamma$ subunit RNA that was 0.5 μg/μl, and 20–60 nl of RNA mixed at the above ratios was injected. The oocytes were maintained in culture at 18°C for at least 2 d in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 2 mg/ml gentamycin.

Electrophysiological recording and experimental treatments. Two-electrode voltage-clamp currents were recorded using a Dagan CA-1 amplifier. Oocytes were clamped at a holding potential of −80 mV, and various electrophysiological protocols were used, as noted. Currents were filtered at 1 or 10 kHz, and a p/4 leak subtraction technique was used. Inhibition of current amplitude was determined by measurements of the peak current attained at any point during the 250 msec test pulse. Analysis was done off-line, using pClamp software version 6.0.2 (Axon Instruments, Foster City, CA). Electrodes contained 3 M KCl and had resistances of 0.5–2 MΩ. Oocytes were placed in a 1 ml chamber and perfused at a rate of 0.5 ml/min. All recordings were made at room temperature using bath solutions containing (in mM): $Ba(OH)_2$, 10; NaOH, 50; CsOH, 2; TEA-OH, 20; *N*-methyl-D-glucamine, 20; and HEPES, 5, titrated to pH 7.5 with methanesulfonic acid. In all experiments, 20 nl of a 100 mM stock solution of K_3-1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA) (Sigma) was injected at least 2 hr before the experiment. The final concentration of BAPTA inside the oocyte was estimated to be between 2 and 5 mM, assuming an oocyte volume of 1 μl. For experiments using *N*-ethylmaleimide (NEM) (Aldrich, Milwaukee, WI), the NEM was dissolved in the external solution to a final concentration of 200 μM and was applied to the oocyte for 2 min. Acetylcholine (ACh) (Sigma) was stored as a 10 mM stock solution in water and dissolved in the recording medium to a final concentration of 50 μM.

RESULTS

Ca^{2+} channel β_3 subunit modulates voltage dependence of M_2-mediated inhibition

A protocol designed to remove tonic G-protein inhibition of α_{1B} Ca^{2+} channels allowed study of the isolated muscarinic M_2 receptor-induced G-protein inhibition of these channels. Briefly, we exposed the oocyte to 50 μM ACh. Immediately after removal of the ACh, there is a large rebound of current amplitude, resulting from temporary loss of tonic G-protein inhibition (Roche and Treistman, 1998). During the period in which tonic inhibition is abolished, ascertained by the loss of prepulse facilitation, the current remains sensitive to muscarinic receptor-induced inhibition. Loss of tonic inhibition occurred, in most cases, after a single 1 min application of ACh; on occasion, multiple ACh applications were required to remove tonic inhibition completely. Using this protocol, we have demonstrated that expression of the Ca^{2+} channel β_3 subunit reduced the magnitude of muscarinic M_2 receptor-induced inhibition (Fig. 1*A*,*B*). However, the reduction in magnitude of inhibition was voltage-dependent, with substantial reductions of G-protein inhibition at voltages more positive than 0 mV, and no effect on calcium current inhibition during voltage steps to −10 or 0 mV (Fig. 1*C*). In addition to the reduced inhibition, a depolarizing prepulse during muscarinic inhibition elicits greater relief of G-protein inhibition after coexpression of the Ca^{2+} channel β_3 subunit (Fig. 1*D*).

The Ca^{2+} channel β_3 subunit increases the rate of voltage-dependent relief of G-protein inhibition of α_{1B} currents

Voltage-dependent relief of G-protein inhibition of N-type currents is thought to result from temporary dissociation of the G-protein from the Ca^{2+} channel (Lopez and Brown, 1991; Golard and Siegelbaum, 1993). Thus, the heightened relief of the inhibited current by depolarizing voltages after coexpression of the Ca^{2+} channel β_3 subunit suggests that the rate of G-protein dissociation has changed. An increase in the G-protein dissociation rate could explain the reduced inhibition of current by M_2 receptor activation when the Ca^{2+} channel β_3 subunit is coexpressed, because the inhibition would be more easily reversed by moderate voltages, such as those in the range normally used to activate the Ca^{2+} channel.

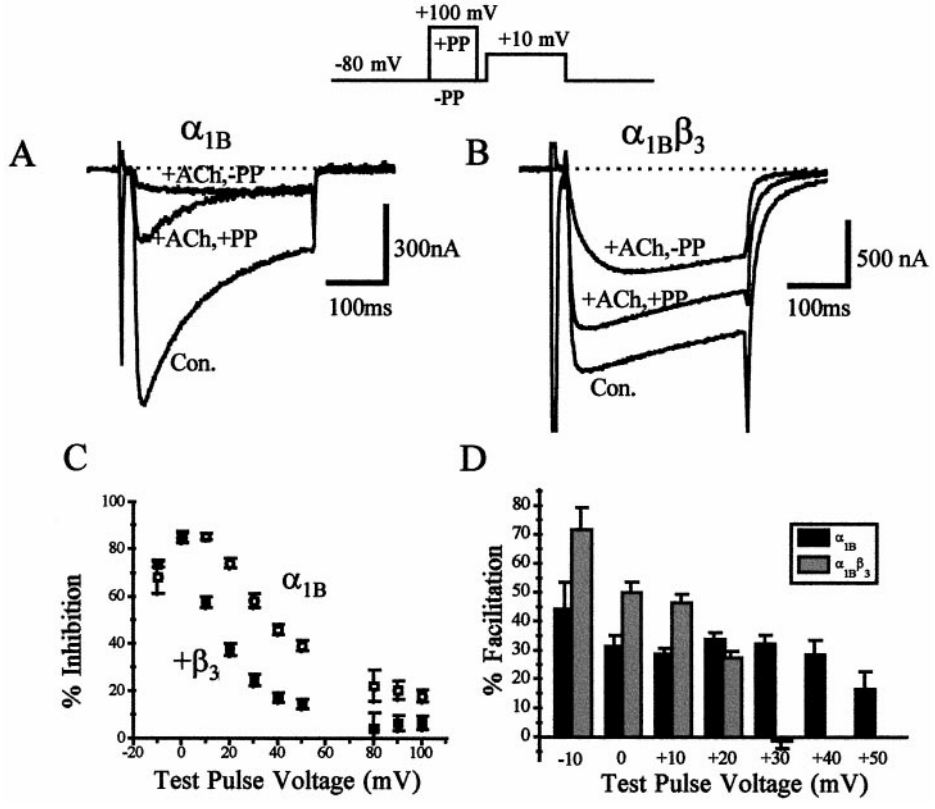

Figure 1. The Ca^{2+} channel β_3 subunit modifies the voltage dependence of muscarinic-induced G-protein inhibition. *A*, *B*, Representative records of α_{1B} and $\alpha_{1B}\beta_3$ Ca^{2+} currents for the control situation (*Con*), as well as after the application of 50 μM ACh and before (*+ACh,−PP*) and after (*+ACh,+PP*) a depolarizing prepulse to +100 mV for 75 msec. Oocytes were held at −80 mV and stepped to a test potential of +10 mV for 250 msec. The M_2 receptor is coupling to G-proteins that are endogenous to the oocyte. *C*, Inhibition of current amplitude at various test potentials for both the α_{1B} (*open*) and $\alpha_{1B}\beta_3$ (*filled*) Ca^{2+} currents. *D*, Relief of M_2 receptor-induced inhibition by a depolarizing prepulse to +100 mV for 75 msec. The prepulse was given 20 msec before the test pulse. Facilitation was measured as the percentage of inhibited current that was reversed by the prepulse voltage protocol.

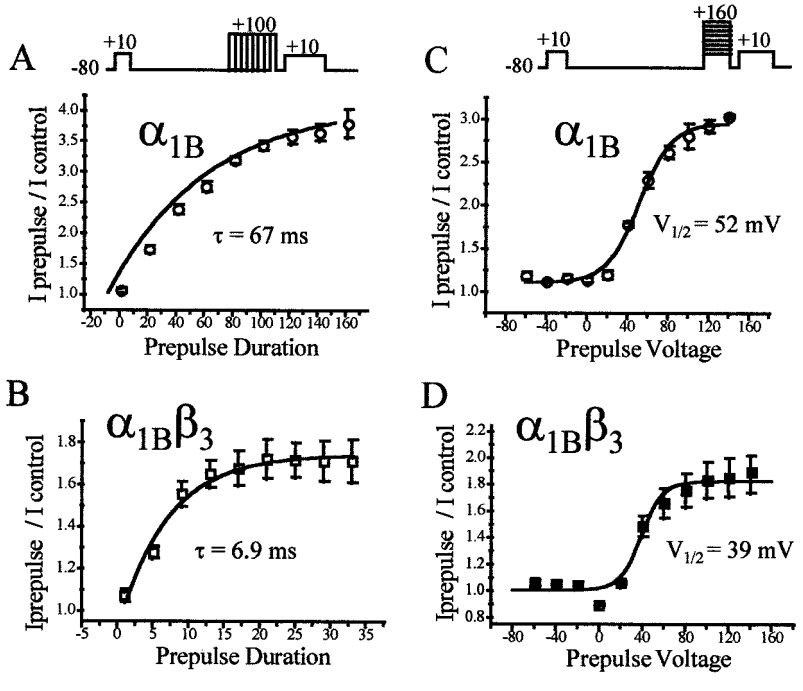

Figure 2. Modulation of time and voltage dependency of prepulse facilitation by the Ca^{2+} channel β_3 subunit. *A*, *B*, Facilitation of current amplitude after G-protein inhibition induced by application of acetylcholine. The prepulse was to +75 mV for varying periods of time, as indicated. The test potential was +10 mV. The data were fit with a single exponential, revealing time constants of 67 msec for the α_{1B} currents and 6.9 msec for the $\alpha_{1B}\beta_3$ currents. *C*, *D*, Facilitation of current amplitude with a 75 msec prepulse of varying voltage, as indicated. The test potential was +10 mV. These data were fit with a Boltzmann curve, revealing $V_{1/2}$ values of 52 mV for the α_{1B} currents and 39 mV for the $\alpha_{1B}\beta_3$ currents.

This model was tested by increasing the duration or voltage of the prepulse incrementally and determining the rate of current facilitation of α_{1B} Ca^{2+} currents both with and without Ca^{2+} channel β_3 subunit associated with the α_{1B} channel. The Ca^{2+} channel β_3 subunit dramatically decreased the duration of the prepulse necessary for maximal facilitation from ~160 msec to <20 msec; a single exponential fit to the data revealed a decrease in the time constant of relief by a voltage step to +100 mV from 67 msec in the absence of β_3 auxiliary subunit to 6.9 msec after coexpression of the Ca^{2+} channel β_3 subunit (Fig. 2*A*,*B*).

We also tested for changes in the voltage dependence of prepulse facilitation. This protocol was similar to the duration protocol used previously except, in this case, the voltage of the prepulse step was increased incrementally, while maintaining a fixed prepulse duration. The data were fitted with Boltzmann curves, revealing a $V_{1/2}$ for current facilitation of 52 mV for the

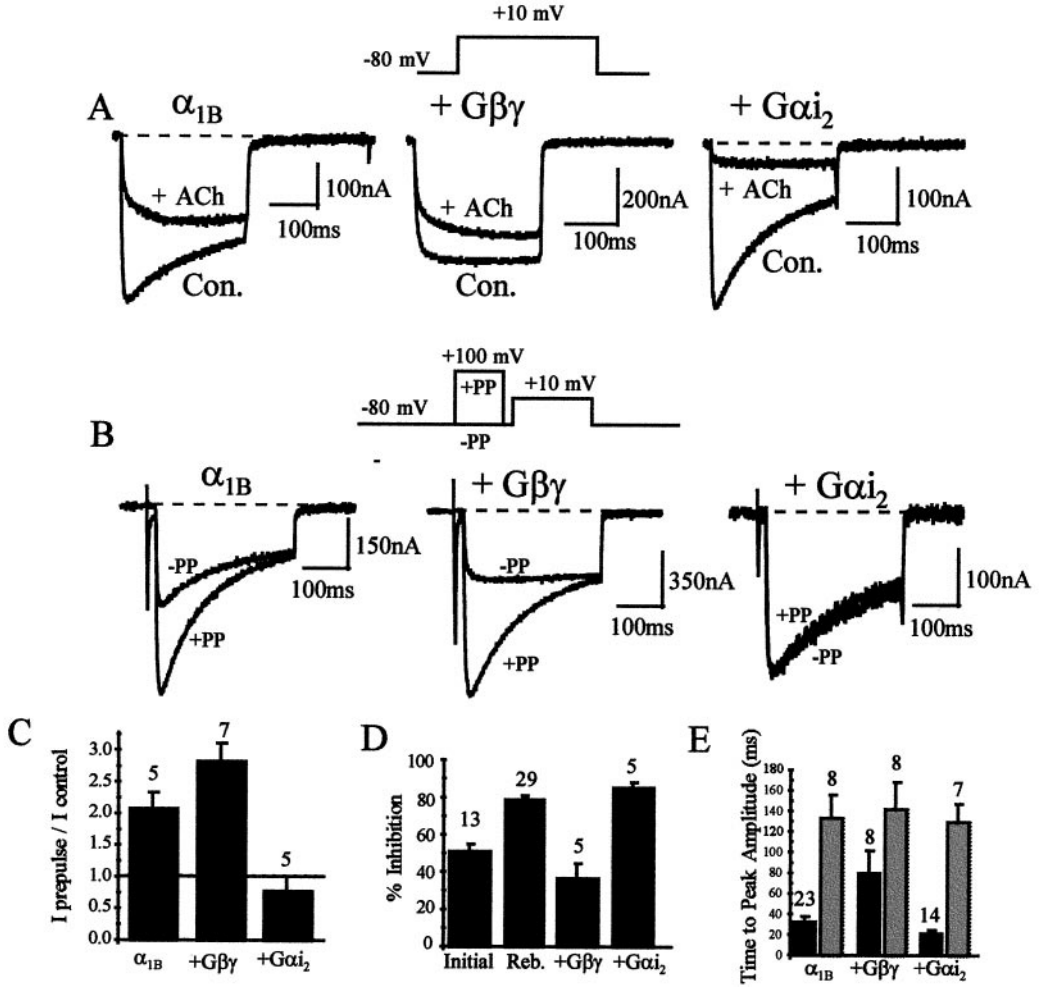

Figure 3. Effects of coexpressed G-protein α and $\beta\gamma$ subunits on α_{1B} Ca^{2+} currents. *A*, Representative currents elicited by a voltage step from a holding potential of −80 mV to a test voltage of +10 mV. Control currents are labeled *Con*, whereas the currents elicited after application of 50 μM ACh are labeled *+ACh*. *B*, Representative currents elicited using the voltage protocol illustrated in oocytes coexpressing the G-protein $\beta\gamma$ ($G_{\beta\gamma}$) or α ($G\alpha i_2$) subunits. Currents elicited without the prepulse are labeled *−PP*, whereas the currents elicited after a voltage step to +100 mV are labeled *+PP*. *C*, Summary of mean voltage-dependent facilitation before and after G-protein subunit coexpression. *D*, Summary of the inhibition of peak current amplitude by the initial application of ACh (*Initial*) and during the rebound phase induced after previous ACh application (*Reb*), as well as after coexpression of the G-protein $\beta\gamma$ ($+G\beta\gamma$) and α ($+G\alpha i_2$) subunits. *E*, Elapsed time from the beginning of the voltage step to the peak amplitude of the elicited current before (*black*) and after (*gray*) application of 50 μM acetylcholine. This was done for α_{1B} alone (α_{1B}), as well as after the coexpression of G-protein $\beta\gamma$ ($+G\beta\gamma$) or α ($+G\alpha i_2$). Sample size indicated above *bars*.

α_{1B} current and 39 mV after coexpression of the Ca^{2+} channel β_3 subunit (Fig. 2*C*,*D*). Thus, the rate of reversal of G-protein inhibition, as well as the voltage that is necessary to reverse the G-protein inhibition of the Ca^{2+} channel, has decreased after coexpression of the Ca^{2+} channel β_3 subunit.

G-protein $\beta\gamma$ subunit mediates inhibition of α_{1B} currents

There is evidence that the voltage-dependent form of G-protein inhibition is mediated by the G-protein $\beta\gamma$ subunit for N-type currents in rat SCG neurons (Herlitze et al., 1996; Ikeda, 1996). However, it is also clear that this is not the case in chick dorsal root ganglion neurons, in which the $\beta\gamma$ subunit mediates a voltage-independent form of inhibition (Diverse-Pierluissi et al., 1995). We coexpressed subunits of a heterotrimeric G-protein with α_{1B} and $\alpha_{1B}\beta_3$ Ca^{2+} channels to determine the G-protein subunit mediating voltage-dependent inhibition in our system, first examining the tonic inhibition of Ca^{2+} currents produced by exogenously expressed G-proteins. Although α_{1B} currents display a large degree of tonic G-protein-mediated inhibition from G-proteins endogenous to the oocyte, activation of a coexpressed M_2 receptor results in both a further decrease in current amplitude and a slowing of activation kinetics (Roche and Treistman, 1998), suggesting that we should be able to detect any further G-protein inhibition induced by coexpression of a G-protein subunit. We first examine the results of $G_{\beta\gamma}$ coexpression and then the influence of the G_α subunit.

Coexpression of the G-protein $\beta\gamma$ subunit slowed current activation kinetics in comparison with current in oocytes in which no exogenous $\beta\gamma$ subunits were expressed (Fig. 3*A*), similar to the slowing of activation kinetics seen after muscarinic receptor-induced inhibition. Coexpression of the G-protein $\beta\gamma$ subunit complex significantly increased the time necessary to reach peak current levels from 31.5 ± 2.2 to 70.0 ± 24.0 msec ($p \leq 0.05$, Student's *t* test) (Fig. 3*A*,*E*). Facilitation of current amplitude by depolarizing prepulses dramatically increased after coexpression of the G-protein $\beta\gamma$ subunit. Figure 3*B* shows the currents elicited

both before (*−PP*) and after (*+PP*) a depolarizing prepulse for oocytes expressing the G-protein $\beta\gamma$ subunit (*+Gβγ*). The mean facilitation of current amplitude was significantly increased from 108 ± 11 to 183 ± 10% after coexpression of the G-protein $\beta\gamma$ subunit ($p \leq 0.05$, Student's *t* test) (Fig. 3*B,C*). Slowed activation kinetics and increased prepulse facilitation are both consistent with increased voltage-dependent G-protein inhibition.

We next examined the effect of overexpression of the $\beta\gamma$ subunit on M_2-mediated inhibition. The magnitude of inhibition of current amplitude after activation of the M_2 receptor was reduced after coexpression of the G-protein $\beta\gamma$ subunit, from a value of 51 ± 3% inhibition for oocytes that were tonically inhibited but expressed no exogenous G-protein subunits (data not shown) to a value of 37 ± 8% inhibition after coexpression of the G-protein $\beta\gamma$ subunit (Fig. 3*D*). This partial occlusion of the M_2-mediated inhibition is consistent with a common pathway for M_2- and exogenous $\beta\gamma$ subunit-mediated inhibition. Further support for this conclusion is provided by examination of another measure of G-protein inhibition, the slowing of I_{Ba} activation kinetics measured as the time-to-peak current. The effects of M_2 activation and exogenous $G_{\beta\gamma}$ were nonadditive, with similar values for maximal slowing obtained by M_2 receptor activation in the absence and presence of coexpressed $G_{\beta\gamma}$ (Fig. 3*E*). These data suggest a common pathway, consistent with voltage-dependent G-protein inhibition of α_{1B} Ca^{2+} currents mediated by the G-protein $\beta\gamma$ subunit.

G-protein α subunit blocks tonic G-protein inhibition

If $G_{\beta\gamma}$ mediates the voltage-dependent inhibition of α_{1B} currents, we might expect that coexpression of the G-protein α subunit would block G-protein inhibition by acting as a "sink" for free $\beta\gamma$ subunit. Such an effect of exogenous G-protein α subunit on G-protein $\beta\gamma$ signaling has been suggested previously (Ikeda, 1996). Coexpression of G_α resulted in a significant decrease in the amount of tonic inhibition. We have shown previously that application of the alkylating agent NEM causes a potentiation of current amplitude (Roche et al., 1995), resulting from uncoupling of the basally active G-protein population. Coexpression of the G-protein α subunit should also eliminate potentiation of current amplitude by NEM, if the exogenous G-protein α subunit has blocked the tonic G-protein pathway. This is, indeed, the case. Potentiation of current amplitude by application of NEM to oocytes expressing α_{1B} currents and no exogenous G-protein subunits was 225 ± 25%, whereas the potentiation was reduced to 29 ± 9% after coexpression of the G-protein α subunit (data not shown). These data are consistent with the assumption that the G-protein α subunit acts as a sink for the tonically active $\beta\gamma$ subunit, thus blocking the inhibition mediated by the G-protein $\beta\gamma$ subunit. The G-protein α subunit did not, however, buffer M_2 receptor-induced inhibition (79 ± 1.8% inhibition for control vs 86 ± 2.4% inhibition after coexpression of $G\alpha i_2$) (Fig. 3*A,D*).

Figure 3*B* shows representative currents elicited before and after a depolarizing prepulse to +100 mV, demonstrating the loss of prepulse facilitation after coexpression of G_α. Facilitation of current amplitude was reduced from 108 ± 11% facilitation for oocytes that expressed no exogenous G-protein subunits to −23 ± 10% facilitation after coexpression of exogenous G-protein α subunit (Fig. 3*C*). This loss of prepulse current facilitation is another indicator of the loss of voltage-dependent G-protein inhibition, supporting the conclusion that $G_{\beta\gamma}$ mediates voltage-dependent inhibition of α_{1B} Ca^{2+} current.

Influence of Ca^{2+} channel β_3 subunit on G-protein $\beta\gamma$ subunit-mediated inhibition

The Ca^{2+} channel β subunit has been shown to significantly modify G-protein modulation of Ca^{2+} channels, and we examined its influence on $G_{\beta\gamma}$-induced inhibition. The expression of exogenous G-protein $\beta\gamma$ subunit was also effective in mediating voltage-dependent inhibition of $\alpha_{1B}\beta_3$ Ca^{2+} currents. Similar to our results for the α_{1B} currents, the activation kinetics of the $\alpha_{1B}\beta_3$ currents was significantly slowed by coexpression of the G-protein $\beta\gamma$ subunit. Figure 4*A* shows representative currents in the presence of exogenous G-protein subunits, demonstrating the slowed activation kinetics of the $\alpha_{1B}\beta_3$ currents after coexpression of the G-protein $\beta\gamma$ subunit. In addition, the G-protein $\beta\gamma$ subunit also occludes the M_2 receptor-mediated inhibition (Fig. 4*A,C*), again suggesting that $\beta\gamma$-induced inhibition is acting via the same mechanism as M_2-induced inhibition.

There was very little facilitation of $\alpha_{1B}\beta_3$ current amplitude by depolarizing prepulses in the absence of exogenous G-protein subunits (Fig. 4*D*). However, after we coexpressed the G-protein $\beta\gamma$ subunit, the facilitation of current amplitude by depolarizing prepulses was significantly increased. Figure 4*B* shows representative $\alpha_{1B}\beta_3$ currents, elicited before and after a depolarizing prepulse to +100 mV, in the absence and presence of coexpressed $G_{\beta\gamma}$. Prepulse facilitation using this protocol increased from 4 ± 4% when no exogenous G-protein subunits were expressed to 57 ± 8% after coexpression of the G-protein $\beta\gamma$ subunit (Fig. 4*D*), indicative of a substantial increase in the amount of voltage-dependent inhibition.

Influence of Ca^{2+} channel β_3 subunit on the ability of G-protein α subunit to block tonic G-protein inhibition

As with the α_{1B} current, the G-protein α subunit caused a small but significant increase in the magnitude of M_2-mediated inhibition of $\alpha_{1B}\beta_3$ current (Fig. 4*C*), from 55 ± 2.3% inhibition in oocytes that expressed no exogenous G-proteins to 68 ± 2% inhibition in oocytes that expressed exogenous G_α subunit. Although the G-protein α subunit did not reduce the magnitude of M_2-induced inhibition, the G-protein α subunit did block a small tonic inhibition, evidenced by a decrease in the small amount of facilitation that was seen in the control (Fig. 4*D*).

The Ca^{2+} channel β_3 subunit modulates the voltage sensitivity of G-protein $\beta\gamma$ subunit-induced inhibition

A model for membrane-delimited voltage-dependent inhibition in which the G-protein $\beta\gamma$ subunit binds directly to the α_{1B} Ca^{2+} channel has recently received experimental support (De Waard et al., 1997; Zamponi et al., 1997). Modulation of the inhibition mediated by exogenous $G_{\beta\gamma}$ by coexpression of the Ca^{2+} channel β_3 subunit, therefore, would likely result from changes in the effectiveness of the interaction of $G_{\beta\gamma}$ with the Ca^{2+} channel. We examined the influence of the Ca^{2+} channel β_3 subunit on the rate of voltage-dependent relief of G-protein $\beta\gamma$ subunit-mediated inhibition. Figure 5 demonstrates that the Ca^{2+} channel β_3 subunit also dramatically increases the rate of relief of the inhibition produced by the coexpressed $G_{\beta\gamma}$ subunit. A single exponential fit to the data revealed a shift in the rate at which the G-protein $\beta\gamma$-induced inhibition is reversed by depolarizing prepulses, from a time constant of 58 msec for α_{1B} alone to 6 msec after coexpression of the Ca^{2+} channel β_3 subunit (Fig. 5*A,B*). This was similar to the increase in the rate of current facilitation produced by the Ca^{2+} channel β_3 subunit for M_2 receptor-induced inhibition of α_{1B} and $\alpha_{1B}\beta_3$ currents (67 and 7 msec, respectively).

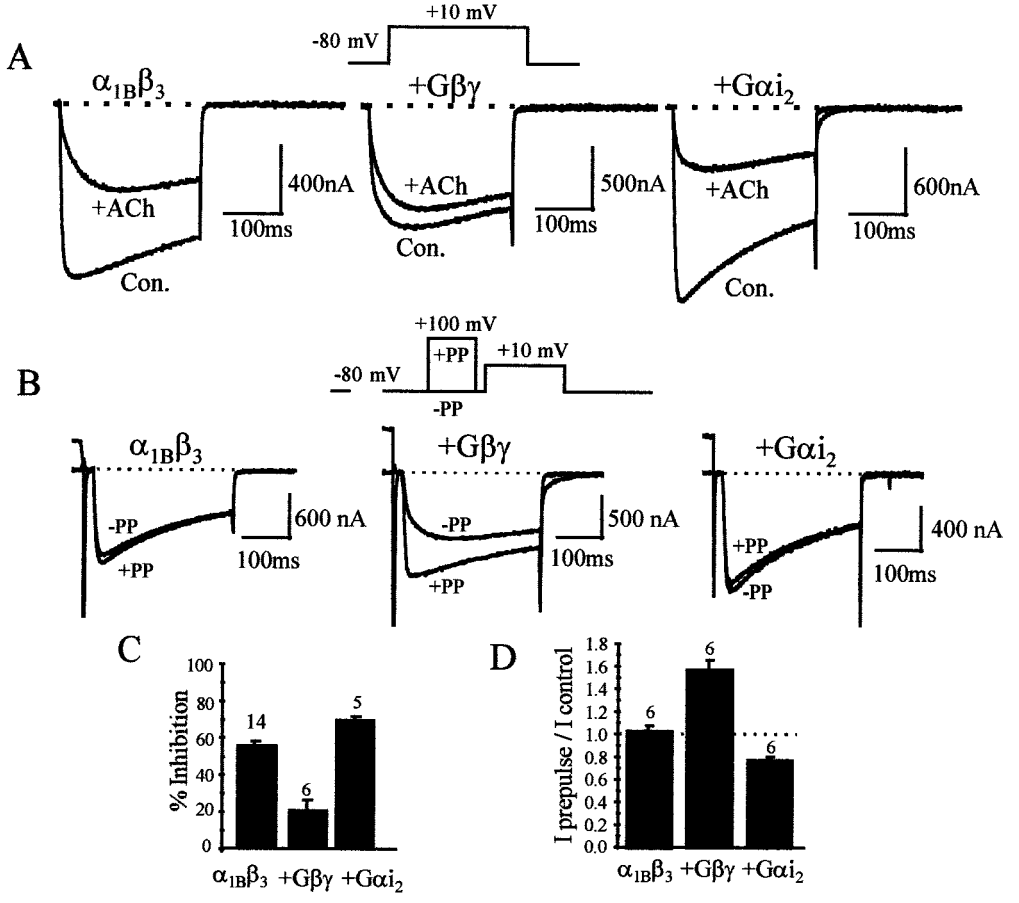

Figure 4. Effects of G-protein α and $\beta\gamma$ subunit coexpression on $\alpha_{1B}\beta_3$ Ca^{2+} currents. *A*, Currents elicited by a voltage step from a holding potential of −80 mV to a test voltage of +10 mV. Control currents are labeled *Con*, whereas the currents elicited after application of 50 μM ACh are labeled *+ACh*. *B*, Representative currents elicited both before (*−PP*) and after (*+PP*) a depolarizing prepulse to +100 mV with and without coexpression of G-protein $\beta\gamma$ (*+Gβγ*) and α (*+Gαi$_2$*) subunits. *C*, Summary of the inhibition of peak current amplitude by the initial application of ACh for $\alpha_{1B}\beta_3$ alone and after coexpression of the G-protein α (*+Gαi$_2$*) and $\beta\gamma$ (*+Gβγ*) subunits. *D*, Summary of prepulse facilitation of current amplitude before and after coexpression of G-protein subunits. Sample size indicated above *bars*.

Figure 5, *C* and *D*, shows also shows the voltage dependence of the relief of G-protein $\beta\gamma$ subunit-induced current inhibition. A Boltzmann fit of the data revealed an ~10 mV leftward shift in voltage sensitivity, similar to the shift in voltage-dependent relief of M_2 receptor-induced inhibition. Thus, the Ca^{2+} channel β subunit increases the rate and decreases the voltage necessary for facilitation of $G_{\beta\gamma}$-inhibited Ca^{2+} currents.

DISCUSSION

Our data demonstrate that the rate of reversal of M_2-mediated inhibition by depolarizing prepulses dramatically increases and the voltage necessary for reversal decreases after coexpression of the Ca^{2+} channel β_3 subunit. We have also confirmed that the G-protein $\beta\gamma$ subunit mediates the inhibition of N-type currents and have extended this observation to include both α_{1B} and $\alpha_{1B}\beta_3$ Ca^{2+} currents. In addition, we have demonstrated that the Ca^{2+} channel β_3 subunit increases the rate and decreases the voltage necessary for voltage-dependent reversal of $G_{\beta\gamma}$-induced inhibition. This results in voltage-dependent relief of inhibition at the moderate voltages used to activate the channel during voltage-clamp experiments and likely explains the reduction in the magnitude of G-protein inhibition of α_{1B} current after coexpression of the Ca^{2+} channel β_3 subunit. Although the leftward shift in the voltage dependence of facilitation is most likely the result of more rapid unbinding of the G-protein in the presence of the Ca^{2+} channel β_3 subunit, caution should be used when interpreting this shift, because the Ca^{2+} channel β_3 subunit also causes a 10 mV leftward shift of peak current in the *I–V* relation (Roche and Treistman, 1998). Because reversal of G-protein inhibition is thought to result from a conformational change in the channel, produced by voltage, the apparent steeper voltage dependence of activation produced by the Ca^{2+} channel β_3 subunit may contribute to the leftward shift in voltage dependence of facilitation.

Recent findings suggest that some characteristics of voltage-dependent inhibition are a result of the G-protein $\beta\gamma$ subunit binding to its consensus site next to the Ca^{2+} channel β subunit binding site (De Waard et al., 1997). The critical amino acids responsible for binding of the Ca^{2+} channel β subunit are not critical for G-protein $\beta\gamma$ subunit binding and vice versa. The close proximity of the two sites, however, make modification of the G-protein $\beta\gamma$ binding site by the bound Ca^{2+} channel β_3 subunit a likely possibility. However, it should be noted that some groups have reported that the $G_{\beta\gamma}$ consensus binding sequence on the I–II loop of the Ca^{2+} channel is not responsible for mediating the effects of G-proteins (Zhang et al., 1996; Qin et al., 1997). Adjacent proximity of the $G_{\beta\gamma}$ and calcium channel β_3 binding sites within the protein is not a requirement for the model we are proposing. The most reasonable interpretation, combining the information from mutagenesis studies and the results presented here, is that the bound β_3 subunit enhances the $G_{\beta\gamma}$ dissociation rate and thus reduces the magnitude of G-protein inhibition of α_{1B} Ca^{2+} channels.

It is interesting that coexpression of the G-protein α subunit eliminates tonic G-protein inhibition but not M_2-mediated inhibition of α_{1B} Ca^{2+} channel current. The differential effect of G_α might be explained by a variety of mechanisms. One possibility is that the endogenous free $\beta\gamma$ subunit, a portion of which is responsible for tonic inhibition, exists at levels that saturate the exogenous free α subunit, so that the expressed G_α subunit cannot

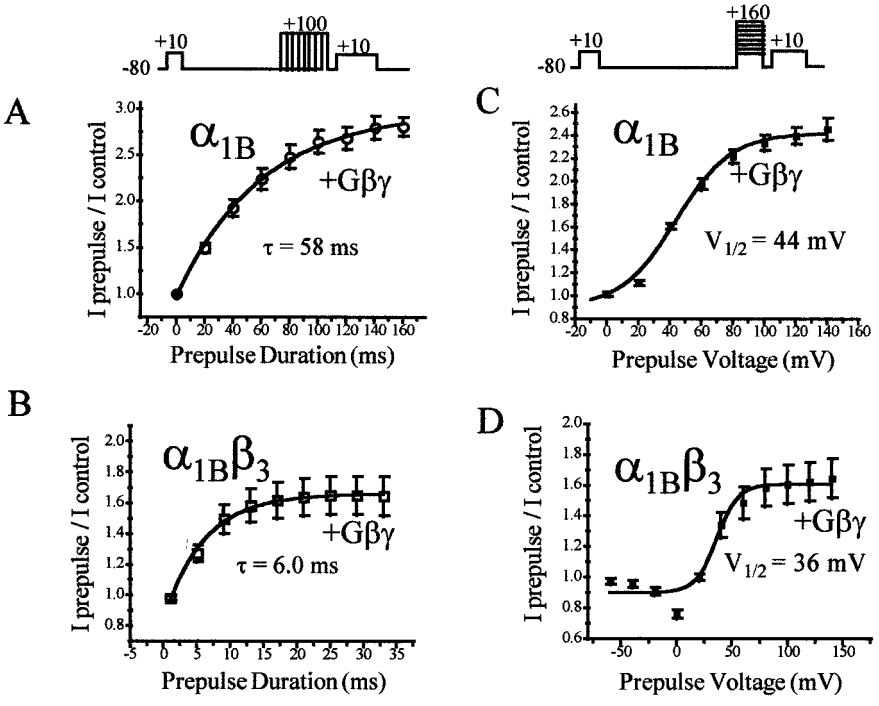

Figure 5. Effects of G-protein $\beta\gamma$ subunit coexpression on rate and voltage dependence of prepulse facilitation. *A*, *B*, Exponential fit of prepulse facilitation by 100 mV prepulse of varying duration in the presence of the G-protein $\beta\gamma$ subunit coexpressed with α_{1B} or $\alpha_{1B}\beta_3$. *C*, *D*, Boltzmann fit of facilitation of α_{1B} current amplitude by a 75 msec prepulse to varying voltages in the presence of the G-protein $\beta\gamma$ subunit coexpressed with α_{1B} or $\alpha_{1B}\beta_3$.

buffer the additional $\beta\gamma$ subunit liberated by activation of the muscarinic receptor. In support of this mechanism, we find that M_2-mediated inhibition is only partially blocked by NEM, an agent that uncouples the G-protein α subunit from receptor activation (Jakobs et al., 1982; Nakajima et al., 1990), when no exogenous G-protein subunits are present. However, after coexpression of the NEM-sensitive Gαi (Shapiro et al., 1994b), the M_2-mediated inhibition is almost entirely blocked by NEM (data not shown). This result is predicted by a model in which exogenous G_α subunits form inactive heterotrimers with the tonically active endogenous $G_{\beta\gamma}$ subunits. These newly formed heterotrimers are then activated after binding of an agonist to the M_2 receptor, liberating the $G_{\beta\gamma}$ subunit, and overwhelming the buffering capacity of the coexpressed G_α subunits.

Regulation of responsiveness to G-proteins at the level of the ultimate target may be a widely used mechanism, enabling a channel or other protein to regulate its sensitivity to modulation while maintaining its basal properties. This mechanism may be necessary in situations in which a modulatory signal is greatly amplified or when the signal has a large number of ultimate targets. In such situations, downregulation of the receptor itself may have unwanted consequences or may be ineffective because of amplification of the signal.

Functional Ca^{2+} channels may exist in the absence of a component auxiliary β subunit (De Waard and Campbell, 1995). Additionally, a recent report (Qin et al., 1997) suggests that a second calcium channel β subunit binding site is located on the C terminal of α_{1A}, α_{1B}, and α_{1E} calcium channels and that this site is responsible for the antagonism of G-protein inhibition of these channels by the calcium channel β subunit. This site is distinct from that believed to be responsible for high expression and insertion of channels. Thus, it is possible that differential occupancy of this second site by the channel β subunit could serve a regulatory function, consistent with our observations with cloned channels. The increased voltage sensitivity of the inhibition observed after coexpression of the Ca^{2+} channel β_3 subunit may play an important role in the regulation of transmitter release in response to high-frequency or long-duration action potentials (Brody et al., 1997), in which depolarization of the presynaptic terminal would reach levels sufficient to relieve G-protein inhibition of Ca^{2+} channels controlling release.

REFERENCES

Anwyl R (1991) Modulation of vertebrate neuronal calcium channels by transmitters. Brain Res Rev 16:265–281.

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153–156.

Beech DJ, Bernheim L, Mathie A, Hille B (1991) Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. Proc Natl Acad Sci USA 88:652–656.

Beech DJ, Bernheim L, Hille B (1992) Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. Neuron 8:97–106.

Bernheim L, Beech DJ, Hille B (1991) A diffusible second messenger mediates one of the pathways coupling receptors to Ca^{2+} channels in rat sympathetic neurons. Neuron 6:859–867.

Bourinet E, Soong TW, Stea A, Snutch TP (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. Proc Natl Acad Sci USA 93:1486–1491.

Brody DL, Patil PG, Mulle JG, Snutch TP, Yue DT (1997) Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca^{2+} channels in HEK 293 cells. J Physiol (Lond) 499:637–644.

Brown AM, Birnbaumer L (1990) Ionic channels and their regulation by G protein subunits. Annu Rev Physiol 52:197–213.

Campbell V, Berrow NS, Fitzgerald EM, Brickley K, Dolphin AC (1995) Inhibition of the interaction of G protein Go with calcium channels by the calcium channel β subunit in rat neurones. J Physiol (Lond) 485:365–372.

Clapham DE (1996) Intracellular signalling: more jobs for Gβ-gamma. Curr Biol 6:814–816.

De Waard M, Campbell KP (1995) Subunit regulation of the neuronal α_{1A} Ca^{2+} channel expressed in *Xenopus* oocytes. J Physiol (Lond) 485:619–634.

De Waard M, Lui H, Walker D, Scott VES, Gurnett CA, Campbell KP (1997) Direct binding of the G protein β-gamma complex to voltage-dependent calcium channels. Nature 385:446–450.

Dittman JS, Regehr WG (1996) Contributions of calcium-dependent

and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. J Neurosci 16:1623–1633.

Diverse-Pierluissi M, Goldsmith PK, Dunlap K (1995) Transmitter-mediated inhibition of N-type calcium channels in sensory neurons involves multiple GTP-binding proteins and subunits. Neuron 14:191–200.

Dolphin AC (1991) Regulation of calcium channel activity by GTP binding proteins and second messengers. Biochim Biophys Acta 1091:68–90.

Elmslie KS, Zhou W, Jones SW (1990) LHRH and GTP-γ-S modify calcium current activation in bullfrog sympathetic neurons. Neuron 5:75–80.

Golard A, Siegelbaum SA (1993) Kinetic basis for the voltage-dependent inhibition of N-type calcium current by somatostatin and norepinephrine in chick sympathetic neurons. J Neurosci 13:3884–3894.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} channels by G-protein β-gamma subunits. Nature 380:258–261.

Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:530–536.

Holz GG, Rane SG, Dunlap K (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. Nature 319:670–672.

Ikeda SR (1991) Double-pulse calcium channel current facilitation in adult rat sympathetic neurons. J Physiol (Lond) 439:181–214.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein β-gamma subunits. Nature 380:255–258.

Jakobs KH, Lasch P, Minuth M, Aktories K, Schultz G (1982) Uncoupling of α-adrenoceptor-mediated inhibition of human platelet adenylate cyclase by *N*-ethylmaleimide. J Biol Chem 257:2829–2833.

Kasai H, Aosaki T (1989) Modulation of Ca-channel current by an adenosine analog mediated by a GTP-binding protein in chick sensory neurons. Pflügers Arch 414:145–149.

Lechleiter J, Girard S, Clapham D, Peralta E (1991) Subcellular patterns of calcium release determined by G protein-specific residues of muscarinic receptors. Nature 350:505–508.

Lopez HS, Brown AM (1991) Correlation between G protein activation and reblocking kinetics of Ca^{2+} channel currents in rat sensory neurons. Neuron 7:1061–1068.

Marchetti C, Carbone E, Lux HD (1986) Effects of dopamine and noradrenaline on Ca^{2+} channels of cultured sensory and sympathetic neurons of chick. Pflügers Arch 406:104–111.

McAllister-Williams RH, Kelly JS (1995) The modulation of calcium channel currents recorded from adult rat dorsal raphe neurones by 5-HT_{1A} receptor or direct G protein activation. Neuropharmacology 34:1491–1506.

Nakajima T, Irisawa H, Giles W (1990) *N*-Ethylmaleimide uncouples muscarinic receptors from acetylcholine-sensitive potassium channels in bullfrog atrium. J Gen Physiol 96:887–903.

Pragnell M, DeWaard M, Mori Y, Tanabe T, Snutch TP, Campbell KP (1994) Calcium channel β-subunit binds to a conserved motif in the I–II cytoplasmic linker of the α_1-subunit. Nature 368:67–70.

Qin N, Platano D, Olcese R, Stefani E, Birnbaumer L (1997) Direct interaction of Gβγ with a C-terminal Gβγ-binding domain of the Ca^{2+} channel α_1 subunit is responsible for channel inhibition by G protein-coupled receptors. Proc Natl Acad Sci USA 94:8866–8871.

Roche JP, Treistman SN (1998) The Ca^{2+} channel β_3 subunit differentially modulates G protein sensitivity of α_{1A} and α_{1B} Ca^{2+} channels. J Neurosci 18:878–866.

Roche JP, Anantharam V, Treistman SN (1995) Abolition of G protein inhibition of α_{1A} and α_{1B} calcium channels by co-expression of the β_3 subunit. FEBS Lett 371:43–46.

Schultz G, Rosenthal W, Hescheler J, Trautwein W (1990) Role of G proteins in calcium channel modulation. Annu Rev Physiol 52:275–292.

Shapiro MS, Hille B (1993) Substance P and somatostatin inhibit calcium channels in rat sympathetic neurons via different G protein pathways. Neuron 10:11–20.

Shapiro MS, Wollmuth LP, Hille B (1994a) Angiotensin II inhibits calcium and M current channels in rat sympathetic neurons via G proteins. Neuron 12:1319–1329.

Shapiro MS, Wollmuth LP, Hille B (1994b) Modulation of Ca^{2+} channels by PTX-sensitive G-proteins is blocked by *N*-ethylmaleimide in rat sympathetic neurons. J Neurosci 14:7109–7116.

Takahashi T, Forsythe ID, Tsujimoto T, Barnes-Davies M, Onodera K (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. Science 274:594–597.

Wanke E, Ferroni A, Malgaroli A, Ambrosini A, Pozzan T, Meldolesi J (1987) Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. Proc Natl Acad Sci USA 84:4313–4317.

Wheeler DB, Randall A, Tsien RW (1994) Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. Science 264:107–111.

Wollmuth LP, Shapiro MS, Hille B (1995) Pancreatic polypeptide inhibits calcium channels in rat sympathetic neurons via two signaling pathways. J Neurophysiol 73:1323–1328.

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. Nature 385:442–446.

Zhang J-F, Ellinor PT, Aldrich RW, Tsien RW (1996) Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. Neuron 17:991–1003.