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Decay of prepulse facilitation of N type calcium channels during G protein inhibition is consistent with binding of a single $G_{\beta\gamma}$ subunit

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ABSTRACT **We have examined the modulation of cloned and stably expressed rat brain N type calcium channels (α_{1B} + β_{1b} + $\alpha_2\delta$ subunits) by exogenously applied purified G protein $\beta\gamma$ subunits. In the absence of $G_{\beta\gamma}$, barium currents through N type channels are unaffected by application of strong depolarizing prepulses. In contrast, inclusion of purified $G_{\beta\gamma}$ in the patch pipette results in N type currents that initially facilitated upon application of positive prepulses followed by rapid reinhibition. Examination of the kinetics of $G_{\beta\gamma}$-dependent reinhibition showed that as the duration between the test pulse and the prepulse was increased, the degree of facilitation was attenuated in a monoexponential fashion. The time constant τ for the recovery from facilitation was sensitive to exogenous $G_{\beta\gamma}$, so that the inverse of τ linearly depended on the $G_{\beta\gamma}$ concentration. Overall, the data are consistent with a model whereby a single $G_{\beta\gamma}$ molecule dissociates from the channel during the prepulse, and that reassociation of $G_{\beta\gamma}$ with the channel after the prepulse occurs as a bimolecular reaction.**

Calcium influx into neurons through voltage-dependent calcium channels mediates a range of intracellular responses including modulation of calcium-dependent enzymes, activation of gene transcription, and mediation of neuronal excitability and synaptic transmission (1, 2). Most neurons express multiple calcium channel types with distinct physiological and pharmacological properties (T, L, N, and P/Q types), and molecular cloning has identified genes encoding five neuronal calcium channel α_1 subunits (termed α_{1A} through α_{1E}; for review, see ref. 3). Transient expression in *Xenopus* oocytes and human embryonic kidney 293 (HEK 293) cells shows that α_{1A} encodes P/Q type calcium channels (4–6), α_{1B} encodes an ω conotoxin GVIA-sensitive N type channel (7–9), α_{1C} and α_{1D} encode L type calcium channels (10, 11), and α_{1E} is a unique channel that shares properties with both high-threshold and low-threshold calcium channels (12–14). The five types of α_1 subunits have differential subcellular localizations, with α_{1C}, α_{1D}, and α_{1E} predominantly located on cell bodies and proximal dendrites and α_{1A} and α_{1B} generally localized to more distal dendritic sites and presynaptic nerve terminals, as well as some cell bodies (15–18).

Native N type and P/Q type calcium currents are inhibited on activation of many types of seven-helix receptors via pertussis toxin-sensitive G protein pathways (G_i and G_o; for reviews see refs. 19 and 20). G protein-dependent inhibition of native currents is strongly voltage-dependent. The inhibitory effect can be partially reversed by application of depolarizing prepulses resulting in an apparent facilitation of inhibited currents (21–23). Similarly, the G protein-dependent inhibition of transiently expressed α_{1A} (P/Q type) and α_{1B} (N type) whole-cell currents resembles that for native channels because both are down-regulated by activation of μ opioid and somatostatin receptors and by application of GTP analogues and also exhibit pronounced prepulse-dependent facilitation (24, 25). Recent studies suggest that G protein inhibition results from the activation of $G_{\beta\gamma}$ (26, 27) and occurs through the direct binding of $G_{\beta\gamma}$ to the calcium channel α_1 subunit domain I–II linker (25, 28–30).

It has been proposed that up to four G protein molecules act simultaneously at the calcium channel complex to promote inhibition (for example, see refs. 31 and 32; for review, see ref. 19). According to these models, prepulse facilitation occurs via dissociation of the G proteins from the channel and recovery from facilitation involves rebinding of the G proteins. It has alternatively been proposed that strong depolarizing prepulses result in a temporary conformational change in the channel protein that decays over time (33). In the former scenario, the time course of recovery from facilitation would be expected to depend directly on the G protein concentration, whereas in the latter scenario, recovery from facilitation would be independent of the G protein concentration. In the original studies, endogenous G protein pathways were stimulated with receptor agonists and GTP analogues. Consequently, it was not possible to precisely control intracellular G protein concentrations. In the present study, we have examined the mechanism of G protein-dependent modulation of stably expressed α_{1B} N type currents by varying the intrapipette concentration of exogenous $G_{\beta\gamma}$. The results show that the time course of recovery from facilitation is strongly dependent on the $G_{\beta\gamma}$ concentration. Furthermore, the inverse of the time constant for recovery from facilitation is linearly dependent on $G_{\beta\gamma}$ concentration. Thus, our results are consistent with the notion that prepulse facilitation involves the complete dissociation of a single $G_{\beta\gamma}$ molecule from the channel and that rebinding occurs via a bimolecular interaction between the channel and a single $G_{\beta\gamma}$.

EXPERIMENTAL PROCEDURES

HEK 293 cells stably expressing α_{1B}, β_{1b}, and $\alpha_2\delta$ (α_{1B} HEK cells; see ref. 25) were grown in standard DMEM, supplemented with 10% fetal bovine serum and neomycin (0.4 mg/ml). Immediately before recording, the medium was removed and replaced with external recording solution (see below). Whole-cell patch clamp recordings were performed by using an Axopatch model 200A amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with PCLAMP version 6.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15) were pulled with a Sutter P-87 microelectrode puller, fire-polished with a Narashige microforge, and showed

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typical resistances of 2–4 MΩ. The external recording solution was 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 40 mM tetraethylammonium hydrochloride, 10 mM glucose, and 65 mM CsCl (pH 7.2). The internal pipette solution was 105 mM CsCl, 25 mM tetraethylammonium hydrochloride, 1 mM $CaCl_2$, 11 mM EGTA, and 10 mM Hepes (pH 7.2). Purified bovine $G_{\beta\gamma}$ (a gift from David Clapham and Grigory Krapivinsky) was stored at −80°C at a stock concentration of 40 mM in 10 mM Hepes, pH 8.0/200 mM NaCl/1 mM DTT/0.5% sodium cholate. The stock was diluted with a solution containing 0.1% CHAPS to a concentration of 1 mM and then directly added to the internal pipette solution to give final $G_{\beta\gamma}$ concentrations of between 1 and 20 nM. Currents were typically elicited from a holding potential of −100 mV to a test potential of +5 mV by using Clampex (Axon Instruments). Prepulses were elicited by stepping from −100 mV to a prepulse potential of +150 mV for 50 ms. In most cells, prepulse (PP) effects were studied cyclically (−PP, +PP, −PP) at any given interpulse duration to eliminate the possibility of contamination of the data due to current rundown. The interpulse duration was varied from a minimum of 2 ms to as high as 300 ms in some instances. Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Data were analyzed by using CLAMPFIT (Axon Instruments) and all curve fitting was carried out in SIGMAPLOT (Jandel, San Rafael, CA).

RESULTS

Fig. 1 depicts whole cell barium currents recorded from α_{1B} HEK cells with 5 nM $G_{\beta\gamma}$ in the patch pipette in the presence and absence of a strong depolarizing prepulse (+150 mV, 50 ms). With a short interpulse interval (Δt = 6 ms; Fig. 1*A*) N type currents increased approximately 35% after the prepulse. In addition, the activation rate is substantially accelerated after the prepulse and is consistent with the relief of G protein-induced inhibition (19–23). When the interpulse duration Δt is increased to 40 ms, the degree of prepulse facilitation is substantially decreased (Fig. 1*B*). This result is consistent with the notion that reinhibition of the calcium currents occurs after the prepulse. Also note that the prepulse-induced speeding of activation after the prepulse is attenuated compared with Fig. 1*A*. In the absence of $G_{\beta\gamma}$, no prepulse facilitation was detected (n = 30).

Fig. 2 shows that the recovery from prepulse facilitation depends on the interpulse duration for a single experiment at a $G_{\beta\gamma}$ concentration of 10 nM. As the interpulse duration was increased, the degree of prepulse facilitation was progressively reduced from about 60% enhancement of α_{1B} currents 4 ms after the prepulse to only a 10% increase 20 ms after the prepulse. The dependence of the prepulse effect on the interpulse duration was well described with a single exponential with a time constant, τ, of 11.7 ms (also see *Discussion* and ref. 34).

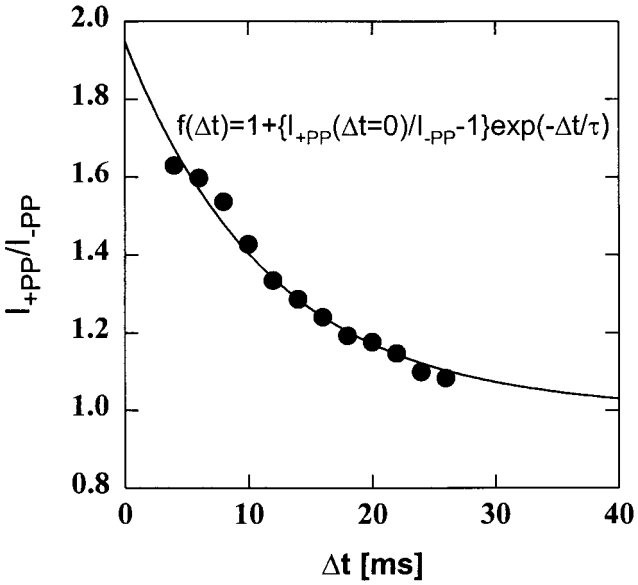

FIG. 2. Time course of recovery from prepulse facilitation for a single cell with an intrapipette $G_{\beta\gamma}$ concentration of 10 nM. The solid line is a monoexponential fit according to the equation $I_{+PP}/I_{-PP} = 1 + I_{max}[\exp(-\Delta t/\tau)]$, where I_{+PP} and I_{-PP} are, respectively, the peak currents in the presence and absence of a prepulse, $1 + I_{max}$ is the maximum ratio of current facilitation, Δt is the interpulse duration, and τ is the time constant for the decay of the prepulse effect. The value for τ obtained from the fit was 11.7 ms.

To determine whether the time constant for reblock was dependent on the $G_{\beta\gamma}$ concentration, we repeated the experiment shown in Fig. 2 for a range of different $G_{\beta\gamma}$ concentrations. Fig. 3 depicts the time course of recovery from facilitation for two $G_{\beta\gamma}$ concentrations (2 nM and 10 nM). Because the maximum degree of facilitation appeared to exhibit some cell to cell variability, the data were scaled so that all of the individual data sets overlap at the same arbitrary value at an interpulse duration of 4 ms. As observed in Fig. 3, the time course of recovery from facilitation is substantially slowed when the $G_{\beta\gamma}$ concentration is decreased from 10 nM to 2 nM.

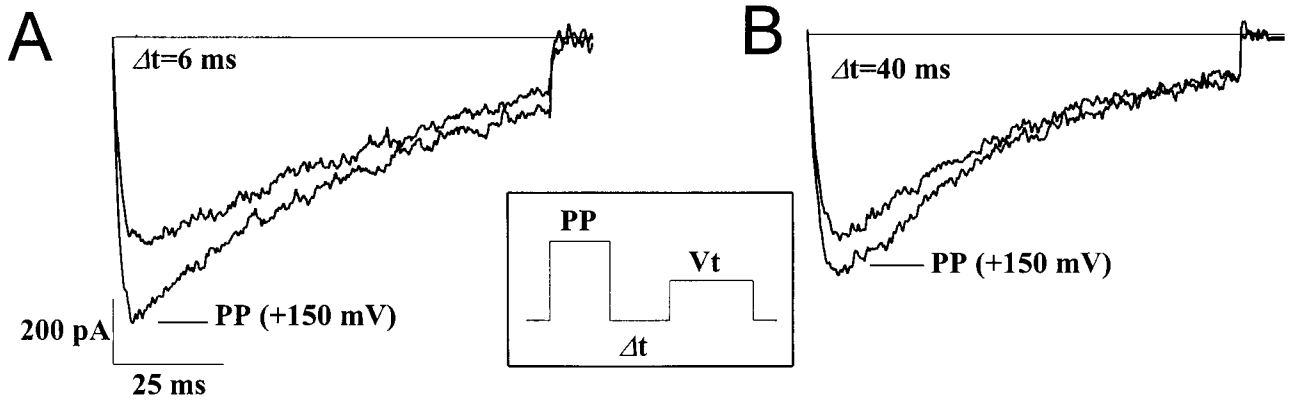

FIG. 1. G protein-dependent modulation of α_{1B} N type whole cell currents stably expressed in HEK 293 cells (coexpressed with β_{1b} + $\alpha_2\delta$ subunits). Inclusion of 5 nM purified $G_{\beta\gamma}$ in the patch pipette in the presence and the absence of a strong depolarizing prepulse (+150 mV for 50 ms). The cell was bathed in 20 mM barium, currents were elicited by stepping from a holding potential of −100 mV to a test potential of +5 mV with or without application of a strong depolarizing prepulse (+150 mV for 50 ms, see *Inset*). The records were leak- and capacitance-subtracted by using a standard p/5 protocol.

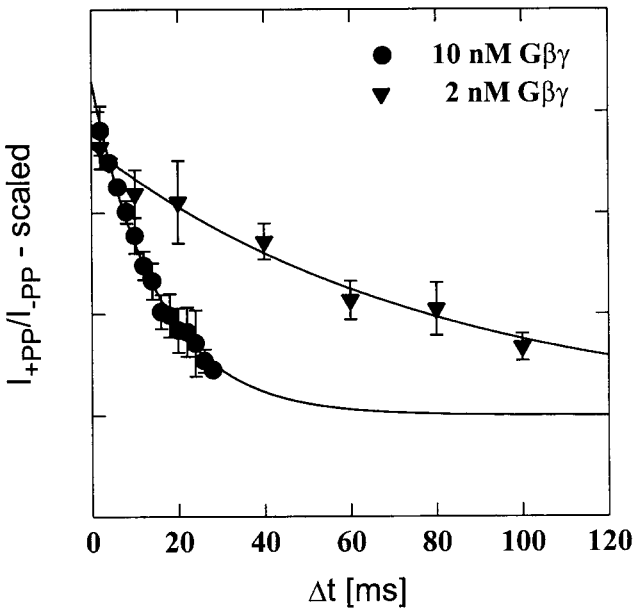

FIG. 3. Time course of recovery from facilitation for $G_{\beta\gamma}$ concentrations of 2 nM (triangles, $n = 5$) and 10 nM (circles, $n = 6$). To facilitate comparison, the data sets for each experiment were scaled to the same arbitrary value at an interpulse interval of 4 ms; hence, no I_{+PP}/I_{-PP} values are provided on the ordinate. Error bars indicate the SEM and the solid lines are fits as outlined in Fig. 2. Note that the time course of recovery from facilitation is considerably slowed when the $G_{\beta\gamma}$ concentration is decreased from 10 nM ($\tau = 15.1$ ms) to 2 nM ($\tau = 79.6$ ms).

The time constants from the ensemble fits were, respectively, 79.6 ms and 15.1 ms in the presence of 2 nM and 10 nM $G_{\beta\gamma}$. These data indicate that the rate of G protein reinhibition of α_{1B} currents after prepulse relief is dependent on the $G_{\beta\gamma}$ concentration and implies that there must be complete dissociation of at least one G protein from the channel.

To address the stoichiometry of the $G_{\beta\gamma}$–calcium channel complex interaction, we examined the time course of recovery from facilitation for five $G_{\beta\gamma}$ concentrations. For a bimolecular interaction between $G_{\beta\gamma}$ and the channel complex, the reciprocal of the time constant for G protein reinhibition, $1/\tau$, equals $k_{on}[G_{\beta\gamma}] + k_{off}$, where k_{on} and k_{off} are, respectively, the $G_{\beta\gamma}$ association and dissociation rate constants for binding and $[G_{\beta\gamma}]$ is the $G_{\beta\gamma}$ concentration. A plot of the inverse of τ as a function of $G_{\beta\gamma}$ concentration would predict a linear relation if binding of a single $G_{\beta\gamma}$ subunit were to mediate reinhibition. In Fig. 4, the data obtained for each cell were individually fitted for τ as described in Fig. 2. The results show that the relation between $1/\tau$ and $[G_{\beta\gamma}]$ is linear over a concentration range from 1 to 20 nM, consistent with a 1:1 interaction between the $G_{\beta\gamma}$ molecule and the channel complex. In this scenario, the slope of the relation would reflect the association rate constant for G protein binding and the intercept at the ordinate would reflect the rate constant for G protein dissociation. As seen from the regression, the G protein dissociation rate constant is essentially zero, which is consistent with modeling data (34) and suggests that at an interpulse voltage of -100 mV $G_{\beta\gamma}$ subunits remain tightly associated with the channel complex. The association rate constant for $G_{\beta\gamma}$ binding to the channel obtained from the linear regression was 0.0075 $ms^{-1}\cdot nM^{-1}$. Overall, the linearity of the data shown in Fig. 4 suggests that G protein inhibition of N type calcium channels during recovery from prepulse facilitation occurs via binding of a single $G_{\beta\gamma}$ molecule to the channel. Furthermore, strong depolarizing prepulses likely result in physical dissociation of at least one $G_{\beta\gamma}$ molecule from the calcium channel.

DISCUSSION

Initial observations indicating that the neurotransmitter-induced inhibition of neuronal calcium currents is rapid, membrane delimited, and strongly voltage-dependent lead to the notion that heterotrimeric G proteins directly interact with the channel complex (19–23, 31–45). In support of this hypothesis, recent biochemical studies have demonstrated the direct binding of $G_{\beta\gamma}$ subunits to the calcium channel α_1 subunit (25, 28). One interesting aspect of direct G protein-dependent inhibition is that it can be temporarily relieved by application of a strong depolarizing prepulse resulting in an apparent facilitation of inhibited currents (21–23, 31–45). At the single channel level, it has been shown that G protein inhibited channels are less likely to open upon a step depolarization leading to the proposed existence of "willing" and "reluctant" states (21). In this scenario, channels are thought to undergo a transition from the reluctant to the willing gating state in response to a strong depolarizing prepulse. A number of authors have since proposed mechanisms by which this type of facilitation occurs (19, 31–33, 44, 45). In one model, based on the lack of neurotransmitter concentration dependence of the slowing of activation kinetics, Kasai and Aosaki (33) proposed that the prepulse might induce a conformational change in the channel protein that results in altered gating behavior (33). In contrast, a number of investigators have proposed that G protein(s) might temporarily dissociate from the channel during prepulses and that recovery from this facilitation over time might involve a reassociation of the channel with the G protein(s) (e.g., see refs. 31, 32, and 46; for review, see ref. 19). However, the precise stoichiometry that underlies G protein modulation and prepulse recovery has been controversial. Elmslie and colleagues (46) were able to simulate G protein inhibition of calcium currents of bullfrog sympathetic neurons and the associated changes in current kinetics by using a simple model in which a single G protein could bind to both open and closed states of the channel. Golard and Siegelbaum (32) investigated the dependence of G protein effects on N type calcium channels of chicken sympathetic neurons through partial desensitization of the somatostatin receptor. The time constant for G protein reinhibition was found to be dependent on the level of receptor desensitization (and thus the free $G_{\beta\gamma}$ concentration) consistent with complete dissociation of one or more G proteins during the prepulse. However, attempts to fit the data based on binding of a single G protein resulted in the rate of G protein dissociation from the closed state becoming G protein-concentration-dependent. Only an expanded model that allowed binding of at least two G proteins could account for the experimental data. Finally, data obtained by Boland and Bean (31) for the LHRH modulation of calcium currents in bullfrog sympathetic neurons were best described when four G proteins were allowed to interact with the channel. The model proposed (31) was able to account for the shape and position of activation curves in the presence of the LHRH stimulus, the dose dependence of the LHRH effect, and both calcium channel activation and tail current kinetics in the presence of various concentrations of LHRH.

The results that we report herein are not consistent with the original model of Kasai and Aosaki (33) whereby the time constant for G protein reinhibition is predicted to be independent of G protein concentration. The results instead support models involving the complete dissociation of $G_{\beta\gamma}$ during the prepulse (19, 31, 32, 46).

Insight into the possible G protein/calcium channel stoichiometry can be gained from the data in Fig. 4. The inverse of the time constant for the decay of the prepulse effect ($1/\tau$) appeared to depend linearly on $G_{\beta\gamma}$ concentration and this result is consistent with a bimolecular reaction between the channel and $G_{\beta\gamma}$. In contrast, when the data were fitted with

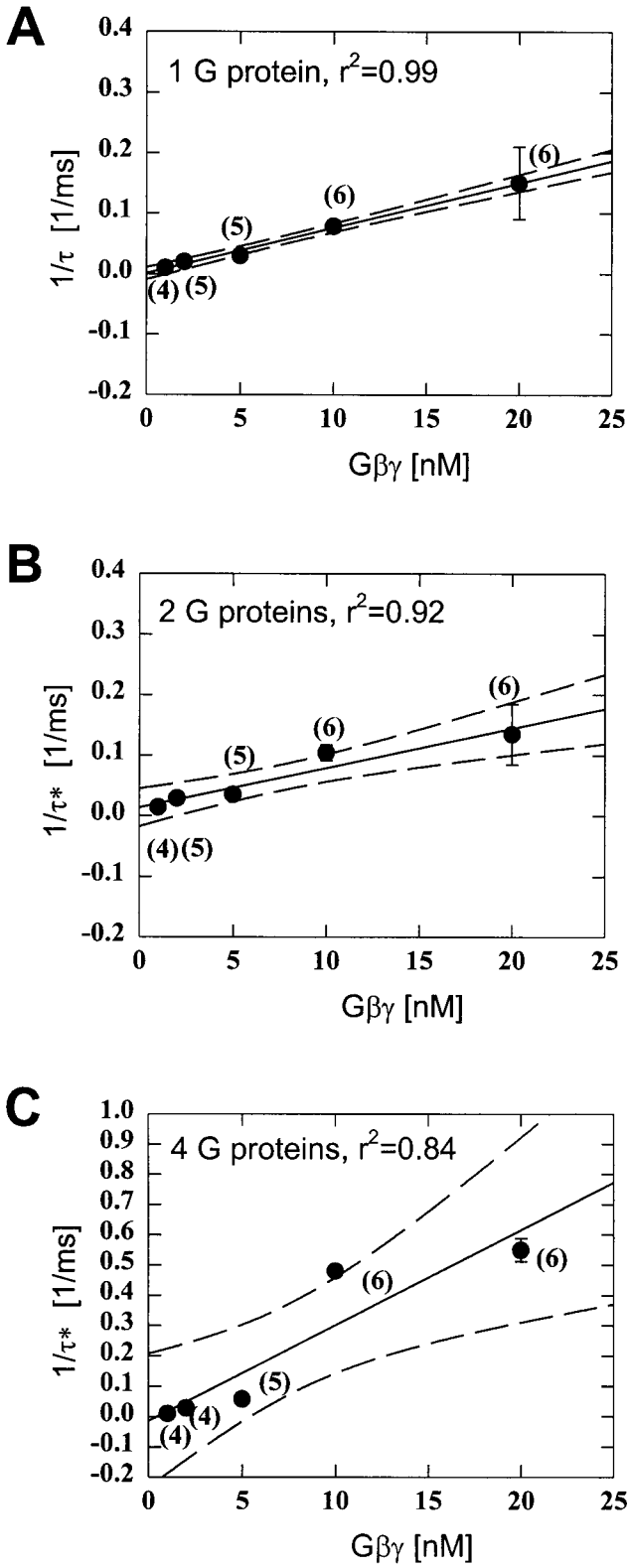

FIG. 4. (*A*) Dependence of the inverse of the time constant for the decay of the prepulse effect τ on the concentration of $G_{\beta\gamma}$. Data from each individual cell were separately fitted according to the equation outlined in Fig. 2. The solid line is a linear regression (correlation coefficient = 0.99) to the data, the error bars indicate the SEM, and the dashed lines indicate 95% confidence intervals. If there is a bimolecular interaction between the channel, then τ is equivalent with the time constant for $G_{\beta\gamma}$ rebinding. Thus, the inverse of τ is expected to linearly depend on the G protein concentration and the slope of the regression line reflects the G protein association rate constant (0.0074 $ms^{-1}{\cdot}nM^{-1}$). (*B*) Dependence of the derived time constant τ^* for $G_{\beta\gamma}$ rebinding on $G_{\beta\gamma}$ concentration for a model based on the binding of two G proteins. Data sets, such as that in Fig. 2, were fitted according to the equation $I_{+PP}/I_{-PP} = 1 + I_{max}\{1 - [1 - \exp(-\Delta t/\tau^*)]^2\}$. This equation reflects a scenario in which consecutive binding of two G proteins occurs with identical time constants, τ^*. Note that τ^* is different from the measured time constant τ shown in *A*, although similar to that for *A*, the inverse of τ^* is expected to linearly depend on the G protein concentration. The regression line used to fit the data in *B* yielded a correlation coefficient of 0.92. (*C*) The data were obtained by fitting individual time courses of G protein reinhibition with a model assuming consecutive binding of four G proteins with identical time constants. The data were fitted by using the equation $I_{+PP}/I_{-PP} = 1 + I_{max}\{1 - [1 - \exp(-\Delta t/\tau^*)]^4\}$. As in *B*, $1/\tau^*$ is expected to depend linearly on the G protein concentration; however, the data are poorly described with a simple regression line (correlation coefficient = 0.84).

a model requiring binding of four $G_{\beta\gamma}$ molecules for reinhibition after the prepulse (to simplify the analysis, binding of each of the four G proteins was assumed to occur with identical kinetics), the inverse of the $G_{\beta\gamma}$ reassociation time constant τ^* (see Fig. 4) did not linearly depend on the $G_{\beta\gamma}$ concentration (a regression line through the data yielded a correlation coefficient of 0.84, Fig. 4*C*). An analogous model based on binding of two G proteins yielded a concentration dependence of $1/\tau^*$ that was fairly linear (Fig. 4*B*); however, the quality of the regression (regression coefficient = 0.92) did not approach that obtained with the model based on a single $G_{\beta\gamma}$. Overall, our data are best described by a model in which a depolarizing prepulse results in the dissociation of a single $G_{\beta\gamma}$ molecule and in which reinhibition occurs as a bimolecular reaction between the channel and the G protein as proposed by Elmslie and coworkers (46).

How can our data be reconciled with studies proposing the binding of multiple G proteins? (*i*) The original studies were carried out by using intact systems and the exact G protein concentrations were both unknown and could not be precisely controlled. (*ii*) Our data do not rule out the possibility that multiple G proteins might be required to initially produce inhibition of N type calcium channels, whereas a depolarizing prepulse results in the dissociation of only a single $G_{\beta\gamma}$ subunit. (*iii*) Although our data did not fit with a model based on the binding of four independent G proteins with identical time constants, we cannot exclude the possibility of very rapid binding of three G proteins followed by a rate limiting slower binding of a fourth G protein. (*iv*) Finally, the kinetic models proposed by Golard and Siegelbaum (32) and by Boland and Bean (31) permit G protein binding to multiple kinetic states of the channel. More recent single channel data (34) indicate that G proteins interact weakly with closed states near the open or inactivated states but interact strongly with deep closed states favored by hyperpolarization. Hence, some of the assumptions of the earlier models may not accurately reflect the state dependence of G protein action.

If $G_{\beta\gamma}$ completely dissociates during the prepulse, then according to the model of Patil and coworkers (34), the channel would have to transit through several closed states near the open conformation before G proteins reassociate appreciably during the interpulse period. At very short interpulse intervals, there might be an initial sigmoidal delay in the time course of reblock, corresponding to the time required for the channel to transit to the deeper closed states. Closer inspection of the experimental data (Fig. 2) might indicate this sort of initial delay before an otherwise monoexponential decay. To examine the extent to which this initial delay could affect our measurement of the reinhibition time constant, we fitted our data with a model in which we required the channels to cycle into the deeper closed state before G protein reassociation (all of the transition rates between the closed states were arbitrarily combined into one collective rate of 0.2 ms^{-1}).

Compared with a simple exponential fit of the reassociation kinetics, the expanded model had little effect at $G_{\beta\gamma}$ concentrations greater than or equal to 10 nM, although the time constant obtained at 20 nM was slightly decreased (data not shown). Nonetheless, the dependence of $1/\tau$ on G protein concentration remained linear suggesting that our data satisfactorily fit with the model introduced by Patil *et al.* (34). Because the introduced delay was somewhat arbitrary (5 ms), all of the data presented herein were obtained by using simple monoexponential fits.

The time course of recovery from facilitation has recently been measured with α_{1B} currents modulated by G protein pathways reconstituted in *Xenopus* oocytes, with a reported time constant of approximately 75 ms (47). Under similar conditions we have measured a value of approximately 40 ms (E. Bourinet and T.P.S., unpublished results). In chromaffin cells, Currie and Fox (48) measured a recovery time constant of approximately 110 ms for both N type and P/Q type channels. Modeling of G protein association rates for α_{1B} currents expressed in HEK cells modulated via activation of transiently expressed M2 receptors indicates a time constant for G protein inhibition of 35 ms (34). On the basis of the blocking rate constant of 0.0074 $ms^{-1}{\cdot}nM^{-1}$ obtained from the fit in Fig. 4, the $G_{\beta\gamma}$ concentration that is liberated after G protein receptor activation would be expected to be on the order of 1.0–3.0 nM. These values are consistent with modulation of $I_{K,ACh}$ channels by $G_{\beta\gamma}$, which occurs with a K_i of 2.6 nM (49).

In summary, our results provide direct evidence for the physical dissociation of a single $G_{\beta\gamma}$ during the prepulse-induced facilitation of N type calcium channels. With biochemical studies (25, 28), the picture emerges that G protein inhibition is mediated by the selective interaction between a single $G_{\beta\gamma}$ molecule and the calcium channel α_1 subunit rather than interaction of multiple $G_{\beta\gamma}$ molecules with multiple independent sites on the channel complex. Further studies will be required to resolve the molecular mechanisms that result in the dissociation of the $G_{\beta\gamma}$ during the depolarizing prepulse.

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