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Regulation of Human Neuronal Calcium Channels by G Protein $\beta\gamma$ Subunits Expressed in Human Embryonic Kidney 293 Cells

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SUMMARY

We examined the ability of different G protein subunits to inhibit the activity of human α1B and α1E Ca^{2+} channels stably expressed in human embryonic kidney (HEK) 293 cells together with β1B and α2Bδ Ca^{2+} channel subunits. Under normal conditions, Ca^{2+} currents in α1B-expressing cells showed little facilitation after a depolarizing prepulse. However, when we overexpressed the β2γ2 subunits of heterotrimeric G proteins, the time course of activation of the Ca^{2+} currents was considerably slowed and a depolarizing prepulse produced a large facilitation of the current as well as an acceleration in its time course of activation. Similar effects were not observed when cells were transfected with constitutively active mutants of the G protein α subunits αs, αi1, and αo or with the G protein β2 and γ2 subunits alone. Studies carried out in cells expressing α1E currents showed that overexpression of β2γ2 subunits produced prepulse facilitation, although this was of lesser magnitude than that observed with Ca^{2+} currents in α1B-expressing cells. The subunits β2 and γ2 alone produced no effects, nor did constitutively active αs, αi1, and αo subunits. Phorbol esters enhanced α1E Ca^{2+} currents but had no effect on α1B currents, suggesting that protein kinase C activation was not responsible for the observed effects. When α1E Ca^{2+} currents were expressed without their β subunits, they exhibited prepulse facilitation. These results demonstrate that α1E Ca^{2+} currents are less susceptible to direct modulation by G proteins than α1B currents and illustrate the antagonistic interactions between Ca^{2+} channel β subunits and G proteins.

One of the characteristic features of voltage-dependent Ca^{2+} channels is their regulation by G proteins and second messengers (1, 2). In neurons, for example, activation of G proteins by a variety of receptors leads to the inhibition of several types of Ca^{2+} channels, and it is likely that this process plays an important role in the phenomenon of presynaptic inhibition (2, 3). In many instances, the receptor/G protein-mediated inhibition of Ca^{2+} channels is rapid and membrane delimited (1, 4). In these cases, the inhibition is not thought to involve the participation of a diffusible second messenger but instead to be due to the direct interaction of the G protein with the Ca^{2+} channel, possibly in a similar manner to that demonstrated for the G protein regulation of the GIRK/CIR class of inwardly rectifying K^+ channels (5). It has recently been demonstrated that the $\beta\gamma$ subunits of heterotrimeric G proteins play a major role in mediating the inhibition of some Ca^{2+} channels, as they also do in the G protein modulation of GIRK channels (6, 7).

The pore-forming α subunits of voltage-dependent Ca^{2+} channels constitute a family of related molecules that at this point in time contains two major branches (8). One subgroup (formed from α1S, α1C, and α1D) is sensitive to dihydropyridine drugs, whereas the other (formed from α1B, α1A, and α1E) is not. These latter channels are particularly well represented in the nervous system, in which they are thought to be expressed as N-, P/Q-, and possibly R-type Ca^{2+} currents (9–11). Among other things, these types of Ca^{2+} currents are known to regulate the release of neurotransmitters at most nerve terminals (12).

The N- and P/Q-type Ca^{2+} channels have frequently been shown to be regulated by receptors and G proteins (1, 13–15), as have Ca^{2+} channels expressed using cloned α1B and α1A subunits (16, 17). Much less is known, however, about the functions and properties of the Ca^{2+} channels formed from the expression of α1E. Recently, we demonstrated that α1E channels showed relatively little receptor or G protein-mediated inhibition in comparison with α1B currents when these Ca^{2+} channels were expressed in cultured cells under identical conditions (16). We now demonstrate that Ca^{2+} chan-

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ABBREVIATIONS: HEK, human embryonic kidney; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I-V, current-voltage; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

nels expressed using both human α1B and α1E are subject to inhibitory regulation by G protein $\beta\gamma$ subunits. In addition, we demonstrate that interaction with Ca^{2+} channel β subunits causes α1E to behave in a manner resembling that observed after removal of G protein-mediated inhibition. These results support suggestions in the literature that G proteins may inhibit Ca^{2+} channels by antagonizing the effects of Ca^{2+} channel β subunits (18, 19).

Experimental Procedures

HEK 293 cell lines. The G1A1 and E-52 HEK 293 cell lines expressing Ca^{2+} channels have been previously described (17) and were kindly provided by SIBIA Neurosciences (La Jolla, CA) (9, 10); in summary, they consist of either α1B-1 (G1A1) or α1E-3 (E-52) along with β1B and α2Bδ.

The G1A1 and E-52 cells were grown onto plastic Falcon dishes in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 5% defined bovine serum (Hyclone Laboratories, Logan, UT) plus 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 500 μg/ml geneticin. One day before recording, cells were dissociated by gentle trituration with fire-polished Pasteur pipettes and replated onto poly-L-lysine (Sigma Chemical, St. Louis, MO)-coated glass coverslips.

Preparation of Ca^{2+} channel expression plasmids. cDNAs encoding the Ca^{2+} channel α1B, β1B, and α2Bδ subunits [kindly provided by SIBIA (9)] and the Ca^{2+} channel α1E subunits [kindly provided by SIBIA (10)] were subcloned into pCMV5 (20) and confirmed by DNA sequencing using a modification of the dideoxy-chain termination method (Sequenase 2.0; United States Biochemical Corp., Cleveland, OH).

Preparation of G protein expression plasmids. cDNAs encoding the G protein β1, β2, and γ2 subunits (kindly provided by SIBIA) were subcloned into pCMV5 (20) and confirmed by DNA sequencing as described above. The constitutively active forms of $G_{o\alpha}$, $G_{i\alpha1}$, and $G_{s\alpha}$ [denoted as $G_{o\alpha}$*, $G_{i\alpha1}$*, and $G_{s\alpha}$*, respectively (21)], were similarly subcloned into pCMV6b and confirmed by sequencing. All constitutively activated α subunits are the Q-to-L mutants [$G_{s\alpha}$ Q227L (22) $G_{i\alpha1}$ Q204L, and $G_{o\alpha}$ Q205L (23)].

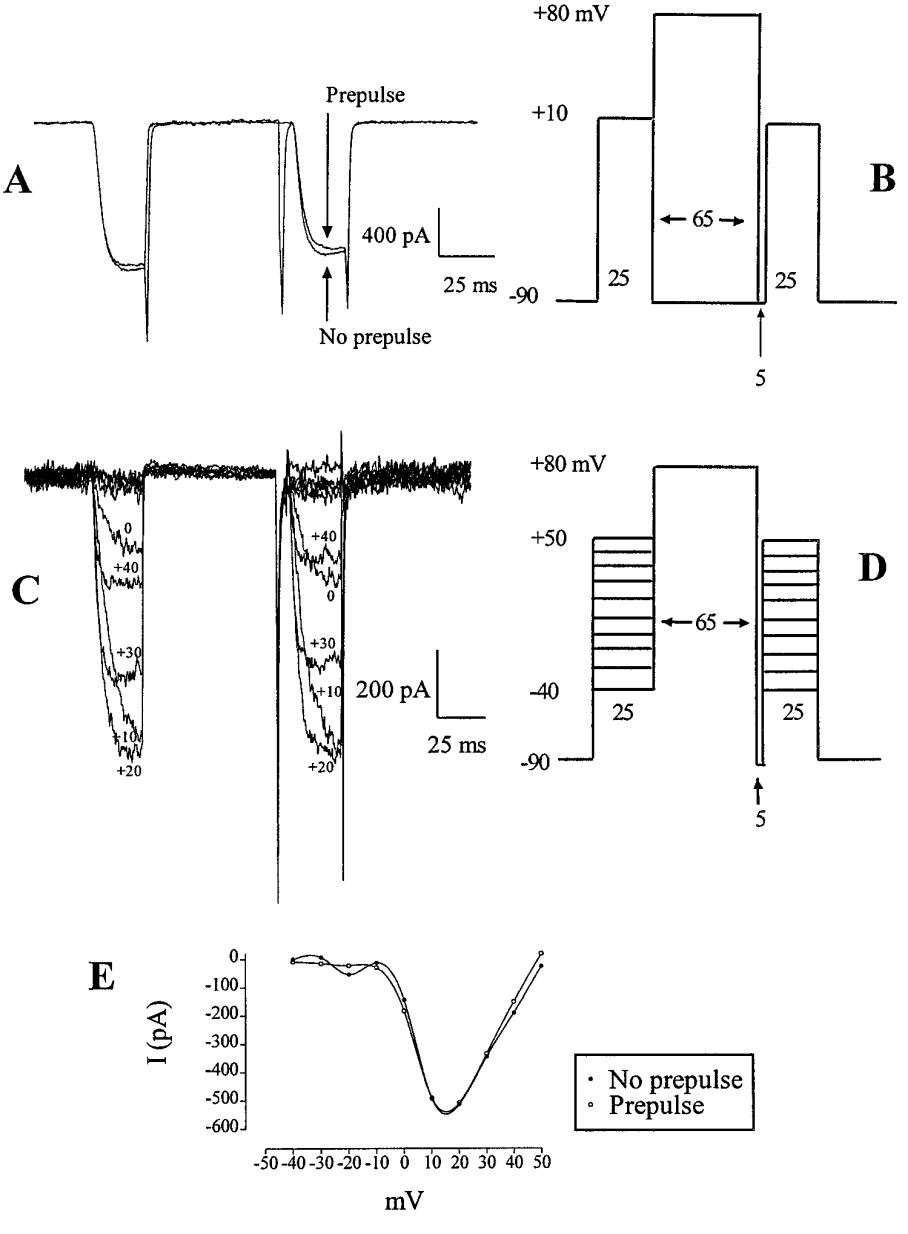

Fig. 1. Ca^{2+} currents in G1A1 cells. A, Depolarization of a control cell was performed using a double-pulse protocol with or without an intervening depolarizing prepulse to +80 mV for 65 msec. Cells were held at −90 mV and stepped to +10 mV for 25 msec and then either stepped back to −90 for 70 msec (no prepulse) or stepped to +80 mV for 65 msec (prepulse) and then −90 mV for 5 msec. A second pulse to +10 mV was then executed for 25 msec. B, Prepulse protocol used in all experiments as in A. C, Example of a cell held at −90 mV before jumping to −40 mV and then stepping by 10-mV increments to a maximum of +50 mV. D, I-V prepulse depolarization protocol used as in C. E, I-V relationship of traces displayed in C.

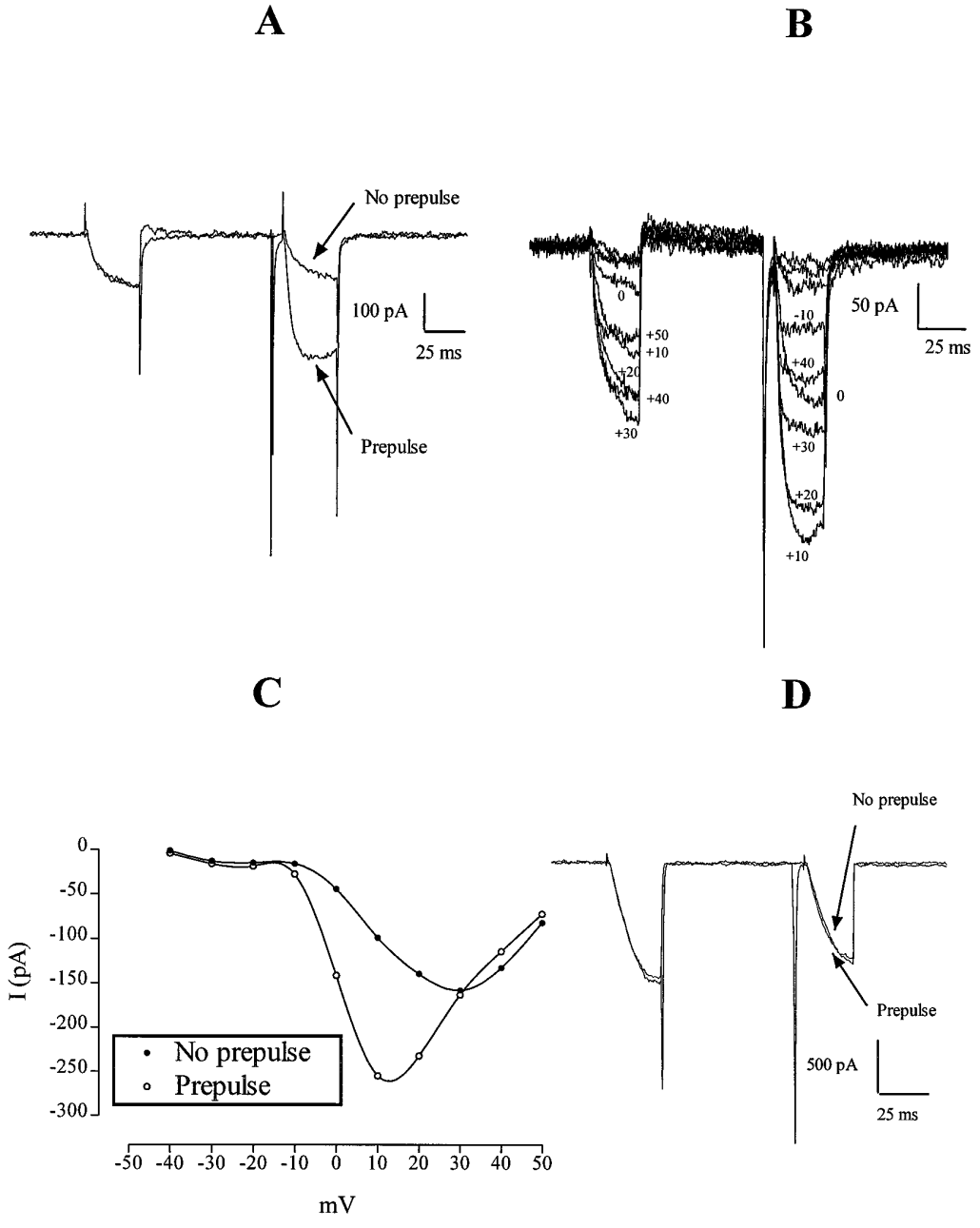

Fig. 2. Effects of overexpression of $G_{\beta 2\gamma 2}$ subunits in the G1A1 cell line. A, Cell displaying characteristic peak current inhibition and slowing, which was then relieved after a prepulse. Ca^{2+} currents were generated as described in Fig. 1B. B, Current traces generated using the I-V protocol described in Fig. 1D. C, I-V relationship of the traces shown in B. D, G1A1 cell similarly transfected with $G_{\beta 2\gamma 2}$ subunits that displayed slowing but very little prepulse facilitation.

Immunohistochemistry. E52–3 cells passage numbers 7, 19, 28, and 35 were shaken off T75 Falcon tissue culture flasks, decanted into centrifuge tubes, and centrifuged at 1500 rpm for 5 min. They were washed in PBS and recentrifuged as described above. Cells were then fixed in 4% paraformaldehyde in PBS (w/v) for 20 min at room temperature. After a washing step as described above, cells were permeabilized in freshly prepared 5% glacial acetic acid in ethanol (5% v/v) at −20° for 20 min. Cells were centrifuged at 1800 rpm for 5 min, washed twice in PBS, and subsequently incubated in 10% goat serum in PBS for 20 min as a blocking step (10% goat serum in PBS was used for antibody dilutions and all further washing steps). Cells were aliquotted into tubes and centrifuged at 1800 rpm for 3 min before antibody application. Primary antibody (β1B) was used at a concentration of 10 μg/ml and incubated for 30 min at room temperature. Cells were washed in PBS containing goat serum and subsequently incubated with goat anti-rabbit IgG-fluorescein isothiocyanate secondary antibody (1:50; Southern Biotechnology Associates, Birmingham, AL) for 30 min at room temperature. Cells were washed as described above and observed using a Leica DM IRB fluorescent microscope.

Transfection of HEK 293 cells. Monolayers of HEK 293 cells of ≤75% confluence were dissociated and replated onto poly-L-lysine-coated glass coverslips. Cells were cotransfected with plasmids containing the cDNAs for the G protein and either β-galactosidase or CD8 using the standard calcium-phosphate precipitation technique (31) or transfection kit (Mammalian Transfection Kit; Stratagene, La Jolla, CA) to detect positively transfected cells. For the 5-bromo-4-chloro-3-indolyl-β-D-galactoside *in situ* staining for β-galactosidase (25), media from the cells were aspirated, and the cells were rinsed twice with 5 ml of PBS. Then, 5 ml of fix (2% formaldehyde and 0.2% glutaraldehyde in PBS) was added and allowed to incubate at room temperature for 5 min. The fix was then removed, and plates were rinsed twice with 5 ml of PBS. Then, 5 ml of reaction mix (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5 mM K-ferricyanide, 5

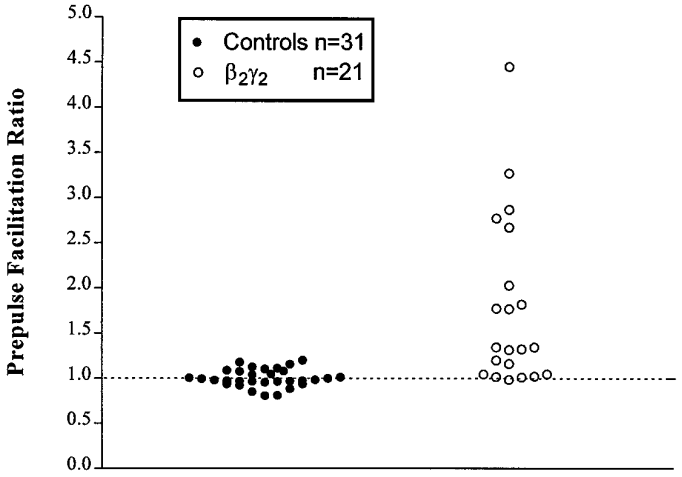

Fig. 3. Magnitude of prepulse facilitation of Ca^{2+} currents in G1A1 cells with and without $G_{\beta 2\gamma 2}$ subunit overexpression. The ratio of the peak Ca^{2+} current before and after the prepulse was calculated and plotted. *Dotted line*, ratio of 1.0.

mM K-ferrocyanide, and 2 mM $MgCl_2$ made up fresh before use) was added and allowed to incubate for 2 hr at 37°. Positive cells stained blue and were visualized under a light microscope.

CD8 transfected cells were washed once in bath solution and then once in bath solution with a 1:1000 dilution of Dynal microspheres coated with a primary monoclonal antibody specific for the CD8 membrane antigen (Dynabeads M-450 CD8; Dynal, Lake Success, NY). Positive cells were identified as those to which beads adhered.

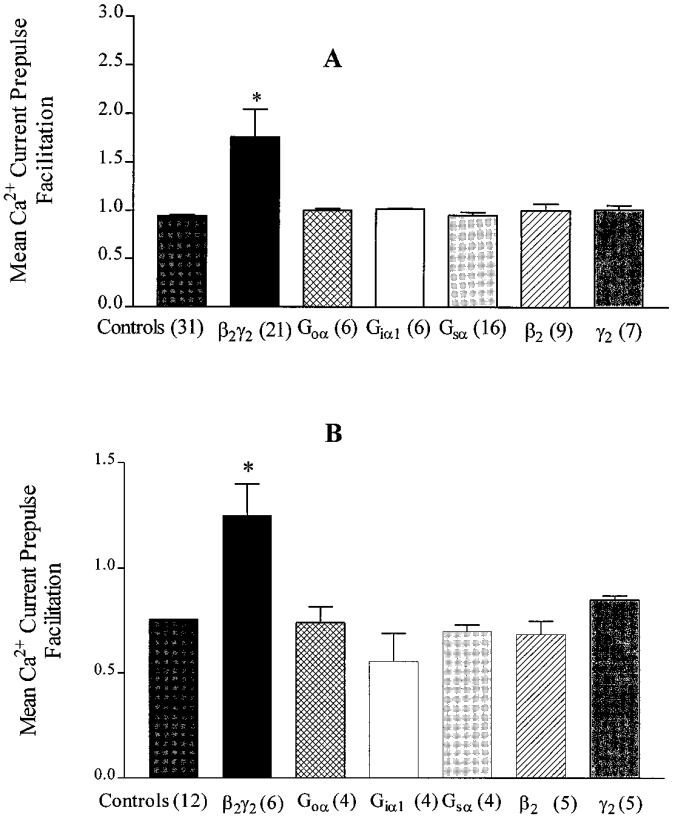

Fig. 4. A, Mean prepulse facilitation for the Ca^{2+} currents in α1B-containing G1A1 cells after transfection of different G protein subunits. *Parentheses*, number of experiments. *, Different from the other groups ($p < 0.05$). B, Mean prepulse facilitation for Ca^{2+} currents in α1E-containing E-52 cells.

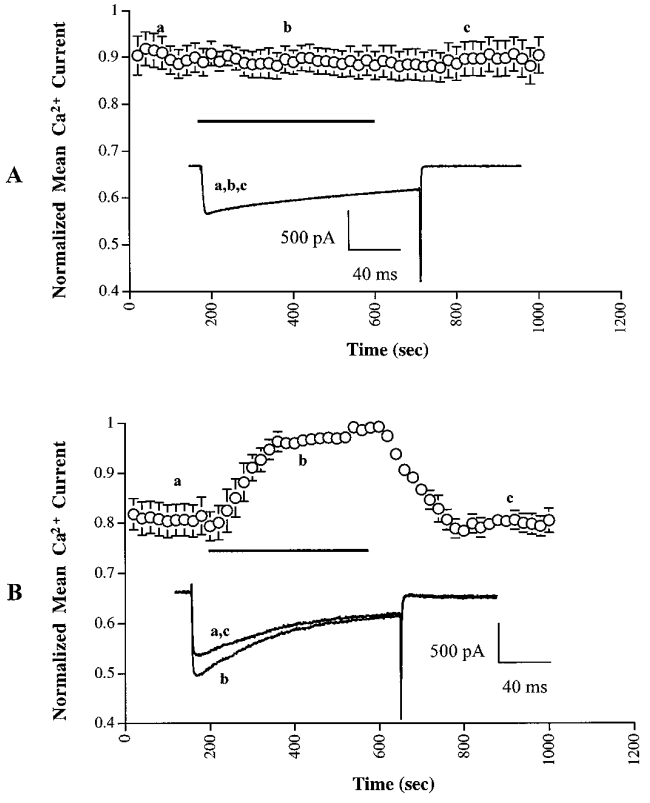

Fig. 5. Time course of PMA-induced effects on Ca^{2+} currents. A, PMA addition (100 nM) had no effect on Ca^{2+} currents in G1A1 cells. B, E-52 cells showed a significant ($p < 0.01$) increase in the magnitude of the Ca^{2+} current after PMA addition that was readily reversible. *Insets*, current traces taken from indicated points in each experiment from the same cell.

Currents were recorded 48–72 hr after transfection. The average transfection efficiencies were 50–80%. All transfections were confirmed by Northern blot analysis.

Whole-cell patch-clamp. The tight-seal whole-cell configuration of the patch-clamp technique (26) was used to record Ca^{2+} currents. Recordings were made at room temperature (21–24°). Currents were recorded using Clampex 6 on an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) filtered at 1 kHz by the built-in filter of the amplifier and stored in the computer. Series resistance compensation of 40–80% was applied based on readings from the amplifier. Leak corrections were performed using a P/N protocol. Two different command pulses were delivered at a 20-sec interval. The first pulses consisted of two 25-msec depolarization steps to +10 mV followed 20 sec later by another two pulses to +10 mV interspersed with a 65-msec pulse to +80 mV. Soft, soda-lime capillary glass was used to make patch pipettes that were coated with Sylgard (Dow Corning, Midland, MI) and had resistances of 1.8–3.5 M (when filled with internal solution). Extracellular buffer solution for whole-cell voltage-clamp experiments was composed of 160 mM tetraethylammonium chloride, 5 mM CaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with tetraethylammonium·OH. The standard internal solution consisted of 100 mM CsCl, 37 mM CsOH, 1 mM $MgCl_2$, 10 mM BAPTA, 10 mM HEPES, 3.6 mM MgATP, 1 mM GTP, 14 mM Tris2CP, and 50 units/ml CPK. The pH was adjusted to 7.3 with CsOH. The osmolarity of the pipette solution was 300 mOsM, and the osmolarity of the extracellular solution was 315–323 mOsM. PMA (Sigma Chemical) was dissolved in dimethylsulfoxide and used at a working concentration of 100 nM. 4-α-PMA (Research Biochemicals, Natick, MA) was similarly dissolved in dimethylsulfoxide and used at a working concentration of 100 nM. The protein kinase C pseudosubstrate (19–31) inhibitor (BIOMOL Research Laboratories,

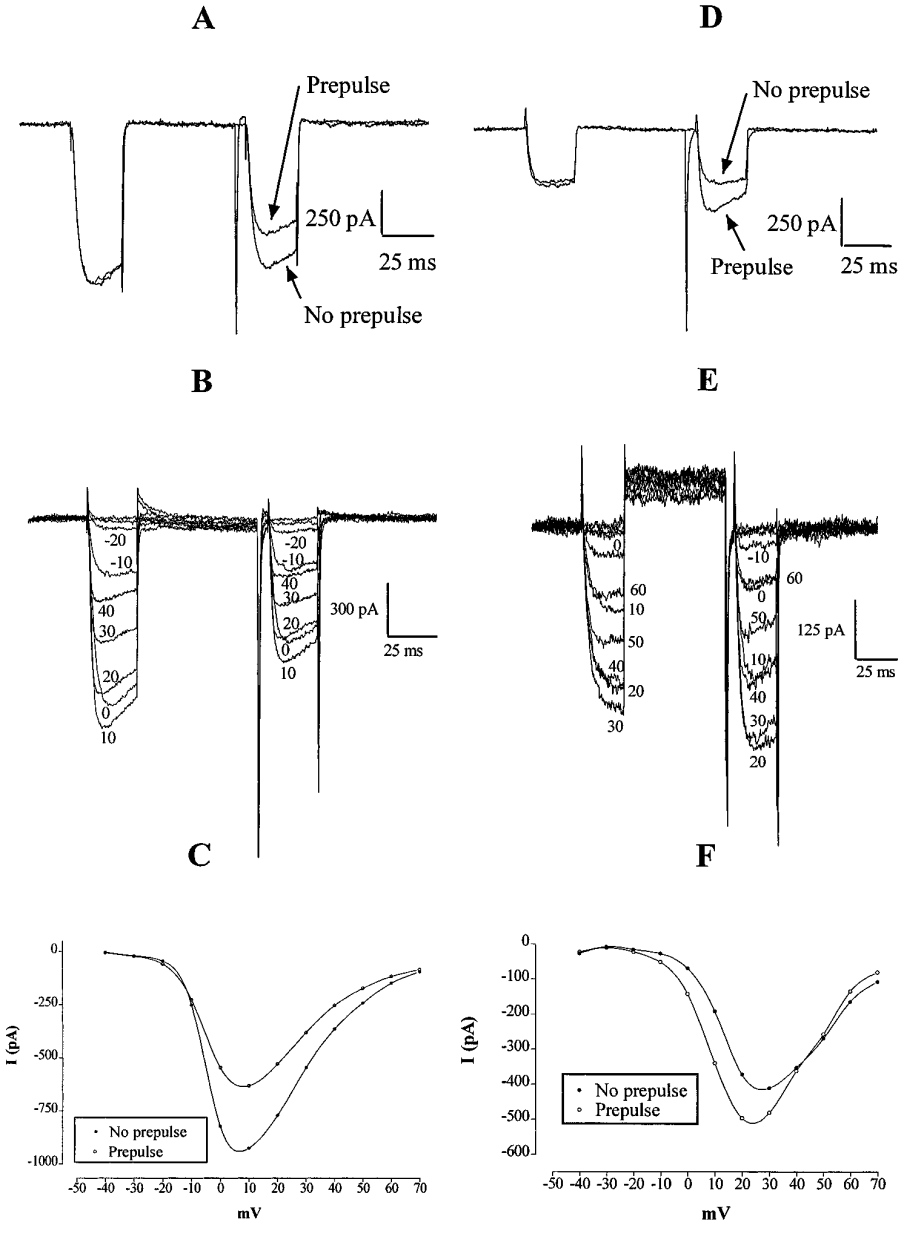

Fig. 6. Effects of $G_{\beta 2\gamma 2}$ overexpression on Ca^{2+} currents in the E-52 cell line. A, Depolarization of a control cell was performed as in Fig. 1A. The cell displays characteristic voltage-dependent inactivation. B, Ca^{2+} current traces generated from an I-V protocol showing inhibition of the Ca^{2+} current after the prepulse. C, I-V relationship for traces shown in B demonstrating the absence of any shift in the peak current after the prepulse. D, $G_{\beta 2\gamma 2}$-transfected cell showing prepulse Ca^{2+} current facilitation. E, Ca^{2+} current traces from a $G_{\beta 2\gamma 2}$-transfected cell showing facilitation after the prepulse. F, I-V relationship of traces displayed in E.

Plymouth Meeting, PA) was dissolved in internal solution at a concentration of 1 μM and backfilled into the pipette just before recording.

Data analysis. Activation portions of currents before and after the prepulse were fitted with a single exponential curve of the form $y = A * e\ [-(t - k)/\tau] + C$, where A is the amplitude, relative to the offset, evaluated at the start of the fit region; τ is the time constant; t is the time; k is the time shift in the fit equation, and C is the steady state asymptote. Curve fitting was performed using Clampfit 6 (Axon Instruments).

Statistical analysis comparing two groups was performed using the Wilcoxon signed-rank variant of the Student's t test for paired, non-parametric data at a significance level of $p = 0.05$. For analyzing three or more unpaired groups the Kruskal-Wallis variant of the ANOVA test was used. A Dunn's *post hoc* test was also carried out to determine which of the groups analyzed in the ANOVA were significantly different from each other. All graphing and statistical analysis were carried out using either Prism 2 (GraphPAD Software, San Diego, CA) or Cricket Graph III 1.5.3 (Computer Associates, Islandia, NY).

Results

As we previously demonstrated (16), Ca^{2+} currents could be elicited by 25-msec depolarizing voltage steps from HEK 293 cells, which stably expressed either α1B (G1A1 cells) or α1E (E-52 cells) Ca^{2+} channel subunits, together with the ancillary subunits β1B and α2Bδ (16). When we applied a double-pulse protocol, in which the two test pulses were applied at a 20-sec interval, currents of nearly identical magnitude were elicited in both cases (Fig. 1, A and B). We examined the cells for evidence of inhibitory G protein-mediated regulation of Ca^{2+} channels using a depolarizing prepulse before the second test depolarization. Under normal conditions, if the test pulse was preceded by a depolarizing prepulse, α1B currents were of similar magnitude, exhibiting no facilitation [0.94 $\pm$ 0.01 of control (n = 31); Fig. 1A]. If we ran an I-V protocol before and after a prepulse, we observed little change in the kinetics or magnitude of the Ca^{2+} cur-

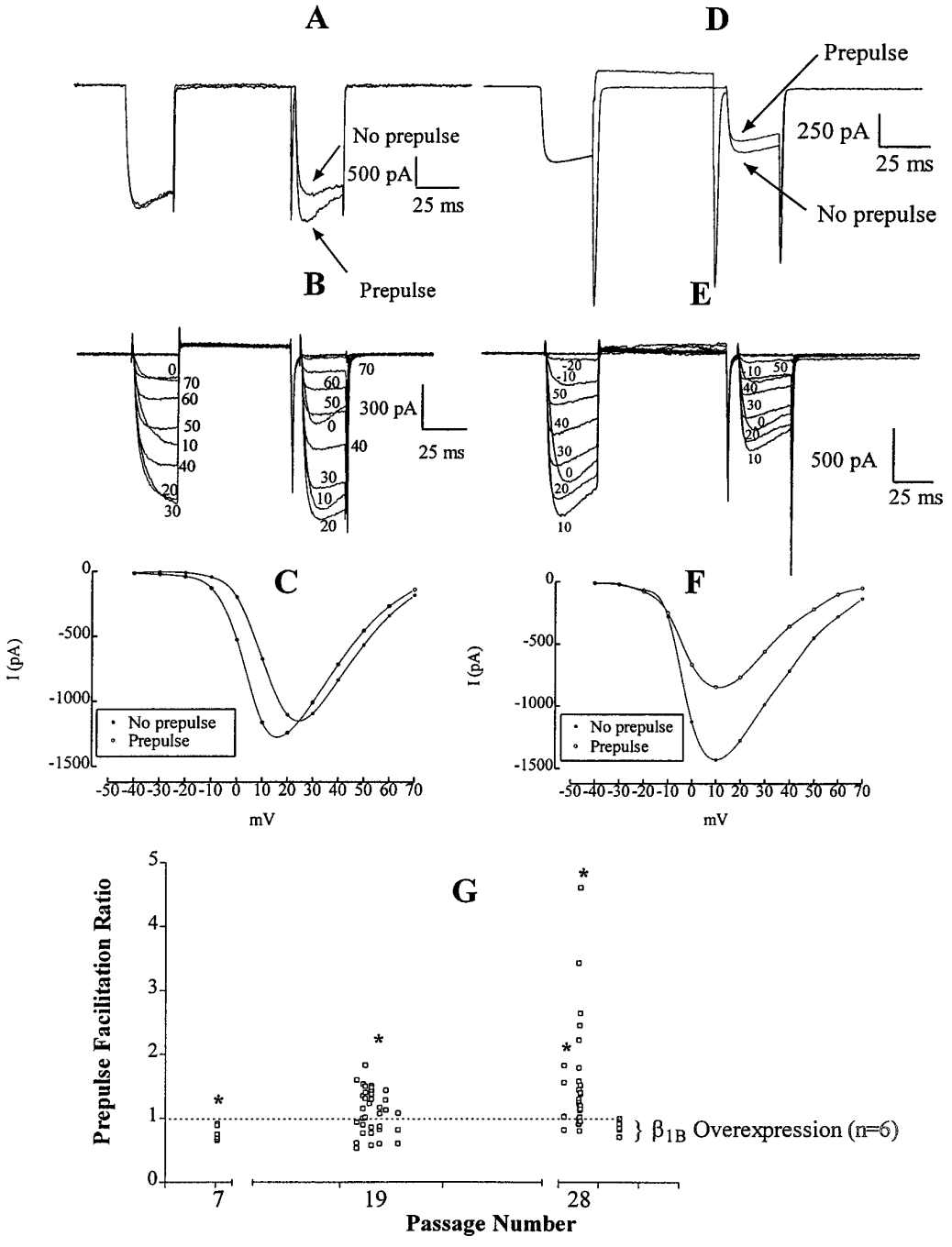

Fig. 7. A, Control high-passage number E-52 cell displaying Ca^{2+} current prepulse facilitation in the absence of $G_{\beta 2\gamma 2}$ overexpression. B, Current traces from an I-V experiment in a high-passage number E-52 cell showing facilitation after the prepulse (also shown in C). D—F, After high-passage number E-52 cells were transfected with the Ca^{2+} channel β1B subunit, the prepulse facilitation was eliminated. D, Ca^{2+} current traces with and without prepulse showing voltage-dependent inactivation as in Fig. 6A. E, Ca^{2+} current traces from an I-V showing the absence of any facilitation. F, I-V plot of peak currents shown in E. G, Summary for prepulse facilitation of E-52 Ca^{2+} currents over time after overexpression of the Ca^{2+} channel subunit β1B. $*$, Different from the other groups ($p < 0.05$).

rents (Fig. 1, C–E). However, when we overexpressed G protein $\beta 2\gamma 2$ subunits in G1A1 cells, we noted two effects. The Ca^{2+} currents in all transfected cells displayed slower rates of activation than did Ca^{2+} currents in control cells [τ = 6.48 ± 0.51 msec (n = 21) for transfected cells versus τ = 1.93 ± 0.19 msec (n = 31) for control cells]. These cells also displayed a wide range of prepulse facilitation (compare Fig. 2, A and D). In cells showing substantial prepulse facilitation, the I-V relationship for the Ca^{2+} currents was shifted in the depolarizing direction by ~+20 mV (Fig. 2C). After a prepulse, the facilitated Ca^{2+} currents in the cells were accelerated, and the I-V curve was shifted in the hyperpolarizing direction to a position similar to that observed in control cells (Fig. 2, A–C). A summary of the facilitation data for all G1A1 control and $G_{\beta 2\gamma 2}$-transfected cells is shown in Fig. 3. A continuum in the degree of facilitation can clearly be seen for the transfected cells. In a separate set of experiments, expression of a second combination of G $\beta\gamma$ subunits ($\beta 1\gamma 2$, n = 4) also produced facilitation of Ca^{2+} currents (2.37 ± 0.37-fold increase). There was no effect of overexpression of three mutant G protein α subunits that are constitutively active (αo^*, n = 6; $\alpha i1^*$, n = 6; and αs^*, n = 16) or of $\beta 2$ (n = 9) or $\gamma 2$ (n = 7) subunits expressed alone (data not shown). A summary of all the data for G1A1 cells is shown in Fig. 4A.

It has been reported that in some cases, the effects of G proteins on N-type Ca^{2+} channels are mediated through activation of PKC (15). This is particularly so when considering non-voltage-dependent components of inhibition. However,

this is unlikely to be of significance in the present situation in that phorbol esters that activate PKC produced no change in the magnitude of the Ca^{2+} currents in these cells. For example, the active phorbol ester PMA (100 nM; $n = 7$) produced no effect on Ca^{2+} currents in G1A1 cells (Fig. 5A). The inactive phorbol ester 4-α-PMA 100 nM; $n = 3$) was similarly ineffective (data not shown). Furthermore, including the PKC pseudosubstrate (19–31) inhibitor (1 μM; $n = 3$) in the patch pipette did not alter the facilitation produced by overexpression of $G_{\beta 2\gamma 2}$ (2.48 ± 0.83-fold; $n = 3$).

We carried out a similar series of experiments using the α1E-expressing cell line E-52. When the second test pulse was preceded by a depolarizing prepulse in these experiments, the test currents evoked were always smaller than those evoked by the test pulse alone (0.76 ± 0.03 of control; $n = 12$) (Fig. 6A). This is due to voltage-dependent inactivation of α1E currents as previously described (10, 17). The peak of the I-V curve was not shifted by the prepulse (Fig. 6, B and C). Overexpression of $G_{\beta 2\gamma 2}$ subunits in these cells produced currents that could now be facilitated by a depolarizing prepulse (1.25 ± 0.15-fold increase; $n = 6$) (Figs. 6D and 4B). Thus, these effects were not as large as those observed with α1B currents under identical conditions. As with the G1A1 cells, we found that overexpression of $G_{\beta 2\gamma 2}$ resulted in a shift in the peak of the I-V curve, typically from 0 to +10 mV (compare Fig. 6, B and C with E and F). Overexpression of any of the three mutant G protein α subunits or of β2 and γ2 alone had no effect (Fig. 4B). We also found that the α1E-based currents in E-52 cells were enhanced by the active phorbol ester PMA [Fig. 5B (100 nM); see also Ref. 29] but not by the inactive phorbol ester 4-α-PMA (100 nM; $n = 3$). Furthermore, dialyzing E-52 cells with the PKC pseudosubstrate (19–31) inhibitor (1 μM; $n = 3$) did not block the effects of $G_{\beta 2\gamma 2}$ (1.13 ± 0.33-fold; $n = 3$), although it completely prevented the effects of treatment with PMA. These results indicate that the effects of $G_{\beta 2\gamma 2}$ were not mediated by PKC activation. In addition, these studies indicate that G protein $\beta\gamma$ subunits, but not α subunits, can produce inhibition of α1B and α1E Ca^{2+} channels. The effects of $G_{\beta 2\gamma 2}$ on α1B seem to be larger than those on α1E Ca^{2+} channels. Such data are consistent with our previous studies using GTP-γ-S (17).

While these experiments were in progress, we made what seemed at first to be a very curious observation. We started to observe a population of E-52 cells in which the Ca^{2+} currents spontaneously exhibited robust prepulse facilitation (Fig. 7A). Thus, we did not overexpress G protein $\beta\gamma$ subunits, we did not coexpress and activate a G protein-linked receptor, and we did not introduce guanosine-5′-*O*-(3-thio)triphosphate into these cells. In addition to facilitation, the cells exhibited I-V curves whose peaks shifted ~10 mV in the depolarizing direction (Fig. 7, B and C). In all respects, these Ca^{2+} currents behaved like those described above as being regulated by G protein $\beta\gamma$ subunits. As the passage number of the cells increased, this population of cells grew until most of the cells behaved in this manner (Fig. 7G). How might such observations be explained? One hypothesis that we considered was that although we were using a "stable" cell line, it was possible that one of the ancillary Ca^{2+} channel subunits was being lost with increasing passage number. This proved to be the case. We observed that when we transiently transfected the Ca^{2+} channel β1B subunit into the cells, they behaved precisely as they had previously (Fig. 7, D–G). Overexpression of the β1B subunit "cured" all aspects of the apparently aberrant behavior that was displayed by the α1E Ca^{2+} channels. Ca^{2+} currents no longer facilitated [0.84 ± 0.04 of control ($n = 6$) versus 1.39 ± 0.26-fold increase for the untransfected ($n = 6$)], and the peak of the I-V curve was no longer shifted in the depolarized direction (Fig. 7, E and F). Immunohistochemical staining for the β1B subunit clearly demonstrated a 4-fold decrease in positive cells over this same time period as the recordings (Table 1).

It therefore seemed that α1E Ca^{2+} channels in E-52 cells that had lost their β1B subunit behaved like Ca^{2+} channels that have been described as being inhibited by G proteins. One possible basis for this behavior was that under conditions in which the β1B subunit was lost, the Ca^{2+} channels were subject to inhibition by the low levels of tonically activated G proteins normally found in the cells. We tested this idea by substituting 1 mM guanosine-5′-*O*-(2-thio)diphosphate for the 1 mM GTP normally found in the intracellular solution to inhibit any endogenous G protein activation. The presence of guanosine-5′-*O*-(2-thio)diphosphate blocks any tonic or receptor-mediated regulation of Ca^{2+} channels in these cells (16). Under these conditions, the high-passage number cells still behaved as if they were inhibited, exhibiting facilitation and other features (1.22 ± 0.50-fold increase; $n = 5$). Thus, we conclude that α1E Ca^{2+} channels that are devoid of β subunits do not need to interact with G protein subunits to exhibit the behavior described. These conclusions were further strengthened by transiently expressing α1E channels in HEK 293 cells with different subunits. When we expressed the combination α1E/β1B/α2Bδ, the currents behaved as they did in low-passage number E-52 cells, in which this combination of subunits was stably expressed (Fig. 8, A and B). Thus, the currents generated by the second test pulse were smaller than those generated by the first test pulse (0.64 ± 0.07 of control; $n = 6$). However, when we expressed α1E/α2Bδ subunits alone, the currents exhibited features such as facilitation (1.47 ± 0.11-fold increase; $n = 3$) and a depolarizing shift in the peak I-V curve, properties similar to those described above for high-passage number E-52 cells, which had apparently lost their β subunits (Fig. 8, C and D).

Discussion

The Ca^{2+} channel subunits α1B and α1E are highly homologous in terms of their primary sequences and are members of the same subfamily of Ca^{2+} channel α1 subunits (8–10). Little is known about the normal functions and regulation of α1E-based channels, although their dendritic localization may indicate a role in the control of excitability of

TABLE 1

Passage number of E-52 cells versus percentage of cells that positively stained for the Ca^{2+} channel β1B subunit antibody

Values represent the percentage of cells expressing β1B above background levels. Measurements were made on three different fields of the same sample.

Passage number	Positive cells	Standard error	*n*
	%		
7	37	6	3
19	32	7	3
28	22	1	3
35	10	4	3

this region of the neuron in particular (28–30). Indeed, it is not yet clear what types of Ca^{2+} currents are normally formed by expression of α1E subunits in neurons. Ca^{2+} currents have been reported in a variety of neurons that are resistant to blockers of N-, L-, and P/Q-type Ca^{2+} channels (11, 32). However, with certain exceptions, these currents do not necessarily display all the biophysical characteristics of α1E currents expressed *in vitro*. On the other hand, a great deal is known about α1B-based Ca^{2+} channels, which are widely believed to give rise to N-type Ca^{2+} channels in neurons (8, 10).

It has been frequently shown that N channels can be inhibited by the activation of "serpentine" receptors and G proteins (1, 14). Receptor/G protein-mediated inhibition of N currents seems to constitute several different processes. One type of inhibition that has been widely described seems to involve the direct interaction of G protein subunits with the Ca^{2+} channel (1, 4). This type of inhibition has been reported to be substantially voltage dependent and is manifest as a slowing of the activation kinetics of the current. Recent studies have indicated that the $\beta\gamma$ subunits of G proteins may play the major role in mediating this type of inhibition (6, 7). However, non-voltage-dependent inhibition of N channels has also been reported (15). It is not entirely clear how such effects are produced. It has been suggested that in this case, the effects of $\beta\gamma$ subunits might be indirect and mediated through activation of the enzyme protein kinase C (15). Although this may be the case in some circumstances, it is not true in the present series of studies; stimulation of PKC produced enhancement of α1E currents yet had no effect on α1B currents.

We previously demonstrated that activation of SRIF or κ-opioid receptors produces inhibition of α1B and α1E currents in the same cell lines as used in the current study (16). We showed that the inhibition produced was much larger in the case of α1B than in the case of α1E. Similar results have been reported when Ca^{2+} channels have been expressed in oocytes (17). Little inhibition of α1E was seen after its coexpression in oocytes with the μ-opioid receptor, although robust inhibition of α1B (N) and α1A (P/Q) currents was observed under the same circumstances. Modest inhibitory effects were observed in response to SRIF and cat-

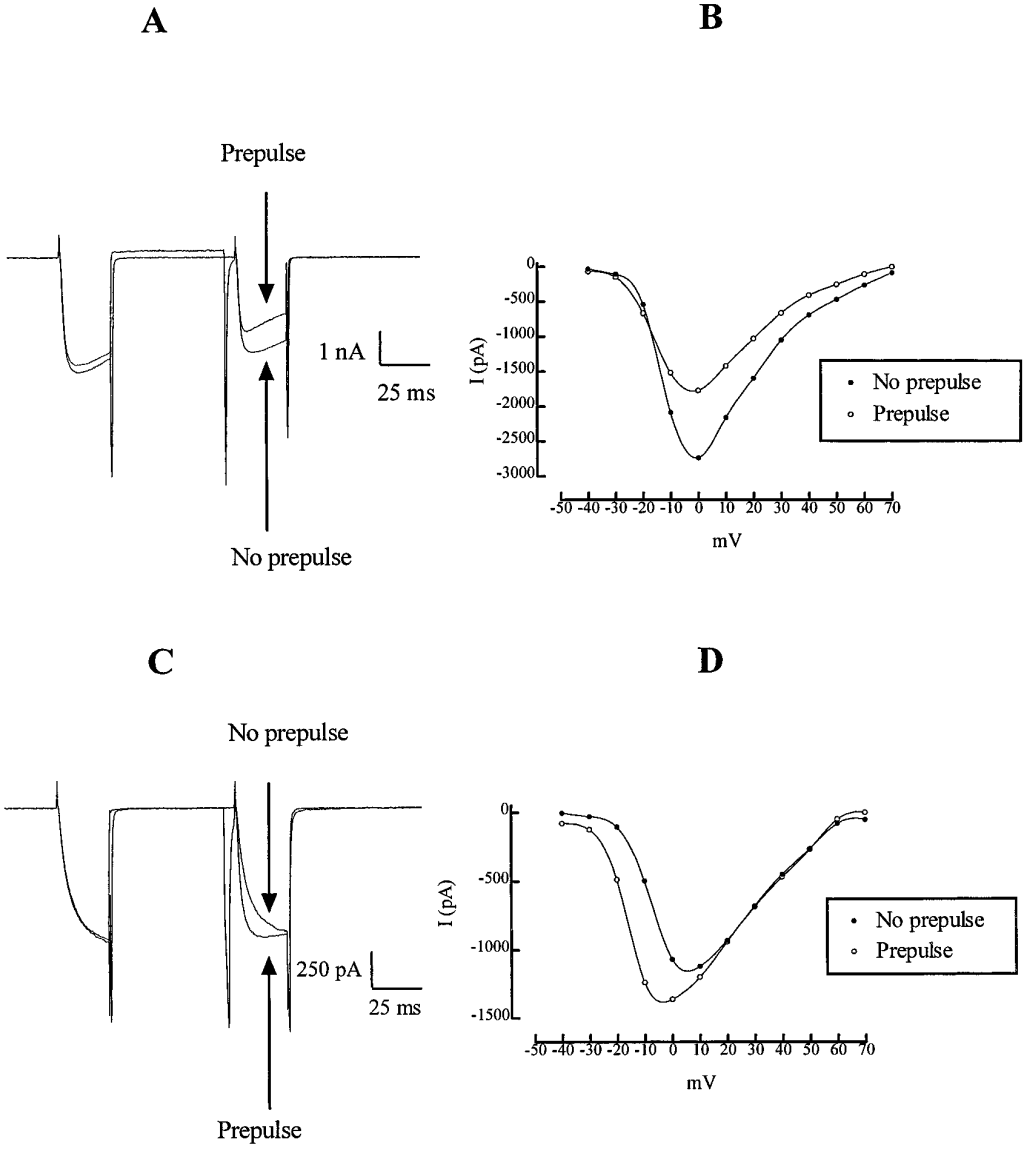

Fig. 8. A, Example of a transiently transfected HEK 293 cell expressing α1E/β1B/α2Bδ Ca^{2+} channel subunits displaying characteristics of a low-passage number E-52 cell. B, I-V relationship showing absence of shift in peak current but distinct voltage-dependent inactivation. C, Example of a transiently transfected HEK 293 cell expressing α1E/α2Bδ showing prepulse facilitation. D, I-V relationship for the cell shown in C displaying a shift in peak current from +10 mV to 0 mV.

echolamines when α1E was expressed in the GH3 cell line (33). When the effects of activating G proteins directly with guanosine-5′-*O*-(3-thio)triphosphate have been examined, voltage-dependent inhibition of α1E currents has been observed, but again these effects are smaller than those seen with α1B under the same circumstances (16).

The results of the current study further define the mechanisms by which these effects occur. As in other recent studies (6, 7), we found that expression of G protein $\beta\gamma$ subunits reduced the magnitude of the peak current and slowed the rate of current activation, which is consistent with $G_{\beta\gamma}$ having an inhibitory effect on α1B-based Ca^{2+} currents. Overexpression of constitutively active α subunits did not produce inhibition. Expression of the β or γ subunits alone was also ineffective, which is in contrast to the observations of Herlitze *et al*. (7). Such observations support suggestions that it is the $\beta\gamma$ subunits of heterotrimeric G proteins that are responsible for mediating inhibitory regulation of N-type Ca^{2+} currents. As shown in Fig. 3, Ca^{2+} currents in G1A1 cells showed a wide range of facilitation after overexpression of $G_{\beta2\gamma2}$. Indeed, in some cells, currents seemed to activate slowly, but no facilitation was observed after a prepulse. How can these observations be explained? It is probable that the basis of the voltage dependence of the α1B channel inhibition results from a reduction in the affinity of the relevant G protein subunits (presumably $\beta\gamma$) for the channel (34, 35). Presumably, if the concentration of these subunits were high enough, the rate of rebinding would be so great that the inhibition might seem to be non-voltage dependent. Thus, it is possible that in cells of this type, the overexpression of $\beta\gamma$ subunits could reach very high levels. Consequently, one reason that voltage-dependent and -independent inhibition of N channels has been observed to varying extents in neurons may relate to the available concentration of G protein $\beta\gamma$ subunits rather than to the existence of diverse mechanisms of channel inhibition. Another possibility is that there is more than one binding site for $\beta\gamma$ subunits on N channels and that these mediate slowing of current activation and steady state inhibition, respectively (35–37). Thus, in the population of cells in which only slowing was observed, it may be that the concentration of $\beta\gamma$ subunits reaches only levels at which one site is occupied. In addition, it seems likely that other forms of N channel-mediated inhibition exist that are not membrane delimited and not voltage dependent (1, 15).

Results obtained with α1E channels further clarify the mechanisms in which Ca^{2+} channels may be regulated. When we overexpressed G protein subunits in α1E-expressing cells, the results were predictable. We observed that α1E channels were inhibited by G protein $\beta\gamma$ subunits but not α subunits, as observed for α1B. The characteristics of this inhibition were similar, although the effects were smaller in magnitude. Such results closely parallel our own and other data in the literature showing that α1E currents are not very susceptible to G protein-induced modulation (16, 17). However, we were subsequently surprised to observe the behavior of α1E currents in the absence of their β subunits. We found that under these circumstances, α1E currents behaved in a similar way to G protein-inhibited currents. Because we were unable to block these effects with guanosine-5′-*O*-(2-thio)-diphosphate, we conclude that the combination of α1E and α2Bδ subunits behaves in a similar way to a G protein-inhibited channel. This type of behavior can be functionally antagonized by a Ca^{2+} channel β subunit, as previously suggested (17, 18, 36, 38). In addition, it is possible that some aspects of the observed behavior of α1E in the presence or absence of its β subunit are related to the effects of this subunit of the voltage dependence of channel inactivation (39).

The behavior of α1E we have observed could be predicted on the basis of the results of Olcese *et al.* (39), who demonstrated that expression of α1E in oocytes in the absence of a β subunit resulted in a biphasic current activation curve. This was shifted to a monophasic curve on coexpression of a β subunit. One way of looking at the situation is that the role of the G protein is to stabilize the Ca^{2+} channel α1 subunit in the "inhibited" or "unwilling" conformation, whereas the Ca^{2+} channel β subunit stabilizes the channel in the "activated" or "willing" conformation. It is not clear how the mutual functional antagonism between Ca^{2+} channel β subunits and G proteins operates at a structural level. It has been shown that the Ca^{2+} channel β subunit interacts with the Ca^{2+} channel at a site in the cytoplasmic loop linking domains 1 and 2 (36, 40). It is possible that G protein β and γ subunits also interact at that site (17, 36). The interaction could be allosteric in nature. In summary, these data also lead to the tentative hypothesis that the degree of Ca^{2+} channel inhibition produced by a receptor/G protein may depend on the type of Ca^{2+} channel β subunit found in the Ca^{2+} channel complex as well as on other factors.

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