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Identification of an Integration Center for Cross-talk between Protein Kinase C and G Protein Modulation of N-type Calcium Channels*

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The modulation of presynaptic calcium channel activity by second messengers provides a fine tuning mechanism for neurotransmitter release. In neurons, the activation of certain G protein-coupled receptors reduces N-type channel activity by ~60%. In contrast, activation of protein kinase C (PKC) results in an approximately 50% increase in N-type channel activity, and subsequent G protein inhibition is antagonized. Here, we describe the molecular determinants that control the dual effects of PKC-dependent phosphorylation. The double substitution of two adjacent PKC consensus sites in the calcium channel domain I-II linker (Thr^{422}, Ser^{425}) to alanines abolished both PKC-dependent up-regulation and the PKC-G protein cross-talk. The single substitution of Ser^{425} to glutamic acid abolished PKC up-regulation but had no effect on G protein modulation. Replacement of Thr^{422} with glutamic acid eliminated PKC-dependent up-regulation and mimicked the effects of PKC phosphorylation on G protein inhibition. Our data suggest that Thr^{422} mediates the antagonistic effect of PKC on G protein modulation, while phosphorylation of either Thr^{422} or Ser^{425} are sufficient to increase N-type channel activity. Thus, Thr^{422} serves as a molecular switch by which PKC is able to simultaneously trigger the up-regulation of channel activity and antagonize G protein inhibition.

Calcium influx through neuronal voltage-dependent calcium channels mediates a range of cytoplasmic responses, such as neurotransmitter release, proliferation, and the activation of calcium-dependent enzymes. Most neurons express multiple calcium channel types with distinct functional properties, and molecular cloning has identified genes encoding at least eight different neuronal calcium channel α_1 subunits (termed α_{1A} through α_{1H}). Functional expression in heterologous expression systems has revealed that α_{1A} encodes for P/Q-type calcium channels (1–3); α_{1B} defines an ω-conotoxin GVIA-sensitive N-type channel (4–6); α_{1C}, α_{1D} and α_{1F} are L-type calcium channels (7–9); and α_{1E} is a unique calcium channel with properties common to both high threshold and low threshold calcium channels (10, 11). More recently, α_{1G} and α_{1H} have been shown to encode members of the family of T-type calcium channels (12). Among the eight types of α_1 subunits, α_{1A} and α_{1B} are predominantly located at more distal dendritic and presynaptic nerve terminals (13, 14) and are directly coupled to the presynaptic vesicle release machinery (15, 16).

The physiological properties of presynaptic calcium channels are extensively modulated by second messenger molecules, including protein kinase C and G protein $\beta\gamma$ subunits (17–21). The activation of certain G protein-coupled seven-helix transmembrane receptors mediates a pronounced voltage-dependent inhibition of both N-type and P/Q-type calcium currents (Refs. 22–25; for a review, see Ref. 26). This inhibition is probably caused by direct 1:1 binding of G protein $\beta\gamma$ subunits to the calcium channel α_1 subunit, resulting in a reluctance of the channels to undergo opening in response to membrane depolarization (27, 28). In contrast, stimulation of protein kinase C-dependent phosphorylation results in a substantial up-regulation of N-type channel activity (19). PKC[1] and $G_{\beta\gamma}$ modulation are functionally coupled (termed cross-talk), such that PKC-dependent phosphorylation of the channel antagonizes subsequent G protein inhibition (20, 21, 29).

We have previously shown that cross-talk between G protein and PKC pathways mainly occurs at the level of the calcium channel α_1 subunit (29). In particular, the cytoplasmic linker connecting domains I and II of the α_{1B} subunit is a crucial determinant of both G protein inhibition and PKC-G protein cross-talk. This region has also been implicated in PKC-dependent up-regulation of N-type calcium channels (19), suggesting the possibility of a common mechanism underlying the dual effects of protein kinase C-dependent phosphorylation. Here, we identify individual amino acid residues within the α_{1B} domain I-II linker that mediate up-regulation by PKC-dependent phosphorylation as well as the cross-talk between PKC and G protein pathways. Using site-directed mutagenesis in combination with functional expression in human embryonic kidney cells, we show that phosphorylation of either threonine 422 or serine 425 is sufficient to mediate PKC-dependent up-regulation of the channel. Whereas the nature of serine 425 does not affect G protein inhibition, substitution of the threonine residue to glutamic acid to create a permanent phosphoform drastically reduces the degree of G protein inhibition. We propose a

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[1] The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

model whereby threonine 422 acts as a molecular switch by which protein kinase C up-regulates the activity of N-type calcium channels and concomitantly antagonizes their inhibition by G protein $\beta\gamma$ subunits. The remaining (~20%) degree of G protein inhibition is further reduced upon deletion of the 3′ third of the α_{1B} carboxyl terminus, suggesting that the calcium channel domain I-II linker and the carboxyl terminus might cooperatively interact with G protein $\beta\gamma$ subunits.

MATERIALS AND METHODS

Molecular Biology—DNA encoding wild type α_{1B} channels was subcloned into the cytomegalovirus expression vector. The α_{1B}-cytomegalovirus construct was cut with *Spe*I and *Spl*I, and the *Spe*I–*Spl*I fragment was then subcloned into a modified pSL1180 Bluescript vector (which had been cut with *Stu*I and *Sma*I and recircularized to eliminate a *Kpn*2I site in the polylinker). A *Kpn*2I fragment was excised from this construct and subcloned into a modified pSL1180 vector (in which a *Nar*I–*Nar*I fragment was deleted). Site-directed mutagenesis of PKC consensus sites was carried out on this construct using the Quick Change site-directed mutagenesis kit (Stratagene). The mutations were confirmed via DNA sequencing. The *Kpn*2I fragment was subsequently subcloned into the *Spe*I–*Spl*I construct in pSL1180, and the *Spe*I–*Spl*I fragment was ligated into the full-length α_{1B} construct in cytomegalovirus. After completion of subcloning, the 900-base pair *Kpn*2I fragment contained in the full-length sequence was completely sequenced to confirm the presence of the mutations and to eliminate the possibility of cloning and polymerase chain reaction artifacts.

A carboxyl-terminal deletion mutant lacking amino acid residues 1955–2336 was constructed by eliminating an *Xba*I fragment contained between an *Xba*I site in the α_{1B} carboxyl terminus and a second *Xba*I site in the 3′ portion of the polylinker of the cytomegalovirus expression vector. The construct was cut with *Xba*I and recircularized, and the successful elimination of the *Xba*I fragment was confirmed via enzyme analysis and DNA sequencing.

Transient Transfection—Human embryonic kidney TSA 201 cells were grown in standard Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 0.4 mg/ml neomycin. The cells were grown to 85% confluency, split with trypsin EDTA, and plated on glass coverslips at 10% confluency 12 h prior to transfection. Immediately prior to transfection, the medium was replaced, and the cells were transiently transfected with cDNAs encoding for calcium channel α_{1B}, β_{1b}, and α_2 subunits (at a 1:1:1 molar ratio) using a standard calcium phosphate protocol. After 12 h, the medium was replaced with fresh Dulbecco's modified Eagle's medium, and the cells were allowed to recover for 12 h. Subsequently, the cells were incubated at 28 °C in 5% CO_2 for 1–2 days prior to recording.

Electrophysiology—Immediately prior to recording, individual coverslips were transferred to a 3-cm culture dish containing recording solution composed of 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM triethanolamine chloride, 10 mM glucose, 65 mM CsCl (pH 7.2). Whole cell patch clamp recordings were performed using an Axopatch 200B 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 using a Sutter P-87 microelectrode puller, fire-polished using a Narashige microforge, and showed typical resistances of 2–3 megaohms. The internal pipette solution contained 105 mM CsCl, 25 mM triethanolamine chloride, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES (pH 7.2), supplemented with nystatin. Nystatin was dissolved in Me_2SO at 100 mg/ml and diluted directly into the pipette solution. After seal formation, nystatin was allowed to equilibrate into the patch for 5–10 min to permit electrical access.

Currents were typically elicited from a holding potential of −100 mV to various test potentials using Clampex software (Axon Instruments). Current-voltage relations were generated by utilizing a ramp protocol (dV/dt = 1 mV/ms) as reported previously (30). Somatostatin (RBI Chemicals) was dissolved in water to give a stock solution of 1 mM, and PMA (RBI) was dissolved in Me_2SO at a stock concentration of 2 mM. These compounds were diluted into the external recording solution at the appropriate final concentrations and perfused directly onto the cell using a gravity-driven microperfusion system. At the applicable concentrations, Me_2SO by itself had no effect on calcium channel activity. In every case, peak current inhibition was assessed 15 s after somatostatin application. Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Data were analyzed using Clampfit (Axon Instruments). All curve fitting was carried out in Sigmaplot 4.0 (Jandel Scientific). Unless stated otherwise, all error bars represent S.E. values, numbers in parentheses displayed in the *figures* reflect numbers of experiments, and p values given reflect Student's t tests.

RESULTS

The Domain I-II Linker Mediates both PKC and G Protein Modulatory Effects on N-type Channels—We have previously shown that N-type (α_{1B} + $\alpha_2\delta$ + β_{1b}) calcium channels transiently expressed in human embryonic kidney cells are reversibly inhibited by 50–70% via activation of endogenous somatostatin receptors (Ref. 29; see Fig. 1*C*). In contrast, activation of protein kinase C with 100–400 nM of the phorbol ester PMA results in a pronounced (50–60%) up-regulation of α_{1B} N-type currents (Ref. 29; see Fig. 1*D*). Subsequent to PMA treatment, inhibition of N-type channel activity by somatostatin is dramatically reduced (see Fig. 1*D*), consistent with previous observations in intact neurons (20, 21). Synthetic peptides directed against two subregions of the α_{1B} calcium channel domain I-II linker block the modulatory effects of exogenously applied $G_{\beta\gamma}$ subunits (29). One of the peptides is a substrate for *in vitro* phosphorylation by protein kinase C, and when phosphorylated *in vitro*, it loses the ability to interfere with G protein modulation (29). These results suggested that cross-talk between protein kinase C and G protein pathways might occur in this subregion of the α_{1B} domain I-II linker.

Two putative protein kinase C consensus sites, Thr^{422} and Ser^{425}, are contained with this region of the channel (Fig. 1, *inset*). To test whether one or both of these residues might mediate PKC/$G_{\beta\gamma}$ cross-talk, we replaced both sites with alanine residues. Fig. 1*A* depicts current records obtained from the double alanine mutant in the absence and the presence of 100 nM somatostatin. Similar to that observed with the wild type channel (Fig. 1), activation of somatostatin receptors mediates a reversible and pronounced inhibition of channel activity, paired with a slowing of activation kinetics and an apparent slowing of inactivation. Fig. 1*B* illustrates the effect of PMA on channel activity and on somatostatin modulation of the alanine double mutant. While application of 100 nM PMA had no detectable effect on channel activity, the subsequent application of 100 nM somatostatin produced the same degree of G protein inhibition as that observed in the absence of PMA. Fig. 1, *C* and *D*, illustrates the effect of alanine substitution for a number of experiments. There are two effects evident. First, the alanine mutation *per se* significantly reduces the degree of somatostatin inhibition seen with the wild type channel from 53 ± 5 to 36 ± 5% (p = 0.02) (Fig. 1*C*). Second, while PMA treatment reduced the somatostatin effect for the wild type channel, the somatostatin sensitivity of the double mutant was not altered by PMA (36 ± 5 *versus* 37 ± 5%, p = 0.86; Fig. 1*C*), indicating that cross-talk between PKC and G protein pathways is blocked by the double alanine substitution. In addition, the PMA-induced up-regulation observed with the wild type channels was reduced from 49 ± 14 to 6 ± 5% when both PKC consensus sites were simultaneously replaced with alanines (Fig. 1*D*). Thus, the critical structures mediating up-regulation of channel activity and inhibition of direct G protein action appear to reside within overlapping regions of the calcium channel domain I-II linker and contain one or both of Thr^{422} and Ser^{425}.

To elucidate which of the two residues mediate the effects of PKC, we "permanently phosphorylated" either Thr^{422} or Ser^{425} by replacing them individually with glutamic acid residues. As shown in Fig. 2*A*, the S425E mutant exhibits a somatostatin response comparable with that of the wild type channel (p = 0.41), suggesting that phosphorylation of Ser^{425} does not antagonize G protein action. PMA treatment had no significant effect on current amplitude (p = 0.83, paired t test; see also Fig.

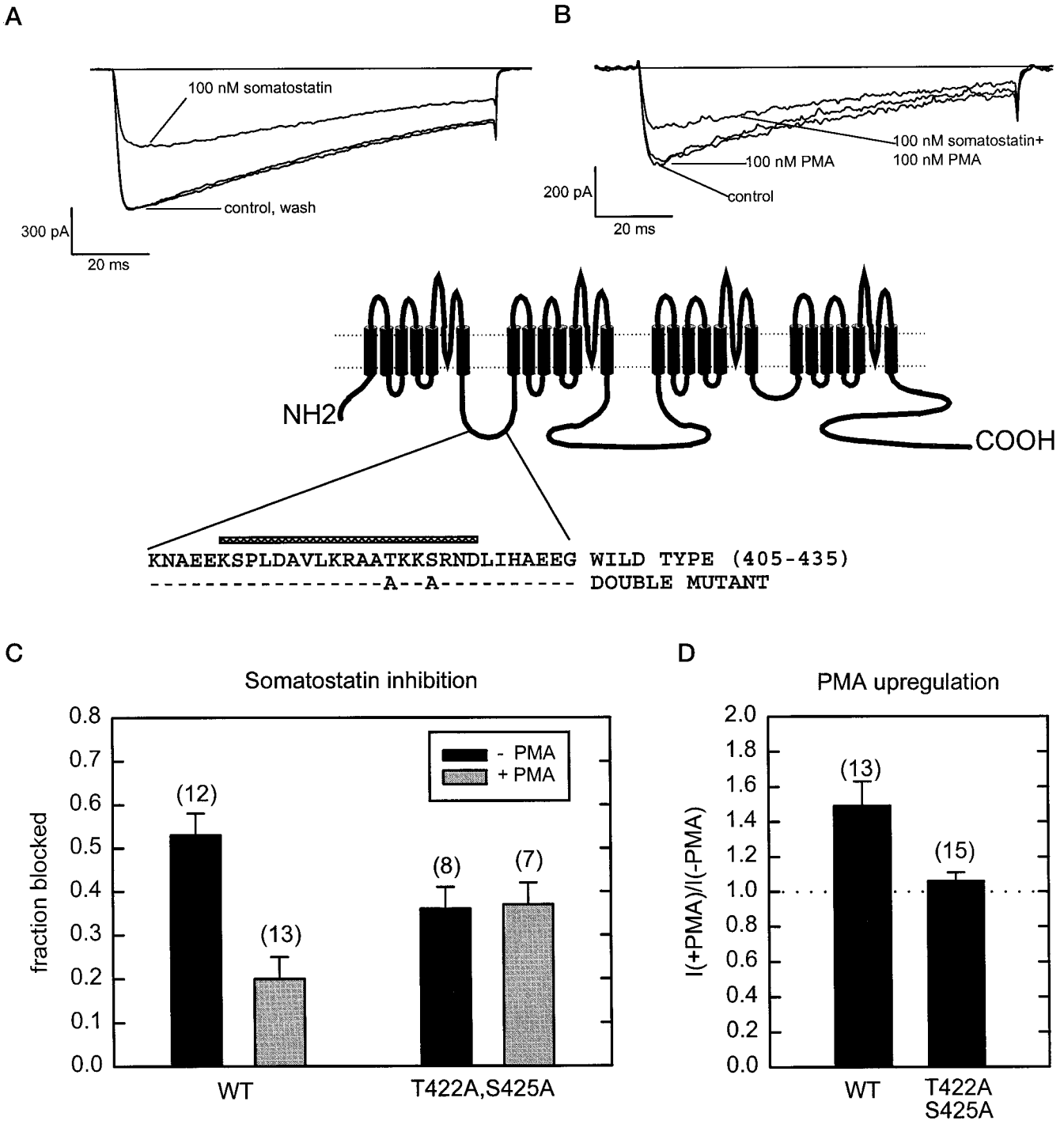

FIG. 1. **Effect of a double substitution of I-II linker protein kinase C consensus sites for alanines on PKC and G protein action.** *A*, current records illustrating G protein inhibition of the double alanine mutant. Somatostatin reversibly reduces the peak current amplitude of the mutant channel to 65% of its control value. The currents were leak-subtracted using a *p*/5 protocol. The holding potential was −100 mV, and the test potential was +20 mV. *B*, application of PMA does not affect current amplitude and has no adverse effects on G protein inhibition mediated by somatostatin (holding potential −100 mV, test potential = +20 mV). *Inset*, proposed transmembrane topology of voltage-dependent calcium channels and amino acid sequence of part of the α_{1B} channel I-II linker. The *bar above* the amino acid sequence indicates a previously identified putative target region for G protein/PKC cross-talk. The Thr^{422} and Ser^{425} residues were substituted to alanine residues. *C*, degree of somatostatin (100 nM) inhibition of wild type α_{1B} channel and the alanine (422 and 425) mutant channels (each coexpressed with β_{1b} and α_2) with or without prior application of 100 nM PMA. Note that the alanine substitution slightly but significantly reduces the degree of somatostatin inhibition. While PMA reduces the G protein sensitivity of the wild type channel, the G protein inhibition of the double mutant is not affected. *D*, up-regulation of channel activity by 100 nM PMA. Note that the up-regulation seen with the wild type channel is blocked by the double alanine substitution. *Error bars* represent S.E. values; the test potential in *C* and *D* was +20 mV.

3*B*), suggesting that replacing Ser^{425} with glutamic acid mimics a permanently up-regulated state of the channel. Nonetheless, PMA treatment significantly attenuated the degree of somatostatin inhibition from 46 ± 7 to 15 ± 4% ($p = 0.004$) (Figs. 2*B* and 3*A*), suggesting that replacement of Ser^{425} with glutamic acid does not preclude phosphorylation of the adjacent Thr^{422} residue and that cross-talk between PKC and $G_{\beta\gamma}$ modulation is probably mediated by Thr^{422} rather than Ser^{425}. If this is correct, then mimicking phosphorylation of Thr^{422} (*i.e.* T422E) should reduce the degree of G protein inhibition to

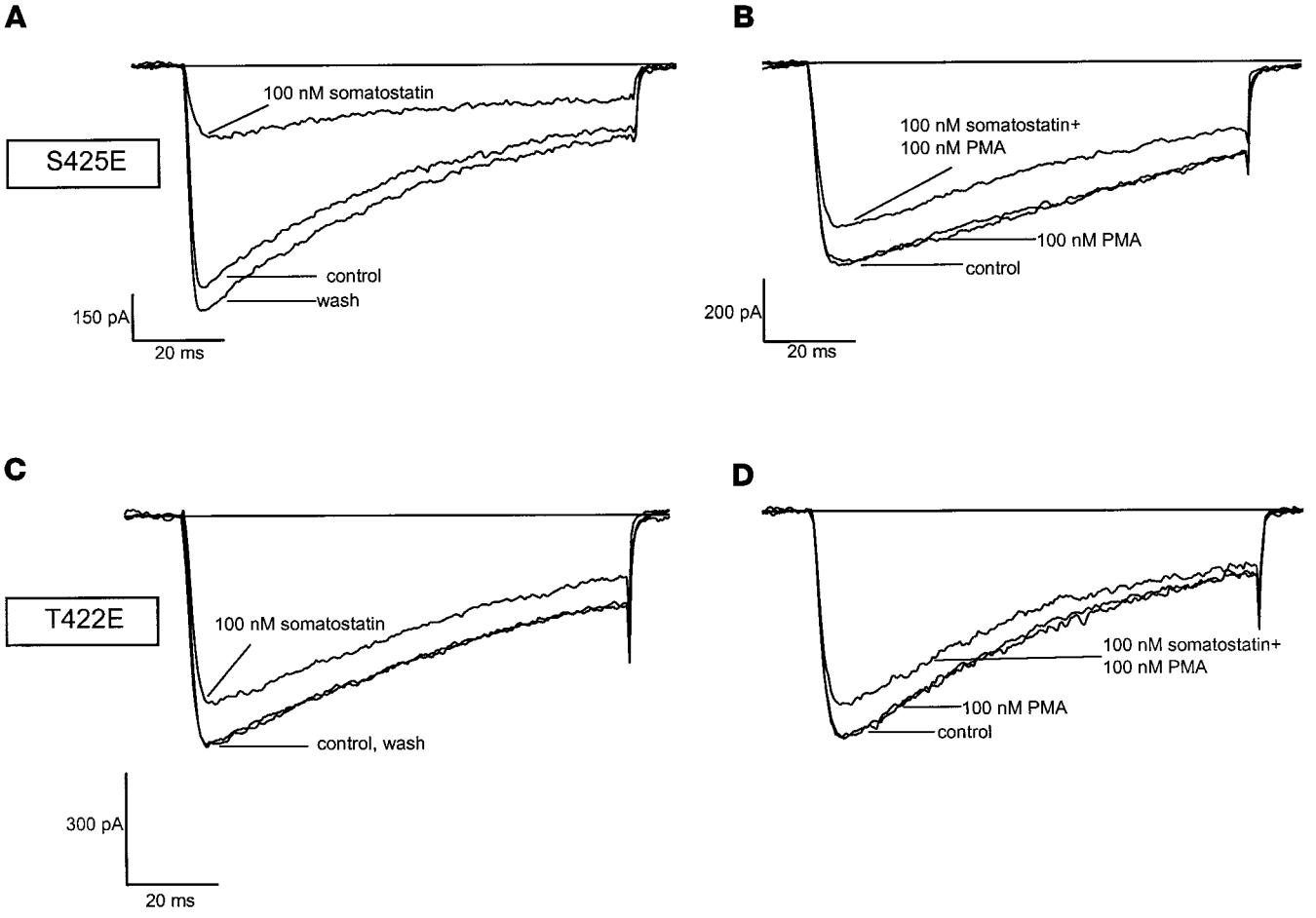

FIG. 2. **Effect of individual replacement of I-II linker PKC consensus sites with glutamic acid residues.** The holding potential was −100 mV, the test potential was +20 mV, and currents were leak-subtracted using a *p*/5 protocol. *A* and *C*, inhibition of mutant channels by 100 nM somatostatin. Note that the threonine substitution dramatically reduces G protein sensitivity. *B* and *D*, effect of PMA on channel activity and on the degree of somatostatin inhibition. Either mutation blocks up-regulation of the channel by PMA. S425E shows a reduced sensitivity to somatostatin inhibition following application of PMA (*i.e.* cross-talk remains intact), while no additional effect of PMA on G protein inhibition of T422E is evident.

those levels observed after PKC phosphorylation of the wild-type channel. This is supported by the current records shown in Fig. 2, *C* and *D*, and the data presented in Fig. 3*A*. The T422E mutant showed a significantly reduced somatostatin sensitivity, which was comparable in magnitude with that observed with the wild type channel after PMA treatment (WT (with PMA) = 20 ± 5%; T422E (without PMA) = 20 ± 3%, $p = 0.91$). These data indicate that replacing Thr^{422} with a negatively charged side group mimics the antagonistic effect of PKC on G protein inhibition. PMA application did not further affect somatostatin sensitivity of T422E (T422E (with PMA) = 14 ± 3%, $p = 0.29$). Also, PMA failed to increase the peak current amplitude of T422E ($p = 0.71$) (Figs. 2*D* and 3*B*), suggesting that similar to S425E, the T422E construct is likely be tonically up-regulated. Overall, the data suggest that whereas only Thr^{422} is capable of mediating the cross-talk effect, phosphorylation of either Ser^{425} or Thr^{422} is sufficient to fully up-regulate channel activity.

To confirm this hypothesis, we created two additional mutants in which Thr^{422} and Ser^{425} were substituted individually by alanines in order to further define the relative contributions of the individual PKC consensus sites to the overall action of PKC. As shown in Fig. 4*A*, T422A exhibits a somatostatin sensitivity that closely parallels that seen with the T422A/S425A double mutant shown in Fig. 1. After treatment with 100 nM PMA, the degree of somatostatin inhibition of T422A did not decrease significantly (37 ± 5% (without PMA) *versus* 32 ± 3% (with PMA), $p = 0.42$) and remained significantly ($p < 0.003$) larger than that of the PMA-treated wild type channel (20 ± 5%). These data indicate that cross-talk between PKC and G protein pathways is blocked upon selective removal of the Thr^{442} PKC substrate. Consistent with this notion, removal of the Ser^{425} PKC substrate (*i.e.* S425A) did not significantly change G protein sensitivity ($p = 0.49$), nor did it affect cross-talk between the G protein and PKC pathways (48 ± 7% (without PMA) *versus* 18 ± 3% (with PMA), $p < 0.002$, Fig. 4*A*). Both T422A and S425A exhibited a similar degree of PKC-dependent up-regulation (Fig. 4*B*), which did not differ significantly from that observed with the wild type channel ($p > 0.79$). This further supports the notion that N-type channel activity is fully up-regulated upon phosphorylation of either Thr^{422} or Ser^{425}, whereas only Thr^{422} is capable of mediating the cross-talk between PKC and G protein pathways.

Voltage Dependence of G Protein Modulation—To examine the voltage dependence of the T422E mutant, we utilized a ramp protocol to allow the acquisition of complete current-voltage relations without contamination from receptor desensitization. Fig. 5 compares the somatostatin response of wild type channels to that of the T422E mutant at a number of test potentials. In both cases, the effect of somatostatin is dependent on membrane potential and is consistent with the direct inhibition of native N-type calcium channels by G proteins (18, 23, 24, 31, 32). As evident from Fig. 5, somatostatin produced a significantly greater inhibition of the wild type channels at all

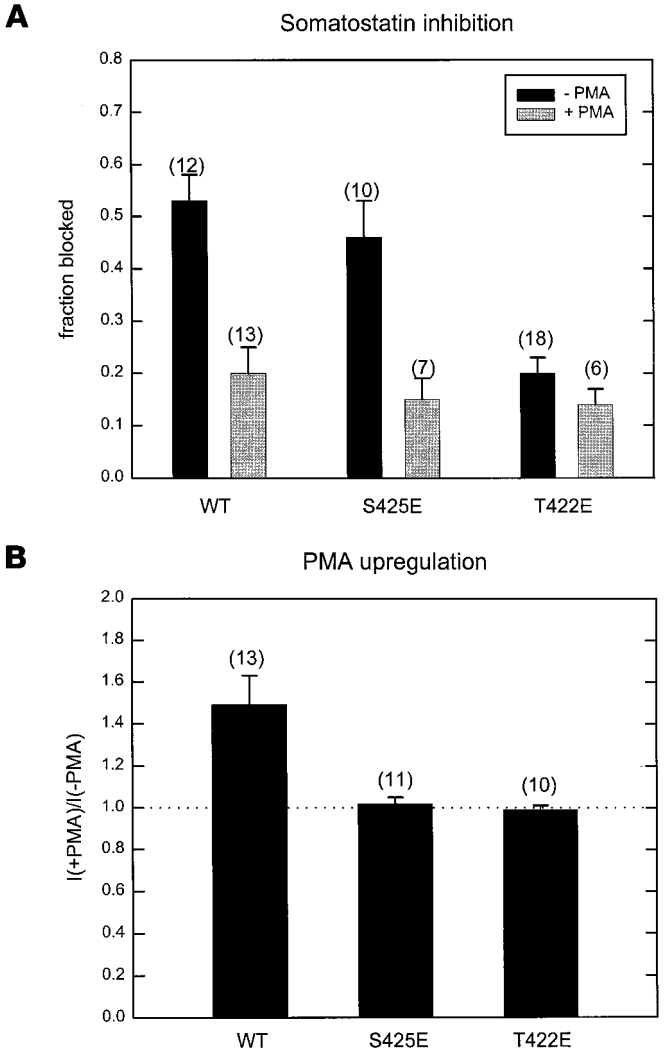

FIG. 3. *A*, effect of somatostatin on calcium channel N-type channel activity with or without prior application of 100 nM PMA. The S425E mutant exhibits a somatostatin sensitivity that is not significantly different from that of the wild type channel. With prior application of PMA, the degree of somatostatin inhibition of both the wild type and the S425E mutant is reduced to similar levels. In contrast, even in the absence of PMA, the T422E mutant exhibits a somatostatin sensitivity that parallels that of a PMA-treated wild type channel, and PMA does not further effect somatostatin sensitivity. The holding potential was −100 mV, and the test potential was typically +20 mV. *Error bars* represent S.E. values. *B*, up-regulation of wild type and mutant channels by 100 nM PMA. Note that the glutamic acid substitution of either Thr^{422} or Ser^{425} blocks up-regulation by PMA. Experimental conditions were as described for *A*.

potentials. Although the T422E mutant is capable of undergoing G protein modulation, the voltage dependence of $G_{\beta\gamma}$ dissociation appears be shifted toward more hyperpolarized potentials in the mutant channel.

Contribution of the α_{1B} Carboxyl Region in PKC and G Protein Modulation—Several studies have implicated the carboxyl terminus in the direct G protein modulation of voltage-dependent calcium channels (33, 34). Furthermore, the carboxyl terminus contains several putative protein kinase C consensus sites. To examine the possibility that the carboxyl terminus might contribute to the modulation of N-type calcium channels by PKC and G proteins, we examined a deletion mutant in which the last third of the carboxyl terminus of α_{1B} (residues 1955–2336) was deleted ($\alpha_{1B}\Delta$COOH). Fig. 6*B* depicts a current record obtained with the deletion mutant in the presence and absence of somatostatin. Although somatostatin reduces the peak current amplitude and mediates the slowing of activation kinetics typical of direct $G_{\beta\gamma}$ modulation, the degree of inhibition is reduced compared with the wild type channel (from 53 ± 5% to 32 ± 2%, $p = 0.02$). In contrast, up-regulation by PMA remains intact (Fig. 6*C* and *inset*), suggesting that the deleted portion of the carboxyl-terminal does not directly mediate PKC-dependent changes in channel activity. Following pretreatment with PMA, somatostatin application resulted in only a small effect on peak current amplitude (4 ± 2% inhibition), at a test potential of +20 mV (Fig. 6*D*). Hence, PKC-dependent phosphorylation in combination with deletion of the carboxyl terminus further reduces the degree of $G_{\beta\gamma}$ modulation. To further examine this observation, we deleted the carboxyl-terminal region of the T422E mutant. As seen from Fig. 6*D*, this mutant showed only a ~10% inhibition in response to somatostatin at a test potential of +20 mV. The degree of inhibition did not differ significantly from that seen with mutant T422E after PMA treatment ($p = 0.1$) but was significantly lower than the sensitivity of the $\alpha_{1B}\Delta$COOH construct ($p = 0.025$). These data indicate that deletion of the carboxyl terminus and replacement of Thr^{422} with glutamic acid produce additive effects on G protein sensitivity. This particular construct did not express well in HEK cells, and we were unable to systematically examine the voltage dependence of somatostatin action.

Overall, the data suggest that the carboxyl region mediates an important role in stabilizing the $G_{\beta\gamma}$ interaction with the calcium channel α_1 subunit, especially when PKC sites in the α_{1B} domain I-II linker are phosphorylated.

DISCUSSION

Protein Kinase C-dependent Up-regulation Is Mediated by the Calcium Channel I-II Linker—Whole cell currents of exogenously expressed α_{1B} N-type channels are up-regulated by activation of protein kinase C by either phorbol esters (such as PMA) or activation of coexpressed metabotropic glutamate receptors (19). Here, we have used application of 100 nM PMA to stimulate protein kinase C in human embryonic kidney cells expressing α_{1B} channels. Consistent with the results of Stea and co-workers (19), PMA application resulted in a pronounced increase in channel activity for wild type α_{1B} channels that was blocked by pretreatment with staurosporine. In their study, Stea and co-workers (19) were able to confer aspects of PKC sensitivity of α_{1B} onto the less sensitive α_{1A} channels by inserting the domain I-II linker of α_{1B} into α_{1A}. We have previously shown that a fusion protein directed against the α_{1B} I-II linker region is a substrate for PKC-dependent phosphorylation (29). Two considerations have led us to focus on a pair of PKC consensus sites (Thr^{422} and Ser^{425}) located within a 20-amino acid stretch (residues 410–428) of the α_{1B} I-II linker. First, this stretch of residues is both a substrate for *in vitro* phosphorylation by PKC and has also been implicated in the PKC-mediated antagonism of G protein inhibition of wild type α_{1B} channels (29). Second, certain amino acid substitutions in the vicinity of the corresponding region in α_{1A} increases PKC sensitivity of α_{1A}.[2]

Here, we show that the double substitution of Thr^{422} and Ser^{425} for alanines abolishes the PKC-dependent up-regulation of α_{1B} channels, suggesting that phosphorylation of one or both of these residues is sufficient to mediate up-regulation. Individual substitutions of these residues for glutamic acid also precluded the effect of PKC stimulation. In contrast, individual substitution of these two residues for alanines had no adverse effect on PKC-dependent up-regulation. These data imply that the effects of phosphorylation of the two PKC consensus sites are nonadditive and that phosphorylation of either Thr^{422} or

[2] G. W. Zamponi and T. P. Snutch, unpublished observations.

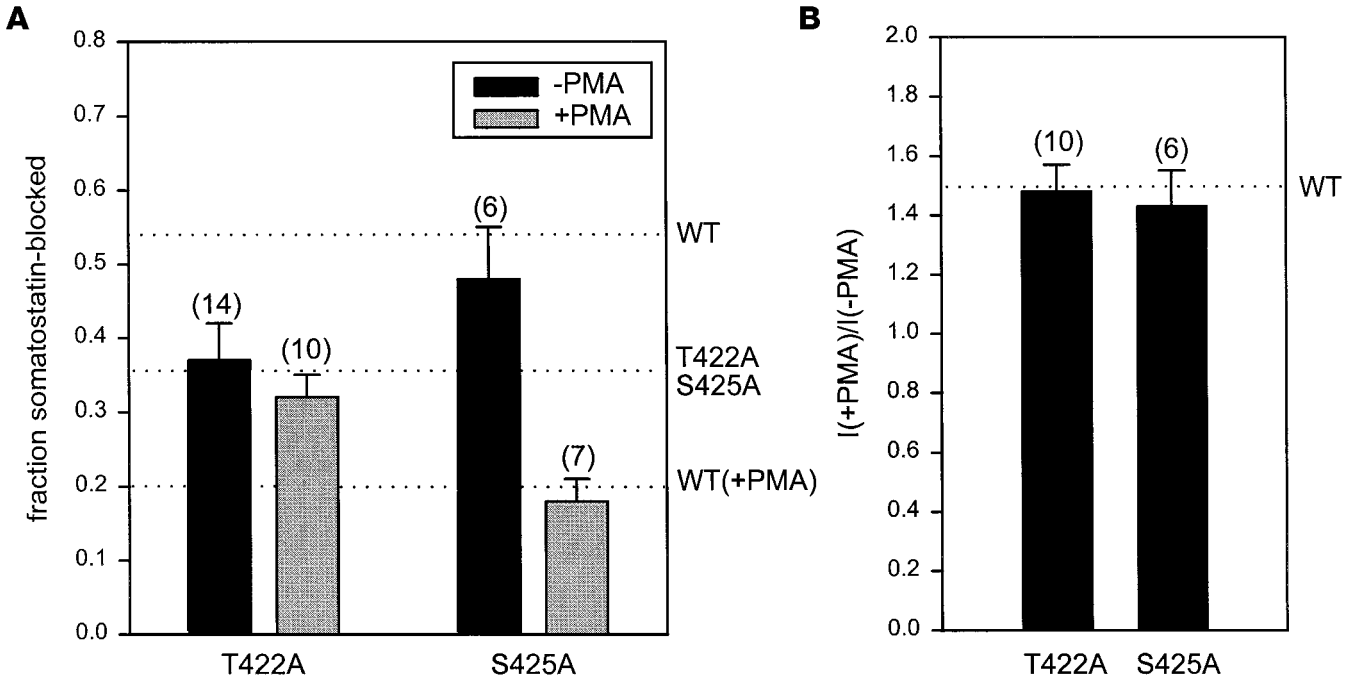

FIG. 4. **Effect of individual alanine substitutions in the I-II linker PKC consensus sites on PKC and G protein inhibition of N-type calcium channels.** *Error bars* represent S.E. values, and *numbers in parentheses* indicate the numbers of experiments. The *dotted lines* indicate the levels of G protein inhibition (*A*) or PKC-dependent up-regulation (*B*) depicted in Fig. 1. *A*, inhibition of T422A and S425A by 100 nM somatostatin with and without prior application of 100 nM PMA. Note that T422A does not permit PKC activation to antagonize somatostatin inhibition, whereas the S425A mutant exhibits the behavior seen with the wild type channel. The level of somatostatin-induced inhibition of T422A closely parallels that observed with the T422A/S425A double mutant examined in Fig. 1*B*. The PKC-dependent up-regulation observed with T422A and S425A is similar to that observed with the wild type channel. The *dotted line* indicates the level of up-regulation of the wild type channel.

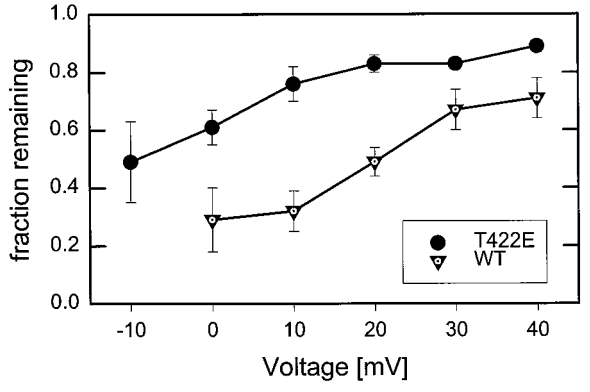

FIG. 5. **Voltage dependence of G protein inhibition for wild type (n = 7) and mutant T422E (n = 12) α_{1B} channels elicited by application of 100 nM somatostatin.** The data were obtained via ramp protocols as described under "Materials and Methods" 15 s after somatostatin application. Note that the voltage dependence of somatostatin inhibition is shifted toward more hyperpolarizing potentials for the mutant channels, resulting in a significantly greater inhibition of the wild type channels at each potential. *Error bars* represent S.E. values.

Ser^{425} is sufficient to mediate complete up-regulation of channel activity in an all or none manner. At present, the molecular mechanisms by which the phosphorylation event affects channel activity remain to be determined. It is possible that phosphorylation induces a conformational change in the domain I-II linker that directly affects activation. Alternatively, phosphorylation of these residues might alter the interaction with the calcium channel β subunit, which in turn may affect channel activation. Such a mechanism would be consistent with data of Stea and co-workers (19), who reported that the calcium channel β subunit is required for PKC-dependent up-regulation.

Recently, Shistik *et al.* (35) showed that deletion of N-terminal residues 2–46 abolished PKC-dependent up-regulation of rabbit heart α_{1C} channels expressed in *Xenopus* oocytes. The N-terminal region of rat brain α_{1B} N-type calcium channel is 51 residues shorter than that of the rabbit heart α_{1C} channel and thus lacks the motif identified by Shistik *et al.* (35). Furthermore, there is no counterpart to Thr^{422} present in the rabbit heart α_{1C} sequence, and the analog to Ser^{425} (Ser^{499} in the rabbit heart α_{1C} sequence) is not part of a PKC consensus motif. This suggests that the molecular mechanism underlying the PKC-dependent modulation of N-type calcium channel activity is fundamentally different from that for the rabbit cardiac L-type isoform.

Model for the G Protein Inhibition of N-type Calcium Channels—Over the past several years, a number of studies have examined the molecular determinants of G protein modulation of presynaptic calcium channels (18, 28, 29, 31–34, 36–40). It is now widely accepted that the $G_{\beta\gamma}$ subunits are the active G protein species mediating the antagonistic effect on presynaptic calcium channel activity (28, 29, 31, 32). The G protein $\beta\gamma$ subunits are able to interact with two separate regions within the calcium channel domain I-II linker (29, 36). We have previously suggested that the PKC dependent phosphorylation of one of these two I-II linker $G_{\beta\gamma}$ binding motifs might mediate the previously identified antagonistic effect of PKC stimulation on G protein sensitivity (29). Here, we present further confirmation for the involvement of the domain I-II linker region in direct G protein modulation of N-type calcium channels. Each, the double alanine mutant and the T422E and T422A constructs, exhibited a significantly reduced sensitivity to somatostatin-induced $G_{\beta\gamma}$ modulation. Neither of these substitutions resulted in significant changes in current kinetics or half-activation potential, minimizing the possibility of an indirect effect due to changes in channel gating. We suggest the possibility that these substitutions more likely reduce the affinity of the channel for binding $G_{\beta\gamma}$.

In addition to the domain I-II linker, the carboxyl terminus

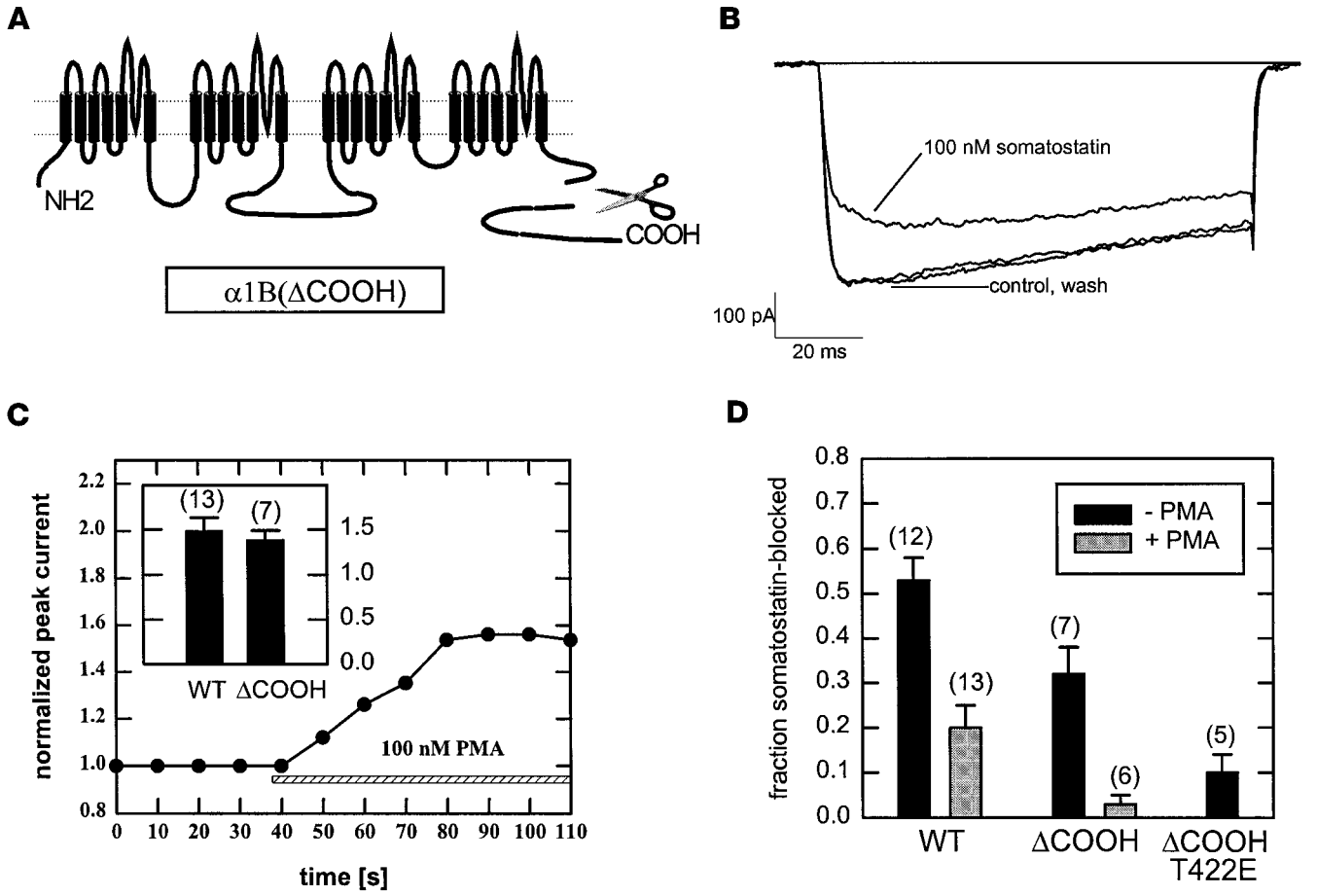

FIG. 6. **Effect of deletion of residues 1955–2336 of the α_{1B} carboxyl terminus on G protein and PKC sensitivity.** *A*, schematic representation of the proposed calcium channel transmembrane topology indicating the deletion of part of the carboxyl terminus region. *B*, current record elicited by a step depolarization from −100 to +20 mV and leak-subtracted via a *p*/5 protocol. Deletion of the 3′ third of the carboxyl terminus region results in a slowing of inactivation kinetics as well as in a reduced degree of G protein inhibition as evident by application of 100 nM somatostatin. *C*, time course of up-regulation of current activity by 100 nM PMA. The *bar graphs* depicted in the *inset* illustrate the degree of PMA up-regulation as defined by the peak current ratio *I* (with PMA)/*I*(without PMA) for the wild type channel and the deletion mutant. *D*, degree of somatostatin-mediated G protein inhibition of the wild type channel and the deletion mutant in the presence and absence of 100 nM PMA. Note that the degree of G protein inhibition is significantly attenuated upon deletion of the 3′ portion of the α_{1B} carboxyl terminus. Pretreatment with 100 nM PMA almost completely abolished G protein inhibition at test potentials of +20 mV. A double mutant (Δ*COOH,T422E*) also exhibited a reduced G protein sensitivity that did not differ significantly from that seen with T422E after PMA treatment. *Error bars* represent S.E. values.

(33, 34) as well as domain I (38) and the amino terminus (39) of the calcium channel α_1 subunit have all been implicated in direct G protein modulation. Here, we present corroborating evidence that the carboxyl terminus contributes to a portion of the overall G protein inhibition of N-type channels. A deletion of one-third of the carboxyl-terminal region significantly reduced but did not eliminate the somatostatin-induced inhibition of α_{1B} channels. The deleted portion contains a highly conserved motif that was recently implicated in $G_{\beta\gamma}$ binding to α_{1E} channels (33). Together with our results, it appears that while the carboxyl terminus probably contributes to $G_{\beta\gamma}$ binding to N-type calcium channels, the major determinant of G protein action is the domain I-II linker. That the carboxyl terminus contributes to $G_{\beta\gamma}$ binding could account for the observation that α_{1B} or α_{1E} channel constructs containing the α_{1C} I-II linker remain sensitive to G protein inhibition (33, 34)[3] despite the fact that α_{1C} I-II linker fusion proteins do not bind $G_{\beta\gamma}$. It is possible that the carboxyl region cooperatively enhances binding to the α_{1C} I-II linker in these chimeric constructs.

Kinetic modeling of G protein inhibition of single N-type calcium channels has shown that G protein binding results in a reluctance of channels to undergo transitions from the closed states to channel opening (27). Upon membrane depolarization, G proteins dissociate from the channels prior to opening, and the associated increase in first latency to opening results in a decrease in peak current amplitude. Within the framework of this model, the binding of $G_{\beta\gamma}$ to the domain I-II linker and perhaps the carboxyl terminus mediates the stabilization of the closed state by inducing a conformational change in the channel protein. The translation of G protein binding into a change in channel function may be mediated by residues located in domain I, consistent with previous studies (34, 38, 39).

Overall, a model emerges in which $G_{\beta\gamma}$ interacts with two high affinity regions within the calcium channel domain I-II linker (residues 353–389 and 410–428 (29, 36) and a lower affinity site in the carboxyl terminus (33). PKC-dependent phosphorylation probably destabilizes $G_{\beta\gamma}$ binding to the second site within the I-II linker $G_{\beta\gamma}$ binding domain (*i.e.* residues 410–428), thereby shifting the voltage dependence of $G_{\beta\gamma}$ dissociation to more hyperpolarized potentials and perhaps reducing the increase in first latency to opening associated with $G_{\beta\gamma}$ binding (27). Single channel experiments will ultimately be required to confirm any effects of phosphorylation (and of the T422E substitution) on first latency.

Implications of Dual PKC Sites for Calcium Channel Modu-

[3] E. Bourinet, personal communication.

lation—Our data indicate that up-regulation of N-type channel activity occurs via phosphorylation of either Thr^{422} or Ser^{425}, whereas G protein modulation is antagonized by phosphorylation of only Thr^{422}. The selective phosphorylation of Ser^{425} would result in up-regulation by ~50%, and the subsequent stimulation of the G protein pathway would produce a ~50% inhibition of the PKC-enhanced current, resulting in an overall inhibition of ~25%. Phosphorylation of Thr^{422} (or of both residues simultaneously) would also result in the 50% up-regulation, but subsequent G protein inhibition would be attenuated to ~20% and result in a net up-regulation by ~20%. Since activation of PKC and G protein pathways individually would respectively produce a 50% increase and a 50% decrease in control current levels, Thr^{422} and Ser^{425} may function as an integration center for inputs from PKC and G protein pathways to produce multiple levels of calcium channel activity. Together with recent reports that protein kinase C-dependent phosphorylation disrupts the interactions between syntaxin and the calcium channel II-III linker (16), this convergence of second messenger pathways directly at the level of the calcium channel α_1 subunit would provide a mechanism to precisely control neurotransmitter release at presynaptic nerve terminals.

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