Cross-talk between G-protein and Protein Kinase C Modulation of N-type Calcium Channels Is Dependent on the G-protein β Subunit Isoform

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The modulation of N-type calcium current by protein kinases and G-proteins is a factor in the fine tuning of neurotransmitter release. We have previously shown that phosphorylation of threonine 422 in the α1b calcium channel domain I-II linker region resulted in a dramatic reduction in somatostatin receptor-mediated G-protein inhibition of the channels and that the I-II linker consequently serves as an integration center for cross-talk between protein kinase C (PKC) and G-proteins (Hamid, J., Nelson, D., Spaetgens, R., Dubel, S. J., Snutch, T. P., and Zamponi, G. W. (1999) J. Biol. Chem. 274, 6195–6202). Here we show that opioid receptor-mediated inhibition of N-type channels is affected to a lesser extent compared with that seen with somatostatin receptors, hinting at the possibility that PKC/G-protein cross-talk might be dependent on the G-protein subtype. To address this issue, we have examined the effects of four different types of G-protein β subunits on both wild type and mutant α1b calcium channels in which residue 422 has been replaced by glutamate to mimic PKC-dependent phosphorylation and on channels that have been directly phosphorylated by protein kinase C. Our data show that phosphorylation or mutation of residue 422 antagonizes the effect of Gβ1 on channel activity, whereas Gβ2, Gβ5, and Gβ6 are not affected. Our data therefore suggest that the observed cross-talk between G-proteins and protein kinase C modulation of N-type channels is a selective feature of the Gβ1 subunit.

The modulation of calcium channel activity by activation of intracellular messenger pathways is a key mechanism for fine tuning calcium entry into neurons. For example, the activation of protein kinase C has been shown to mediate an up-regulation of N-type calcium currents in intact neurons (1, 2) and in transient expression systems (3, 4). In contrast, the direct 1:1 binding of G protein βγ subunits to the domain I-II linker region of N-type, P/Q-type, and possibly R-type calcium channels results in a depression of current activity (5–8) (reviewed in Refs. 9 and 10), which can be reversed by strong membrane depolarization (10–12). Different types of calcium channels are modulated by G-proteins to different extents, such that N-type channels are typically inhibited more effectively than P/Q-type channels (13–16). There is also increasing evidence that the degree of inhibition is dependent on the G-protein β subunit species (16–18). Finally, it has been shown that protein kinase C-dependent phosphorylation of the N-type calcium channel α1 subunit antagonizes receptor-mediated G-protein inhibition of the channel (1, 2, 12, 19). This phenomenon (termed PKC/G-protein cross-talk) appears to be mediated by a single threonine residue (Thr-422) in the α1b domain I-II linker region (4). For somatostatin receptor-induced G-protein inhibition of N-type calcium channels, mutation of Thr–422 to glutamic acid mimics the antagonistic effect of protein kinase C on G-protein inhibition, whereas a switch to alanine precludes the occurrence of PKC/G-protein cross-talk (4).

Here we have examined the dependence of PKC/G-protein cross-talk on the nature of the G protein β subunit isoform. Using transient expression of either wild type or mutant (T422E) N-type calcium channels in combination with various G-protein β subunit isoforms (Gβ1, Gβ2, Gβ5, and Gβ6), we show that the effect of only the Gβ1 isoform is reduced in the “permanently phosphorylated” mutant N-type channel. PKC/G-protein cross-talk thus appears to be a selective feature of the Gβ1 subunit isoform. In view of the notion that different types of G-protein-coupled receptors may combine with specific subsets of Goγy combinations (20), this may suggest that the extent of cross-talk occurring in intact neurons could be dependent on the type of neurotransmitter involved, thus providing a mechanism for the fine tuning of calcium homeostasis.

EXPERIMENTAL PROCEDURES

Calcium Channel and G-protein cDNAs—The calcium channel cDNA constructs (α1b, α1b(T422E), β1b, α2γβδ) were the same as those discussed previously in Hamid et al. (4). Wild type constructs were kindly donated by Dr. T. P. Snutch. The cloning of the various Gβ-subunits is described...
PKC/G-protein Cross-talk

Transient Transfection into tsa-201 Cells—Human embryonic kidney tsa-201 cells were grown in standard Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.5 mg/ml penicillin/streptomycin. Cells were grown at 37°C to 85% confluency, split with trypsin-EDTA, and plated on glass coverslips at 5–10% confluency about 12 h before transfection. Immediately before transfection, the medium was replaced with fresh Dulbecco’s modified Eagle’s medium, and a standard calcium phosphate protocol was used to transiently transfect the cells with cDNA constructs encoding for mutant and wild type calcium channel α1, β1, and α2δ subunits and green fluorescent protein as an expression marker and as appropriate with G-protein subunits or the α2δ-opioid receptor. After 12 h, cells were washed with fresh medium, allowed to recover for 12 h, and then incubated at 28°C in 5% CO2 for 1–3 days prior to recording.

Electrophysiology—The exact whole cell recording procedures have been described in detail previously (16). The external recording solution was comprised of 20 mM BaCl2, 1 mM MgCl2, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 65 mM CsCl (pH 7.2), recording pipettes were filled with 108 mM cesium methanesulfonate, 4 mM MgCl2, 9 mM EGTA, 9 mM HEPES (pH 7.2) and showed typical resistances of 2 to 4 megohms. For experiments involving opioid receptor activation, the intracellular solution was supplemented with 40 μM GTP. DAMGO (purchased from RBI) was dissolved in water at a stock concentration of 2 mM, diluted into the recording solution at a final concentration of 1 μM, and perfused directly into the vicinity of the cells via a gravity-driven microperfusion system. Typically, cells were held at −100 mV and currents were elicited upon depolarizations to various test potentials.

Tonic G-protein inhibition of the channels was assessed by application of strong depolarizing prepulses (to +150 mV) followed by a test depolarization to +20 mV (see Refs. 8 and 16). The time course of development of prepulse relief was determined by varying the duration of the prepulse (Δt) while leaving the duration between the prepulse and the test pulse constant at 5 ms (see Fig. 2). The inhibition kinetics were determined by applying a 50-ms prepulse, followed by a test pulse spaced from the prepulse at variable intervals (Δt2). The total degree of prepulse relief was assessed by extrapolating the exponential decay of the prepulse effect back to time t2 = 0 (see Ref. 16), thus allowing us to assess the degree of prepulse relief without contamination from re inhibition. For the experiment in Fig. 2, the data were obtained at a fixed interval of 4 ms between the prepulse and the test pulse.

For experiments involving PKC-dependent phosphorylation of the channels, the phosphor ester PMA was dissolved in Me2SO at a stock concentration of 2 mM, diluted into the recording solution at a final concentration of 30 nM, and perfused directly onto the cell with a gravity-driven microperfusion system.

Western Blot Analysis—Western blots on cell lysates generated from transfected or sham-transfected tsa-201 cells were carried out as described in detail by Jarvis et al. (21). Antibodies to G protein β subunits were purchased from Santa Cruz Laboratories (anti-Gβ1 and anti-Gβ2) and from Transduction Laboratories (anti-Gβδ1). Immunoblots were subjected to chemiluminescence analysis using ECL plus (Amersham Pharmacia Biotech) and detected on film.

Data Analysis and Statistics—Data were analyzed using Clampfit software. Preparation of figures and statistical analysis was performed via SigmaPlot (Jandel Scientific). Error bars are standard errors, p values reflect Student’s t tests.

RESULTS AND DISCUSSION

We have previously shown that activation of protein kinase C in tsa-201 cells reduces a somatostatin receptor-induced G-protein inhibition of N-type calcium channels (4). A point mutation in the domain I-II linker region of the channel (T422E) mimicked the effects of PKC activation, whereas a substitution to alanine precluded the effects of PKC on G-protein inhibition. We concluded that PKC/G-protein cross-talk occurs via phosphorylation of threonine 422 (4). To assess whether this effect was selective for somatostatin receptors, we coexpressed μ-opioid receptors with wild type or mutant (T422E) N-type (α1b, β1, α2δ) calcium channels and applied 1 μM DAMGO to trigger G-protein inhibition of the channels via the receptor. As seen in Fig. 1, wild type channels underwent a robust, reversible inhibition by 56 ± 4% in response to opioid receptor activation. In contrast, the T422E mutant exhibited a reduced degree of G-protein inhibition (32 ± 3%), in accord with our assertion that a negative charge (which permanently mimics phosphorylation) in position 422 reduces G protein efficacy. A comparison with our previous work with somatostatin receptors (4), however, shows that the somatostatin receptor-mediated inhibition of N-type calcium channels was significantly more strongly affected than the μ-opioid response (20 ± 3% versus 32 ± 3%, p < 0.05), whereas the wild type channels were similarly inhibited by both pathways (DAMGO, 56 ± 4%; somatostatin, 53 ± 5%, p > 0.05). Although the latter observation indicates that both receptor types are equally efficiently coupled to wild type N-type channels in tsa-201 cells, the observation with the T422E mutant suggests that there are nonetheless differences in the way the two receptor types couple to the channels. It is possible that the two receptor types couple to the...
channels via different G-protein heterotrimer compositions, which may be differentially affected by the presence of the T422E mutation.

The key G-protein species involved in direct inhibition of N-type calcium channels is the Gβ subunit (5, 6, 16, 18). To date, five different types of Gβ subunits have been identified in mammalian brain (17), and we have recently shown that N-type channels expressed in tsa-201 cells are most effectively inhibited by Gβ0 and Gβ5, whereas Gβ1 and Gβ2 mediate a somewhat smaller inhibition, and Gβ3, is ineffective and is thus not further considered here (16).

To test the possibility that PKC/G-protein cross-talk might preferentially affect a subset of the Gβ subunit isoforms, we coexpressed wild type channels with one of four different Gβ subtypes (+Gγ) and used a strong depolarizing prepulse to compare the resulting tonic G-protein inhibition before and after phosphorylation of the channel by protein kinase C (elicited by 2-min application of 30 nM PMA). As shown in Fig. 2, in cells expressing Gβ6, the degree of prepulse relief was reduced from 2.4 ± 0.3 to 1.4 ± 0.1 (n = 7, p < 0.02, paired t test) following PMA treatment, whereas inhibition by the three other G-protein β subunit subtypes was not significantly altered (Fig. 2B). Thus, these data suggest that PKC/G-protein cross-talk selectively affects Gβ1-mediated responses.

Although N-type calcium channels expressed in tsa-201 cells show only negligible background G-protein inhibition in the absence of exogenous Gαγ (16), it is true that these cells contain endogenous G protein βγ subunits that are presumably complexed as αβγ heterotrimers. To assess the likelihood of contamination of our results by endogenously present Gβ proteins, we carried out Western blot analysis of control cells and cells transfected with either one of the four Gβ subunits. As seen in Fig. 2C, exogenous expression of each of the four subtypes tested resulted in a substantial increase in Gβ levels compared with those found in control cells, confirming that the exogenously expressed subunits are the predominant G-protein species in transfected cells.

To obtain an indication of the change in affinity of the channels for the G-proteins, which occurs after phosphorylation, we utilized dynamic prepulse protocols to determine the time constants of recovery from inhibition (by varying prepulse duration) and reinition after the prepulse (by varying the duration between the prepulse and the test pulse, see Fig. 3A). However, after prolonged application of PMA (>3 min), we noted that current levels began to run down, which would interfere with the accuracy of these types of experiments. Hence, we chose to utilize the T422E mutant for our kinetic measurements.

Fig. 3B shows that the T422E mutant behaved like the phosphorylated wild type channel with regard to the Gβ sub-type dependence of cross-talk, such that the mutation induced a selective decrease in the degree of prepulse relief seen in the presence of Gβ1 (wild type, 2.8 ± 0.3; T422E, 1.9 ± 0.1; p < 0.05) whereas the facilitation ratios of the three remaining G-protein β subunit isoforms did not differ significantly from those obtained with the wild type channels (note, however, that the data shown in Figs. 2B and 3B were obtained by slightly different protocols, and hence, absolute values for the degree of prepulse relief are not directly comparable). As seen in Fig. 3C, the T422E mutation selectively reduced the time constant of recovery from Gβ1 inhibition (wild type, 11.0 ± 0.6 ms; T422E, 8.0 ± 0.6 ms; p < 0.05), indicating that one of the consequences of the T422E mutation is a slight destabilization of the Gβ1-channel complex. In addition, the time course of Gβ1 reinition after the prepulse (Fig. 3D) was slowed 3-fold as a result of the T422E mutation (wild type, 18.5 ± 2.4 ms; T422E, 51.7 ± 4.7 ms; p < 0.05). Interestingly, a small increase in the time course of reinition was also observed with Gβ2 (WT, 46.2 ± 6.3 ms; T422E, 27.8 ± 4.1 ms; p < 0.05), indicating that the T422E mutation may exert subtle effects on this subunit. Overall, however, the data shown in Figs. 2 and 3 indicate that a mutagenically phosphorylated threonine residue in position 422 affects predominantly the inhibition of N-type calcium channels by Gβ1 subunits.

To date, little is known about the association of individual types of seven helix transmembrane receptors with specific subsets of G-protein subunits. It has been shown, however, that antisense depletion of Gβ1 subunits abolishes somatostatin receptor signaling in rat pituitary Gα1 cells (22). It is thus likely that somatostatin receptors exclusively couple to Gβ1 subunits, which can account nicely for our observation that the T422E mutation dramatically reduced the G-protein inhibition induced by both overexpression of Gβ1 and upon activation of somatostatin receptors. In contrast, it is possible that the µ-opioid receptor couples to N-type calcium channels through more than one type of G-protein β subunit. Whereas the observation that the opioid response was reduced in the T422E mutant would suggest that at least part of the opioid signaling...
is mediated by Gβ1, our data showing that the opioid and somatostatin responses were not equally affected by the T422E substitution (Fig. 1D), however, support a mechanism in which μ-opioid receptors may couple to a mixed population of G-protein β subunit isofoms. This scenario would account for the intermediate response we observed.

The data shown in Fig. 3, C and D, provide some insights into the molecular mechanisms by which cross-talk may occur. It is now widely accepted that Gβγ physically interacts with the N-type calcium channel domain I-II linker region (8, 23, 24), thus phosphorylation events or amino acid substitutions occurring in this region may affect the binding interactions between the channel and the Gβγ dimers. Consistent with such an effect, the T422E substitution resulted in a significant decrease in the time constant for development of facilitation during the prepulse and an increase in the time constant for reinhibition after the prepulse. In view of evidence that G proteins must physically dissociate from the channel during the prepulse (8), these changes in kinetics likely reflect a change in the G-protein association and dissociation kinetics, and thus an over-all reduction in the affinity of the channel for Gβγ, which may also account for the reduction in the degree of prepulse relief seen with the T422E mutant. Our experiments do not permit us to provide an absolute value for the PKC-induced changes in the equilibrium dissociation constant between the G proteins and the channels, because the kinetics for recovery from inhibition during the prepulse (+150 mV) and reinhibition after the prepulse (i.e., repolarization to −100 mV) were obtained at different voltages. Nonetheless, based on the 3-fold decrease in reinhibition kinetics and the 1.4-fold speeding of the recovery time constant, we estimate that the presence of the T422E mutation may perhaps result in an ~5-fold change in Gβ1 affinity.

An important aspect to consider is control over the G protein expression levels in our experiments. Our Western blot analysis shows that each of the four Gβ subtypes expressed well in tsα-201 cells, indicating that the exogenously expressed G-protein subunits are much more abundant than endogenously present Gβ, although it is difficult to predict relative expression levels among the four Gβ subtypes from Western blots as antibody sensitivity may vary. In the experiments shown in Fig. 2B, the inhibition of nonphosphorylated and phosphorylated channels by each Gβ subtype was studied in the same cell, and hence, at a constant Gβ concentration. In the experiments shown in Fig. 3B, the effects of each Gβ subtype on wild type and mutant channels was assessed in different cells. However, for each given Gβ subtype, wild type and mutant channels were studied under identical conditions, thus attributing any changes in channel inhibition to residue 422 rather than Gβ levels. Both types of experiments resulted in essentially the same result, namely that only Gβ1-mediated responses were affected by phosphorylation/mutation of the channel.

Although in vivo evidence supporting our conclusions is still lacking, it is tempting to speculate about the implications of observations for neurotransmission: calcium influx through N-type and P/Q-type calcium channels is essential for the fast release of neurotransmitter (25). Regulation of presynaptic calcium channel activity by cytoplasmic messenger molecules is thus an essential means for the precise control of neurotransmission. The activation of opioid receptors, for example, depresses synaptic activity (13, 26), and tonic G-protein inhibition at presynaptic nerve terminal contributes to paired pulse facilitation (27–29). We have previously presented evidence that integration of protein kinase C and Gβγ pathways by the N-type calcium channel α1 subunit can produce multiple levels of current activity (4). If our results are extrapolated to an in vivo situation, the notion that the extent of this integration appears to depend on the type of Gβ subunit isoform present may provide additional avenues by which neurotransmitter receptors may regulate calcium homeostasis in the presynapse. Together with the notion that different types of G-protein β subunits do not solely affect N-type channel activity to different degrees (16–18) but also show pronounced differences in their abilities to inhibit P/Q-type calcium channels (16), the unique activation of specific Gβγ combinations by different types of seven-helix transmembrane receptors could provide a highly complex regulatory mechanism for the fine tuning of presynaptic calcium levels and, thus, neurotransmitter release.

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