Scanning Mutagenesis Reveals a Role for Serine 189 of the Heterotrimeric G-Protein Beta 1 Subunit in the Inhibition of N-Type Calcium Channels

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Methods

cDNAs

cDNAs encoding human Gβ1 and Gγ2, rat Gβs were previously described (Arnot et al. 2000; Feng et al. 2001). Wild-type (WT) rat calcium channel subunit cDNAs were donated by T. Snutch (University of British Columbia). Constructs encoding GIRK1 and GIRK4 subunits were provided by Dr. Hubert van Tol (Center for Addiction and Mental Health, Toronto, Ontario).

Gβ1 mutants

Using overlap extension PCR methodologies (Ho et al. 1989), the following mutants of human Gβ1 were constructed: G141M, G144N, amino terminus, the loop-linking domains I and II, and the carboxy terminal region—all contribute to enable binding of the G-protein βγ complex (Agler et al. 2005; Canti et al. 1999; De Waard et al. 1997; Page et al. 1997; Qin et al. 1997; Zamponi et al. 1997). Complementary efforts to map regions of the G-protein β subunit that are involved in N-type and P/Q-type channel modulation have resulted in the identification of several amino acid residues on the Gβ1, many of which overlapped with the Gγ interaction region (Agler et al. 2003, 2005; Ford et al. 1998; Mirshahi et al. 2002a). By using series of chimeras between Gβ1 and Gβ5 we recently identified a structurally contiguous region of the Gβ1 molecule that, when replaced by the corresponding Gβ5 sequence, resulted in complete loss of inhibitory action against the N-type channel (Doering et al. 2004). This structurally contiguous region of Gβ1 constitutes residues 110–112, 140–168, and 186–204 of the primary Gβ1 structure. Within these regions, mutations of Gβ1 residues Tyr111 and Asp153 resulted in loss of voltage-dependent inhibition, suggesting their importance for direct binding interactions with the N-type channel (Doering et al. 2004).

Here we further characterize the molecular determinants that underlie the inhibitory action of Gβ1 on the N-type channel. We describe construction of a series of Gβ1 mutations within regions 140–168 and 186–204, in which individual residues were replaced with those of Gβ5, and examination of their effects on N-type calcium channel inhibition by paired-pulse facilitation assays. Of all of the mutants examined, only Ser189 of Gβ1 emerged as a crucial residue for voltage-dependent inhibition of the N-type channel, suggesting that modulation of N-type channels involves highly localized Gβ subunit structural determinants.

INTRODUCTION

N-type and P/Q-type calcium channels are inhibited in response to activation of G-protein–coupled receptors (GPCRs), with crucial consequences for synaptic activity, neurotransmitter release, and nociception (Dunlap et al. 1995; Wheeler et al. 1994; Zamponi 2001). It is now known that heterodimers of the G-protein β and γ subunits interact directly with N-type and P/Q-type channels, causing steady-state inhibition of the channels and increasing their time constant of activation. Both aspects of this modulation depend on membrane potential and can be relieved by either application of strong depolarizations or trains of action potentials (Dolphin 2003; Page et al. 1997). The extent of inhibition is dependent on both calcium channel subtype and on the G-protein β subunit isoform (Arnot et al. 2000; Garcia et al. 1998; Ruiz-Velasco and Ikeda 2000), with Gβ5 subunits mediating little or no detectable voltage-dependent inhibition of N-type currents, whereas Gβ1 results in robust inhibition of these channels (Doering et al. 2004).

Efforts to map the inhibitory binding interactions of the N-type and P/Q-type channels with the G-protein β subunit have yielded evidence suggesting that three intracellular regions of the pore-forming Ca2.2 subunits of the channels—the
C148A, R150S, L152T, V158L, S160A, T164G, M188L, S189C, S191D, D195S, L198T, and A203G. In each case, this constitutes a change to the residue at the corresponding position of the primary structure of the rat Gβ1 molecule. Mutagenic PCRs were performed using Pfu ultra polymerase (Stratagene), according to the manufacturer’s suggestions. Full-length mutant Gβ1 cDNAs were digested with XhoI and KpnI (these restriction sites were included in the 5’ ends of the nonmutagenic flanking primers designed to anneal to the 5’ and 3’ ends of the human Gβ1 open reading frame), then subcloned into XhoI- KpnI double-digested pMT2-XS expression vector. All inserts were sequenced to confirm the presence of the mutations and to rule out PCR errors.

**Tissue culture and transient transfection**

Human embryonic kidney tsA-201 cells were grown and transfected with calcium phosphate as previously described in detail (Doering et al. 2004). In each experiment involving calcium channels, wild-type or mutant rat Ca_{2.2} calcium channel α1 subunits were cotransfected with rat β_{1a}, rat α_{2.1}, human Gγ2s, and an enhanced green fluorescent protein (EGFP) expression marker, plus one of wild-type or mutant Gβ6 subunits. For experiments involving GIRK channels, GIRK1 and GIRK4 subunits were used instead of calcium channel subunits. To prevent overgrowth of cells, culture dishes were placed in a 28°C incubator 3–9 h after the washing step performed to remove DNA precipitate from the cultures.

**Voltage-clamp recordings of N-type channel currents**

Glass coverslips carrying transfected cells were transferred to a 3-cm culture dish containing recording solution consisting of (in mM) 20 BaCl₂, 1 MgCl₂, 10 HEPES, 40 tetraethylammonium hydroxide, 10 glucose, and 65 CsCl (pH 7.2 with tetraethylammonium-hydroxide). Whole cell patch-clamp recordings were performed as described previously (Doering et al. 2004), using internal pipette solution consisting of (in mM) 108 cesium methane-sulfonate, 4 MgCl₂, 9 EGTA, and 9 HEPES (pH 7.2). Currents were evoked by stepping from −100 mV to a test potential of +20 mV. G-protein inhibition was assessed by application of a +150-mV prepulse (PP) for 50 ms. Only cells with current amplitudes >50 pA and <1.2 nA were used for analysis. The degree of prepulse relief of tonic G-protein inhibition was determined as the ratio of peak current amplitudes seen after (I_{pp}) and before (I_{pp}) the prepulse—referred to hereinafter as the paired-pulse facilitation (PPF) ratio and reflects the ability of a given G-protein subunit to inhibit N-type current activity. The PP paradigm were programmed using the “train” and “user list” functions in pCLAMP (Axon Instruments).

**Voltage-clamp recordings of GIRK channel currents**

Whole cell recordings were performed using an internal solution of (in mM) 100 potassium gluconate, 40 KCl, 10 HEPES, 5 EGTA, 1 MgCl₂, and 5 NaCl (pH 7.4 with KOH). External solution consisted of (in mM) 25 KCl, 10 HEPES, 10 glucose, and 116 NaCl (pH 7.4 with NaOH). Under these conditions, the predicted reversal potential of potassium is about −30 mV. GIRK channel activity was tested by holding the cells at −35 mV, followed by application of a voltage ramp from −120 to +60 mV over 525 ms. Only cells displaying inward rectification were used for analysis, and whole cell GIRK conductance was obtained by a linear fit to the inward current observed between the potentials of −100 and −60 mV during the voltage ramp. Whole cell capacitance ranged from 10 to 186 pF. In this range, there was no significant correlation between capacitance and whole cell conductance (Pearson correlation coefficients for matched arrays of capacitance and conductance from cells expressing GIRK1/4 channels were 0.233, 0.247, and 0.609 for data from cells coexpressing either no heterologous Gβ, heterologous Gβ1S189C, or heterologous WT Gβ1, respectively). Thus data are plotted in Fig. 2B as whole cell conductance rather than current densities. GIRK channel data presented herein were all recorded from one batch of tsA-201 cells of the same passage and culture, cotransfected simultaneously in separate plates, under identical conditions with the exception of the G-protein–expression vectors used (or omitted) to obtain the desired coexpression pattern in the individual transfections.

**Voltage-clamp data analysis**

All electrophysiological data were analyzed using Clampfit (Axon Instruments) and fitted in Sigmaplot 2000 (SPSS) or Microsoft Excel. Statistical analysis was carried out using SigmaStat 2.03 (SPSS). A Kruskal–Wallis one-way ANOVA on ranks (Dunn’s method) was performed on N-type calcium channel data; assuming normal distribution (based on passed normality test) a one-way ANOVA was performed using a post hoc Tukey test on GIRK channel data.

**RESULTS**

We previously showed that substitution of residues 140–168 or residues 186–204 of Gβ6 with the corresponding residues of Gβ1 yields a chimera that cannot induce voltage-dependent inhibition of the N-type channel (Doering et al. 2004). Alignment of the human Gβ1 sequences for residues 140–168 and 186–204 with the corresponding rat Gβ3 sequences revealed 14 nonconserved residues. To identify which of these individual amino acid residues were critical for G-protein modulation of the N-type channel, responsible for this effect, we systematically replaced Gβ1 residues with those corresponding to the Gβ3 sequence. The resulting mutant Gβ1 constructs were coexpressed in HEK (human embryonic kidney) cells with N-type channel subunits and an EGFP expression marker. Voltage-dependent G-protein inhibition of N-type currents was assessed by using a PPF paradigm (see METHODS), wherein alternating test potentials were recorded after application of +150-mV depolarizing prepulses intended to disrupt inhibitory interaction of Gβ subunits and N-type channels (Fig. 1).

As controls and to establish a baseline, N-type channels were also coexpressed either with heterologous WT Gβ1 or without any heterologous Gβ and PPF was tested under both conditions. As illustrated by current traces shown in Fig. 1A, application of prepulses to cells coexpressing heterologous WT Gβ1 resulted in substantially larger and more rapidly activating N-type currents (Fig. 1A, right traces), but prepulses applied to cells not expressing heterologous Gβ typically resulted in only negligible changes (Fig. 1A, left traces), as expected from the absence of G-protein modulation.

The results of PPF assays performed with the 14 Gβ1 mutants are summarized in Fig. 1B. Among the 14 mutants, 11 yielded PPF ratios that were similar to those obtained with WT channels. Two additional mutants, G144N and M188L, appeared to result in a somewhat reduced degree of prepulse facilitation; however, although a Student’s t-test indicated significant differences for these two residues (P values of 0.003 and 0.004, respectively), the respective distributions of PPF values failed the normality test and analysis by ANOVA on ranks indicated that this reduction did not reach statistical significance in either case. In contrast, the S189C mutant yielded a mean PPF ratio slightly lower than that of negative control assays (1.13 and 1.16, respectively), which was significantly different from that observed with WT Gβ1 (difference of ranks score, 141.9; P < 0.05).
To eliminate the possibility that the S189C mutant might not express, or not fold correctly, we examined the abilities of both $G_{\beta_1}$-$S189C$ and WT $G_{\beta_1}$ to activate GIRK1/4 channels expressed in tsA-201 cells. As illustrated by raw current trace ensembles in Fig. 2, A and B, large inward $K^+$ currents were observed at negative test potentials in cells coexpressing GIRK1/4 channels and either $G_{\beta_1}$-$S189C$ or heterologous WT $G_{\beta_1}$, with significant rectification at more positive test potentials. Inward $K^+$ currents recorded as negative controls from cells with no heterologous $G_{\beta_1}$ were substantially smaller (see Fig. 2C). ANOVA analysis of whole cell conductances (calculated for ramp potentials between $-100$ and $-60$ mV) confirmed a statistically significant difference between $K^+$ conductances of cells with no heterologous $G_{\beta_1}$ compared with cells expressing either $G_{\beta_1}$-$S189C$ or WT $G_{\beta_1}$ (Fig. 2D), but with no statistically significant difference between the latter two conditions. These results thus confirm the expected functionality of the $G_{\beta_1}$-$S189C$ mutant in the activation of GIRK1/4 channels, consistent with the view that Ser$^{189}$ is required for direct modulatory interactions.
with the N-type calcium channel, but not for proper $\beta$-subunit folding and expression.

**Discussion**

Although the bioactive surfaces of peptide toxins and hormones are often studied using comprehensive scanning mutagenesis of all solvent-exposed amino acid residues (Froy et al. 1999; Kristensen et al. 1997; Maggio and King 2002; Nadasdi et al. 1995; Tedford et al. 2001, 2004), the $\beta$ subunit is 10- to 50-fold larger than the typical subjects of these studies—a poor prospect for comprehensive scanning mutagenesis, in view of the resources that would be required. Structure–activity relationship (SAR) studies of the $\beta$ subunit have thus avoided comprehensive scanning approaches, instead imposing limits on the structural variants tested by using chimeras of $\beta$ subtypes (Mirshahi et al. 2002b), or in other cases using targeted mutations of structural regions suspected to be significant based on interactions observed in cocystal structures (Agler et al. 2003).

Here, we used a scanning mutagenesis approach, but limited it to examination of 14 $\beta_1$ residues in sequence regions 140–168 and 186–204, areas previously identified in our tests of a panel of chimeras of $\beta_1$ and $\beta_5$ (Doering et al. 2004). Our data show that only one of these 14 residues (Ser$^{189}$) significantly contributed to voltage-dependent channel modulation. Taken together with our previous results, there appear to be three residues of $\beta_1$—Tyr$^{111}$, Asp$^{153}$, and Ser$^{189}$—that are required for voltage-dependent inhibition of N-type channels (Doering et al. 2004). Consistent with their critical role in modulating N-type channels, these residues are all highly conserved in $\beta_1$ orthologues expressed in human, rat, and mouse. Moreover, sequence alignment indicates that they are also conserved in any of the known human $\beta$ subtypes (i.e., $\beta_1$–$\beta_4$) that are capable of modulating the N-type channel (Arnot et al. 2000). As illustrated in Fig. 3, the three residues are noncontiguous in the three-dimensional structure of $\beta_1$—a situation with many precedents because SAR studies of conotoxins, spider toxins, and also $\beta$-modulation of effectors other than N-type calcium channels have all identified noncontiguous residues presumed to participate directly in functional contacts with their respective targets (Agler et al. 2003; Lew et al. 1997; Maggio and King 2002; Mirshahi et al. 2002b). The side chains of Tyr$^{111}$ and Asp$^{153}$ each protrude outward from the rest of the structure and would presumably be quickly accessible for interactions with intracellular loops of the $\alpha_B$ subunit of the N-type channel. By contrast, Ser$^{189}$ is situated in a concavity in the center of the structure, with only the $\gamma$-OH functionality at the distal end of its side chain exposed to the aqueous solution at the structural surface. Thus the kinetics of its binding interactions with the N-type channel would be slow relative to Tyr$^{111}$ and Asp$^{153}$, although this would have to be confirmed by biochemical means. However, precedents exist in the literature both for binding epitopes that protrude from protein structures and also for less-accessible binding epitopes situated in concavities of protein surfaces (Bogan and Thorn 1998; Kim et al. 1995; Tedford et al. 2004).

We should also note that Ser$^{189}$ does not appear to form a serine kinase consensus site (as evaluated by PROSITE analysis), and thus the loss of N-type channel inhibition is not attributable to altered phosphorylation of $\beta_1$.

Our previous findings with rat $\beta_5$ indicate that this subunit is unable to modulate N-type channel activity (Arnot et al. 2000). Interestingly, human $\beta_5\gamma_2$ has been shown to inhibit heterologously expressed human N-type calcium channels (Zhou et al. 2000), and mouse $\beta_5\gamma_2$ can inhibit native N-type currents in rat superior cervical ganglion neurons (Garcia et al. 1998; Ruiz-Velasco and Ikeda 2000). Considering the propensity of certain $\beta_5$ subunits to regulate N-type channels, why then would replacement of a $\beta_1$ residue with corresponding $\beta_5$ residue result in a loss of N-type channel regulation, in particular when this $\beta_5$ residue is conserved in rat, mouse, and human? One possible explanation may lie in the $\beta_5$ subunit isoforms used in the latter two studies. The N-terminus region of mouse (GenBank accession number P62881) $\beta_5$ contains about 40 additional amino acid residues compared with the rat $\beta_5$ subunit used in our previous work (GenBank accession number AAS9141), and this extended N-terminus is not seen with $\beta_1$. Considering that the remainder of the mouse $\beta_5$ sequences are $>99\%$ identical to the rat isoform, this implies an important role of the extended N-terminus in N-type channel modulation. Because the $\beta_1$ and $\beta_5$ subunits used in our experiments do not contain extended N-termini and our $\beta_5$ subunit is incapable of inhibiting N-type channel activity, the loss of $\beta_1$ subunit regulation of the channel that occurs on substitution of certain key residues with $\beta_5$ sequence is thus expected. The human $\beta_5$ subunit used by Zhou and coworkers (2000) is virtually identical to shorter rat isoform used in our study, although the former study involved human rather than rat Cav2.2 channels, thus making firm comparisons difficult.

Substitution of two additional residues, Gly$^{144}$ and Met$^{188}$ (which are conserved in $\beta_1$ through $\beta_5$), was found to reduce the degree of prepulse relief, although the statistical significance of this effect depended on the statistical analysis used (i.e., ANOVA vs. $t$-test). These two residues are situated on the opposite face of the $\beta$ subunit compared with Tyr$^{111}$, Asp$^{153}$, and Ser$^{189}$ (not shown), and thus they are unlikely to mediate their effects by altering channel interactions with these three residues. However, they are located in close proximity to other $\beta_1$ residues (i.e., Thr$^{142}$, Asp$^{153}$, Met$^{181}$) previously implicated in N-type channel modulation by Ford and col-

![FIG. 3.](image_url)
leagues (1998), thus perhaps accounting for a possible partial effect. It is unlikely that the substitution of these two residues would result in gross alterations in the overall Gβ structure because substitution of larger regions of Gβ2 flanking these residues with Gβ3 sequence preserves the ability of such chimeric constructs to activate GIRK channels (Doering et al. 2004).

Particularly remarkable is the presence of several unique ensembles of modulatory epitopes in the structure of Gβ, all of which contribute to Gβ modulation of a different effector molecule—e.g., N-type and P/Q-type calcium channels, GIRK channels, adenyl cyclase, and phospholipase β2 (Agler et al. 2003; Ford et al. 1998; Mirshahi et al. 2002a). All of these effector interaction sites are localized to the Gα interaction domain of Gβ1 (Agler et al. 2003; Ford et al. 1998; Mirshahi et al. 2002a). However, the N-type channel may be unique among these effectors because the critical modulatory residues identified by our present study are situated on the Gβ surface opposite to that involved in Gα binding (see Fig. 3). The presence of multiple, widely dispersed N-type channel interaction sites on the Gβ subunit is consistent with the notion that several Gβ binding regions (i.e., N-terminus, I–II linker region) have been identified on the channel (Agler et al. 2005; Zamponi et al. 1997). The alignment of these three critical residues highlighted in Fig. 3 in a ribbon-like fashion may be indicative of specific contact points with a single one of these channel regions. Ultimately, mutation complementation experiments may be needed to identify which channel subunits participate in such mutual interactions. Nonetheless, our data point to highly specific sequence differences between Gβ2 and the four other known Gβ subunit subtypes that underlie the Gβ subtype specificity of N-type channel modulation.

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