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Scanning mutagenesis of the I-II loop of the $Ca_V2.2$ calcium channel identifies residues Arginine 376 and Valine 416 as molecular determinants of voltage dependent G protein inhibition

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Abstract

Direct interaction with the β subunit of the heterotrimeric G protein complex causes voltage-dependent inhibition of N-type calcium channels. To further characterize the molecular determinants of this interaction, we performed scanning mutagenesis of residues 372-387 and 410-428 of the N-type channel α_1 subunit, in which individual residues were replaced by either alanine or cysteine. We coexpressed wild type $G\beta_1\gamma_2$ subunits with either wild type or point mutant N-type calcium channels, and voltage-dependent, G protein-mediated inhibition of the channels (VDI) was assessed using patch clamp recordings. The resulting data indicate that Arg^{376} and Val^{416} of the α_1 subunit, residues which are surface-exposed in the presence of the calcium channel β subunit, contribute significantly to the functional inhibition by $G\beta_1$. To further characterize the roles of Arg^{376} and Val^{416} in this interaction, we performed secondary mutagenesis of these residues, coexpressing the resulting mutants with wild type $G\beta_1\gamma_2$ subunits and with several isoforms of the auxiliary β subunit of the N-type channel, again assessing VDI using patch clamp recordings. The results confirm the importance of Arg^{376} for G protein-mediated inhibition and show that a single amino acid substitution to phenylalanine drastically alters the abilities of auxiliary calcium channel subunits to regulate G protein inhibition of the channel.

Background

The mammalian nervous system expresses nine different genes that encode different types of voltage-gated calcium channel (VGCC) α_1 subunits which interact with auxiliary subunits and form classes of VGCCs that are distinct in structure, pharmacology, and physiology [1]. VGCCs containing the α_{1A} and α_{1B} subunits (P/Q- and N-type channels respectively) are distinguished from other types by their localization to pre-synaptic nerve terminals, where they mediate calcium influx which contributes to evoked neurotransmitter release and overall synaptic function [2-4].

Inhibition of P/Q- and N-type channels resulting from activation of G-protein coupled receptors (GPCRs)–a crucial mode of regulation, notably illustrated in the relief of pain sensations in response to opioid drugs [5]–has been studied for over 25 years [6-16]. This mode of regulation is complex and comprises multiple pathways that include direct and indirect actions of G proteins on the channel [17]. During membrane delimited G protein inhibition, GPCR activation releases $G\beta\gamma$ heterodimers which then bind directly to the α_1 subunits of P/Q- and N-type channels, and this interaction stabilizes closed channel conformations and culminates in channel inhibition [18,19].

A recent study suggests that interaction of $G\beta\gamma$ with N-type channels can slow the kinetics of channel transition to inactivated states [20]. However, most studies of the direct $G\beta\gamma$-presynaptic channel interaction have

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investigated the slowing of transition to activated channel states, and have found this mode of inhibition to be more favored at hyperpolarized potentials, thus allowing for activity dependent dis-inhibition [21-24]. Hence, the term "voltage-dependent inhibition" (VDI) has been used to describe two experimental hallmarks of this $G\beta\gamma$-mediated regulation: slowing of presynaptic channel activation, and relief of channel inhibition by a strong, depolarizing pre-pulse.

$G\beta\gamma$-mediated VDI depends on a complex set of structural determinants that contribute to direct interaction between $G\beta\gamma$ and the presynaptic calcium channel. As such, the extent of VDI varies with the isoforms of the channel subunits and the G protein subunits in question [17]. Structure-activity relationship studies of the interaction have revealed roles for three cytosolic regions of the α_1 subunit: the N-terminus, the I-II linker domain, and the C-terminus [25-30]. While the C-terminal region of the channel is thought to play a supporting role as an enhancer of overall $G\beta\gamma$-channel binding affinity [31], both the N-terminus and the I-II linker contribute directly to the inhibitory interaction with $G\beta\gamma$. Furthermore, contact between the N-terminus and the I-II linker is demonstrated to be necessary for $G\beta\gamma$-mediated VDI [25]. Efforts to resolve functionally important $G\beta\gamma$-channel binding interactions have also revealed the direct involvement of two nearby sections of the I-II linker: amino acid residues 372-389 and 410-428 [27,30]. The first of these sections partially overlaps with the α_1 subunit alpha interaction domain (AID) and contains residues known to bind the calcium channel β subunit, presumably in a manner that precludes many of them from interaction with $G\beta\gamma$ [32]. However, the contribution of the other I-II linker residues in question–to direct $G\beta\gamma$-binding and hence to channel inhibition–has remained unclear.

Here we aimed to further resolve the molecular determinants of $G\beta\gamma$-mediated channel inhibition by testing the functional contribution of individual residues in the two above-mentioned sections of the α_{1B} I-II linker. Using a combination of alanine/cysteine scanning mutagenesis and whole-cell electrophysiological recordings from tsA-201 cells, we identify two residues of the I-II linker, Arg^{376} and Val^{416}, as key determinants of $G\beta\gamma$-mediated, voltage-dependent modulation of N-type channels.

Methods

cDNAs

Wild type (WT) rat calcium channel subunit cDNAs encoding $Ca_v2.2$ (α_{1B}), $Ca_v\beta_{1b}$, $Cav\beta_{2a}$, $Ca_{v\beta 3}$, and $Ca_{v\beta 4}$, and α_2-δ_1 subunits were generously donated by Dr.Terry Snutch (University of British Columbia, Vancouver, BC). The construction of cDNAs encoding WT human $G\beta_1$ and $G\gamma_2$ subunits have been described previously [33].

$Ca_v2.2$ α_{1B} mutants

cDNAs encoding single-residue $Ca_v2.2$ α_{1B} mutants were constructed by overlap extension PCR [34], using WT α_{1B} as the DNA template and Pfu turbo DNA polymerase (Stratagene) according to manufacturer's instructions. AarI and BsiWI restriction sites, found in the native sequence at locations flanking the mutagenized I-II loop-encoding sequence, were included in the 5' ends of the non-mutagenic flanking primers. After mutagenizing and overlap extension reactions, mutant α_{1B} cDNA fragments were digested with AarI and BsiWI, and then sub-cloned into the (AarI-, BsiWI-digested) α_{1B} mammalian expression vector, pCMV30-14G. Codons for 27 residues within amino acid sequence regions 372-389 and 410-428 were individually substituted to alanine, and three naturally occurring alanine codons were substituted to cysteine. These 30 mutations included: F372A, L373A, K374A, L375A, R376A, R377A, Q378A, Q379A, E382A, R383A, N386A, K410A, S411A, P412A, L413A, D414A, A415C, V416A, L417A, K418A, R419A, A420C, A421C, T422A, K423A, K424A, S425A, R426A, N427A, and D428A. cDNAs encoding four additional point mutations, R376E, R376F, V416E, and V416K, and a double alanine mutation, (both R376A and V416A), were also constructed using overlap/extension PCR as described above.

Tissue Culture and Transient Transfection

Human embryonic kidney tsA-201 cells were grown and transiently transfected using the calcium phosphate method as described previously (32). Transfection solutions for individual culture dishes contained a mixture of cDNA expression vectors, with the following quantities of each cDNA expression construct used: calcium channel α_{1B} subunit, 6 μg; $Ca_v\beta$ subunit (6 μg), $Ca_v\alpha_2$-δ_1 subunit (6 μg), $G\beta_1$ subunit, 6 μg; $G\gamma_2$ subunit, 6 μg; and 1 μg of a pEGFP marker vector (Clontech). Positive controls contained the WT α_{1B} subunit in place of mutant α_{1B}, and negative controls consisted of the WT α_{1B} subunit in the absence of exogenous $G\beta_1\gamma_2$. 12 hr post-transfection, cells were washed 1× with PBS pH 7.4, supplemented with fresh DMEM, and allowed to recover for an additional 12 hr. To prevent overgrowth, cells were subsequently transferred to a 29°C incubator and maintained for 24-72 hr prior to voltage-clamp recording.

$Ca_v2.2$ Voltage Clamp Recordings

Glass coverslips carrying cells expressing mutant or WT $Ca_v2.2$ channels were transferred to a 3.5-cm culture dish (Corning) containing external recording solution

consisting of 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEA-Cl, 10 mM glucose, and 65 mM CsCl (pH 7.2 adjusted with TEA-OH). Micro-electrode patch pipettes were pulled using a Sutter P-87 micro-electrode puller or a DMZ Universal puller, and manually fire-polished using a Narishige MF-830 Micro Forge to attain a typical resistance of 4-5 MÙ. Internal pipette solution consisted of 108 mM $CsMeSO_4$, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES (pH 7.2 adjusted with CsOH).

Whole cell patch clamp recordings were performed in voltage-clamp mode using an Axopatch 200B amplifier (Axon Instruments) linked to a personal computer with pCLAMP version 9.0 or 9.2 software. Series resistance was compensated by 85%, leak currents were negligible, and the data were filtered at 1 kHz. Individual pEGFP-expressing cells were held at -100 mV, and currents were evoked by stepping to a test potential of +20 mV. Only cells with current amplitudes greater than 50 pA and less than 1.5 nA were used for analysis.

Voltage-dependent G protein inhibition was assessed by application of a strong, depolarizing pre-pulse (PP) to +150 mV for 50 ms, immediately prior to the test potential–during alternating sweeps of an assay. Pre-pulse relief of inhibition was quantified as the ratio of peak current amplitudes observed in paired test pulses performed with (I_{+PP}) and without (I_{-PP}) the prepulse (i.e., I_{+PP}/I_{-PP}).

Data Analysis

All data were analyzed using Clampfit version 9.2 (Axon Instruments) and fitted in Sigmaplot 2000 (SPSS Inc.). Statistical analyses were carried out using SigmaStat 2.03 (SPSS Inc.). All sample means are reported +/- SEM. Statistically significant differences between means were assessed using student's t-test, Mann-Whitney rank sum test, or one-way ANOVA at 95% confidence level as appropriate.

Results

Previously, two sections of the N-type channel I-II linker region, α_{1B} amino acid residues 372-389 and 410-428, were identified as functionally important binding sites for the $G\beta\gamma$ heterodimer [27,30]. To test the contribution of individual residues of these sections to $G\beta\gamma$-mediated channel inhibition, alanine/cysteine scanning mutagensis was performed. Residues 372-389 include six amino acid residues that are predicted, on the basis of crystallographic data, to be unavailable for interaction with $G\beta\gamma$, as their access is likely occluded by the calcium channel β subunit [32]. The remaining residues in this section, and in the second region (residues 410-428) were individually substituted to alanine or cysteine. The resulting mutant channels were coexpressed with human $G\beta_1\gamma_2$, and their respective susceptibilities to $G\beta\gamma$-mediated VDI were quantified using a pre-pulse facilitation (PPF) paradigm (Figure 1).

WT channels displayed the hallmark characteristics of $G\beta\gamma$-mediated channel inhibition (Figure 1A, left), including kinetic slowing of activation and relief of inhibition by a strong depolarizing pre-pulse (PPF ratio for WT channel assays: 2.36 +/- 0.23). When examining PPF ratios obtained with mutant and WT channels, two of the 30 mutants examined, R376A and V416A, showed a significant loss of $G\beta\gamma$-mediated inhibition when compared to wild type channels (PPF ratios 1.64 +/- 0.18 and 1.44 +/- 0.01, respectively; *P = 0.028 and 0.001, respectively; see Figure 1B). To test whether or not the effect of these mutations on $G\beta\gamma$-mediated channel inhibition were additive, a double $Ca_v2.2$ α_{1B} mutant containing both the R376A and V416A substitutions was engineered. Co-expression of this double mutant with exogenous $G\beta_1\gamma_2$, and subsequent electrophysiological analysis using the PPF protocol, found the degree of $G\beta\gamma$-mediated inhibition to be significantly less than that of WT channels, but similar to that observed in the presence of either one of the individual mutations alone (PPF ratio: 1.71 +/- 0.15, *t-test, P = 0.023) (Figure 2A).

To further characterize the roles of Arg^{376} and Val^{416}, additional single mutant α_{1B} subunits, containing R376E, R376F, V416E, and V416K substitutions, were engineered and co-expressed with exogenous $G\beta_1\gamma_2$. Neither of the latter Val^{416} substitutions resulted in significant changes in PPF ratio as compared to WT channels, Fig. 2A). However, the phenylalanine substitution at Arg^{376} significantly increased the PPF ratio for channels coexpressed with Gβ1γ2 (mean values of 2.36 and 3.62 for WT and R376F, respectively, t-test $p < 0.017$, Fig. 2A), suggesting that this amino acid substitution actually increased $G\beta\gamma$-mediated channel inhibition. These data thus further support the notion of residue 376 being an important determinant of VD G protein modulation of N-type channels.

The enhancement of $G\beta\gamma$-mediated VDI by the R376F mutation, and the proximity of this mutation to critical sites of interaction between the α_{1B} and β subunits of the $Ca_v2.2$ channel, led us to ask whether the nature of the $Ca_v\beta$ subunit might affect this enhancement. To examine this issue, we coexpressed the α_2-δ subunit and the R376F mutant with different isoforms of the $Ca_v\beta$ subunit in tsA-201 cells, and for each resulting population of $Ca_v2.2$ channels, we tested the effect of coexpression of heterologous $G\beta_1\gamma_2$ on current densities and PPF ratios. As shown in Fig. 2B, little VD modulation was observed in the absence of exogenously coexpressed $G\beta_1\gamma_2$ irrespective of the type of calcium channel β subunit that was present. For channels containing either $Ca_v\beta_{2a}$ or $Ca_v\beta_4$, coexpression with $G\beta_1\gamma_2$ induced

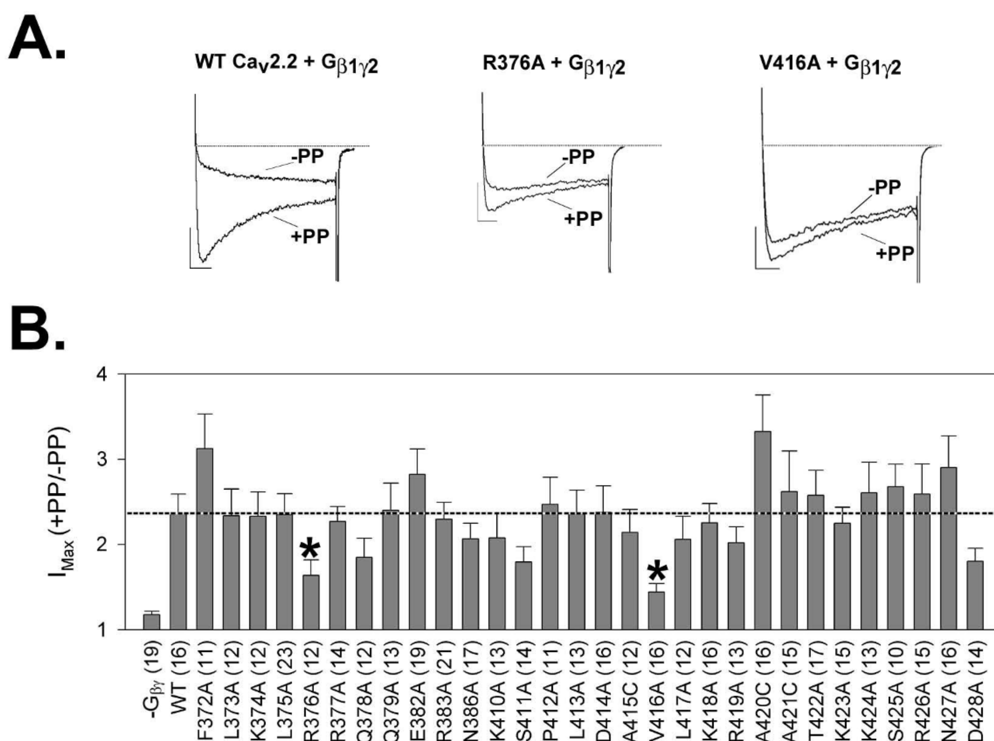

Figure 1 ***A*: Three sets of typical *current traces* from tsA-201 cells expressing WT or mutant rat $Ca_v2.2$ calcium channels and $G\beta_1\gamma_2$, as described in METHODS**. Each is a matched set of *current traces* from sequential test pulses, with the second test pulse preceded by a150-mV depolarizing prepulse. (Vertical and horizontal scale bars represent 15 pA and 15 ms, respectively; in each set the trace with larger current values is from the second test pulse.) *Left*: traces from a cell expressing the WT channel and $G\beta_1\gamma_2$. *Center*: traces from a cell coexpressing the $Ca_v2.2$-R376A mutant channel and $G\beta_1\gamma_2$. *Right*: traces from a cell coexpressing the $Ca_v2.2$-V416A mutant channel and $G\beta_1\gamma_2$. ***B***: Histogram summarizing the results of paired-pulse facilitation (PPF) experiments performed with all Ala/Cys point mutants of $Ca_v2.2$; columns show mean PPF values with SE bars for each condition. Respective positions of mutations in the $Ca_v2.2$ amino acid sequence are indicated by numbers beneath the corresponding columns (see METHODS for full description of the mutations used). Of the 30 individual amino acid residues examined in the $Ca_v2.2$ I-II linker region, Ala mutations at both Arg^{376} and Val^{416} (*$p < 0.05$, t-test) result in a significant loss of $G\beta\gamma$-mediated channel inhibition, as measured by the degree of pre-pulse relief following a depolarizing pre-pulse, when compared to WT control. Numbers in parentheses indicate numbers of cells tested.

robust VD modulation of channel activity, whose magnitude was, however, smaller than that observed with channels containing $Ca_v\beta_1$. Strikingly, virtually no VD modulation was evident in R376F channels containing the $Ca_v\beta_3$ subunit (i.e., there was no significant difference in PPF in the presence and the absence of G proteins). These data are in striking contrast to our previous findings showing that with WT $Ca_v2.2$, $G\beta_1\gamma_2$ most strongly inhibited channels containing $Ca_v\beta_{2a}$, followed by $Ca_v\beta_3$, $Ca_v\beta_4$ and $Ca_v\beta_{1B}$ [35]. Hence, a single amino acid substitution in the $Ca_v2.2$ I-II linker drastically alters the $Ca_v\beta$ subunit dependence of $G\beta_1\gamma_2$ inhibition of the channel.

Discussion

In this manuscript we have narrowly focused on the contribution of individual amino acids in the $Ca_v2.2$ I-II linker region to voltage dependent G protein inhibition of the channel. Among thirty amino acids in the I-II linker of the $Ca_v2.2$ channel, we have identified two, Arg^{376} and Val^{416}, that serve as determinants of G-protein mediated VDI of $Ca_v2.2$ channels, suggesting a highly localized interaction of $G\beta_1\gamma_2$ with the I-II loop. The impact of single amino acid substitutions on G protein inhibition is reminiscent of our earlier findings showing that phosphorylation of a single I-II linker residue, Thr^{422}, can disrupt modulation of $Ca_v2.2$ channels by $G\beta_1$ [36,37].

Arg^{376} is particularly interesting because the R376F mutation drastically altered the impact of $Ca_v\beta$ subunit coexpression on the degree of VDI: whereas $G\beta_1$-mediated inhibition of WT channels is reported to be strongest for channels containing $Cav\beta_{2a}$, followed by $Ca_v\beta_3$, $Ca_v\beta_4$ and $Ca_v\beta_{1B}$, respectively [35], we report here that $G\beta_1$-mediated regulation of R376F channels is strongest for channels containing $Ca_v\beta_{1B}$, followed by $Ca_v\beta_{2a}$, $Ca_v\beta_4$, $Ca_v\beta_3$, respectively, with no significant voltage-dependent inhibition observed for the latter. At

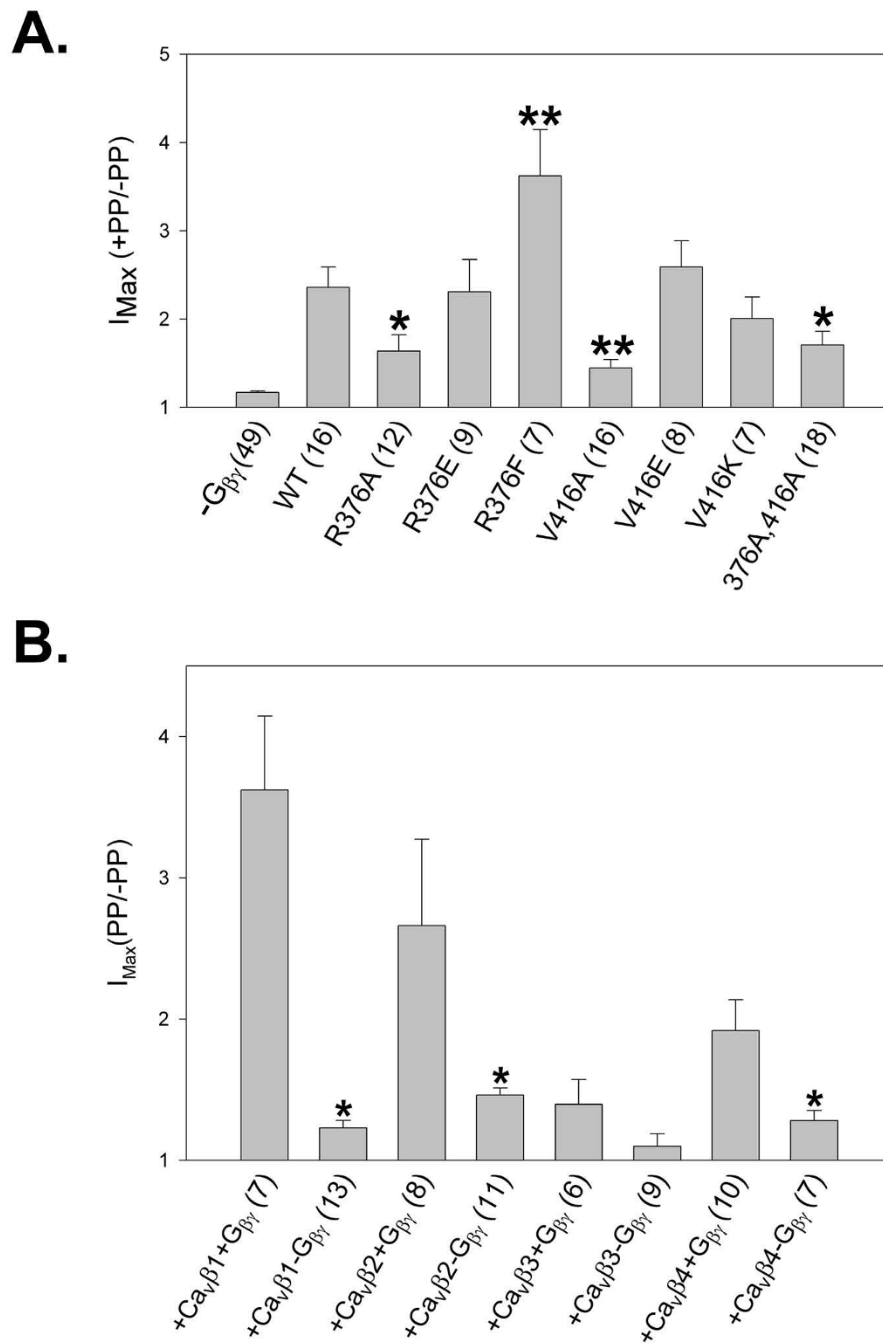

Figure 2 ***A*: Histogram summarizing the results of PPF experiments performed with $Ca_v2.2$ isoforms carrying mutations of α_{1B} residues Arg^{376} and Val^{416}**. Columns show mean PPF values with SE bars for each condition. Human $G\beta_1\gamma_2$ was co-expressed in tsA-201 cells with the $Ca_v2.2$ isoforms for each condition presented except for the negative control ("-$G\beta_1\gamma_2$"). Respective mutations in the $Ca_v2.2$ amino acid sequence are indicated beneath the corresponding columns (see METHODS for full description of the mutations used). Of the various conditions examined only the mutations R376A, R376F, V416A, and the double mutation R376A, V416A resulted in a significant loss of $G\beta\gamma$-mediated channel inhibition, as measured by the degree of pre-pulse relief following a depolarizing pre-pulse, when compared to WT control (*$p < 0.05$, t-test, **$p < 0.05$ one-way ANOVA, Dunnett's method, or Kruskal-Wallis one-way ANOVA on ranks). Numbers in parentheses indicate numbers of cells tested for the respective condition. ***B***: Histogram summarizing the results of PPF experiments using tsA-201 cells co-transfected to express α_{1B} mutant R376F with $Ca_v\beta$ isoforms β_{1B}, β_2, β_3, and β_4, with or without heterologous human $G\beta_1\gamma_2$ as indicated by labels beneath columns. Columns show mean PPF values with SE bars for each condition (see METHODS for full description of the mutations used). Of the conditions examined, coexpression of $Ca_v\beta_{1B}$ and $Ca_v\beta_{2a}$, and $Ca_v\beta_4$ resulted in statistically significant differences in mean current density for R376F channels expressed with and without heterologous $G\beta\gamma$ (*$p < 0.05$ using t-test, Mann-Whitney rank sum test, and t-test, respectively).

this point we do not know how the mutation of residue 376 to phenylalanine increases VD G protein inhibition. When coexpressed with $Ca_v\beta_{1B}$ in the absence of heterologous $G\beta_1\gamma_2$, the R376F mutant had a half activation potential that did not differ significantly from that of the wild type channels (data not shown); moreover, at the majority of test potentials examined, the mutation yielded no significant changes in the rates of activation and inactivation (versus WT channels, data not shown), suggesting that the effects of the mutation on $G\beta\gamma$ modulation are not complicated by changes in biophysical properties of the channel. An increased degree of prepulse relief could occur as a result of several mechanisms. First, the mutation could destabilize the binding of $G\beta\gamma$ to the channel, thus resulting in more effective dissociation of $G\beta\gamma$ from the channel in response to membrane depolarization. This however seems unlikely, because the kinetics of the facilitated current were found to be similar for both the wild type and the mutant channels, indicating that both channels are completely dis-inhibited (and thus dissociated from $G\beta\gamma$) following the application of a prepulse (data not shown). Agler and colleagues [25] reported that the N-terminus of $Ca_v2.2$ is capable of interacting with the domain I-II linker, and that this interaction contributes to G protein inhibition of the channel. It is thus conceivable that the nature of residue 376 could affect G protein inhibition indirectly by virtue of altering the binding of the N-terminus to the I-II linker.

Alternatively, it is possible that residue 376 is involved in transducing $G\beta\gamma$ binding to alter channel gating, such that a stronger voltage dependent inhibition is observed in the mutant channel. Residue 376 is three amino acid residues just upstream of an alpha helical structure (the AID, comprising residues 379-396) that is involved in binding of the $Ca_v\beta$ subunit [38], and could potentially serve as a hinge that links G protein binding to the gating machinery of the channel. However, it has also been proposed for $Ca_v2.1$ channels that I-II linker residues 357-378 are all part of a stable continuation of the alpha helical structure of the AID, and that stability and continuity of this helical structure is required for VD G protein inhibition of the channel [39]. In the latter case the R376F mutation may simply create a more stable binding pocket for $G\beta\gamma$, perhaps in part by eliminating one of the eight positive charges carried by the side chains of I-II residues 357-396, which may render this section of the I-II linker less likely to move in response to a membrane depolarization event. Whatever the actual case, the proximity of Arg^{376} to the $Ca_v\beta$ subunit interaction site also provides for a mechanism by which the nature of the $Ca_v\beta$ subunit could affect the extent of G protein inhibition that is observed.

Although alanine mutagenesis of residues 376 and 416 significantly reduced the effects of $G\beta\gamma$, VDI was not completely eliminated, and the effects of the individual amino acid substitutions were not additive. This suggests that either other amino acid residues in the $Ca_v2.2$ $\alpha1$ subunit might help stabilize the binding of $G\beta\gamma$ to the channel (such as for example, residue in the N-terminus), or that the $Ca_v\beta$ subunit may contribute directly to anchoring $G\beta\gamma$ to the channel. The latter would be consistent with recent findings showing that the presence of the $Ca_v\beta$ subunit is required to permit VDI of $Ca_v2.1$ channels [39].

Altogether, our data further implicate the domain I-II linker region as an important contributor to voltage dependent G protein modulation of N-type calcium channels. Furthermore, our results suggest that the regulation of N-type calcium channels by G proteins involves complex interactions between $G\beta\gamma$, the $Ca_v2.2$ $\alpha1$ subunit, and the auxiliary $Ca_v\beta$ subunit, and reveal that substitution of a single amino acid residue that is conserved in all HVA calcium channels is sufficient to significantly alter the interactions among these players. Although the precise molecular mechanism by which residue 376 couples $G\beta\gamma$ interactions to alterations in channel function remains unknown, the observation that highly localized alteration of a single amino acid residue increased G protein inhibition of the channel may offer potential avenues to enhance the efficacy of therapeutics acting on N-type channels via GPCRs.

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Authors' contributions

HWT performed molecular biology, cell transfection, electrophysiology, data analysis, and proofreading. AEK performed cell transfection, most of the electrophysiology recordings, and contributed data analysis and proofreading. LC performed tissue culture and electrophysiology recordings. LBV and DL performed cell transfection, electrophysiology, and data analysis. GWZ designed and supervised the research project, and provided analysis and proofreading. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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