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Determinants of the voltage dependence of G protein modulation within calcium channel β subunits

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Abstract $Ca_V\beta$ subunits of voltage-gated calcium channels contain two conserved domains, a *src*-homology-3 (SH3) domain and a guanylate kinase-like (GK) domain with an intervening HOOK domain. We have shown in a previous study that, although $G\beta\gamma$-mediated inhibitory modulation of $Ca_V2.2$ channels did not require the interaction of a $Ca_V\beta$ subunit with the $Ca_V\alpha1$ subunit, when such interaction was prevented by a mutation in the $\alpha1$ subunit, G protein modulation could not be removed by a large depolarization and showed voltage-independent properties (Leroy et al., J Neurosci 25:6984–6996, 2005). In this study, we have investigated the ability of mutant and truncated $Ca_V\beta$ subunits to support voltage-dependent G protein modulation in order to determine the minimal domain of the $Ca_V\beta$ subunit that is required for this process. We have coexpressed the $Ca_V\beta$ subunit constructs with $Ca_V2.2$ and $\alpha_2\delta$-2, studied modulation by the activation of the dopamine D2 receptor, and also examined basal tonic modulation. Our main finding is that the $Ca_V\beta$ subunit GK domains, from either β1b or β2, are sufficient to restore voltage dependence to G protein modulation. We also found that the removal of the variable HOOK region from β2a promotes tonic voltage-dependent G protein modulation. We propose that the absence of the HOOK region enhances $G\beta\gamma$ binding affinity, leading to greater tonic modulation by basal levels of $G\beta\gamma$. This tonic modulation requires the presence of an SH3 domain, as tonic modulation is not supported by any of the $Ca_V\beta$ subunit GK domains alone.

Keywords Calcium channel · Beta subunit · Electrophysiology

Introduction

Voltage-gated calcium (Ca_V) channels play a major role in the physiology of all excitable cells. Three families have been identified, Ca_V1–3 (for review, see [17]). The high-voltage-activated (HVA) Ca_V1 and 2 classes are heteromultimers composed of the pore-forming $\alpha1$ subunit, associated with auxiliary $Ca_V\beta$ and $\alpha_2\delta$ subunits (for review, see [12]). Four $Ca_V\beta$ subunit genes have been cloned, and these subunits are important for HVA calcium channel function (for review, see [16]), since they promote the expression of functional channels at the plasma membrane and modulate their biophysical properties [6, 8, 11, 29]. $Ca_V\beta$ subunits bind with high affinity to the α-interaction domain (AID) on the I–II loop of Ca_V1 and 2 channels [29], although other $\alpha1$ subunit interaction sites are also likely to be important in mediating the actions of $Ca_V\beta$ subunits [35, 40].

In a previous study, we investigated the role of $Ca_V\beta$ subunits in the plasma membrane expression and G protein modulation of $Ca_V2.2$ calcium channels, by mutating the AID tryptophan (W391) in the I–II loop of $Ca_V2.2$, and thus disrupting the high-affinity interaction with $Ca_V\beta$ subunits

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[21]. One conclusion was that the $Ca_V2.2$W391A mutant channels lost all modulation by $Ca_V\beta1b$ and showed strongly reduced expression at the plasma membrane. While they still showed G protein modulation following the activation of a coexpressed dopamine D2 receptor, this modulation could not be reversed by depolarization. In contrast, for palmitoylated $Ca_V\beta2a$, only the expression at the plasma membrane was affected when it was coexpressed with the mutant $Ca_V2.2$W391A channels, while all the biophysical properties of the expressed $Ca_V2.2$W391A channels remained normally modulated by $Ca_V\beta2a$. Furthermore, they also showed voltage-dependent G protein modulation. We concluded that the continuing influence of β2a was dependent on its palmitoylation, which increased the local concentration of β2a near the plasma membrane sufficiently to allow lower-affinity interactions to occur between it and the mutant channel α1 subunit, which were effective in modulating the channel properties [21].

$Ca_V\beta$ subunits were originally predicted by structural modeling to contain a *src*-homology-3 (SH3) domain followed by a guanylate kinase-like (GK) domain [18]. The SH3 domain is split with its final (fifth) β-strand separated from the rest of the domain by an intervening sequence termed the HOOK domain, whose sequence varies between $Ca_V\beta$ subunits and which is encoded by either a short or an alternative long exon. X-ray crystallographic studies have now produced detailed information on the domain structure [13, 27, 39]. From these studies, it is clear that the fifth β-strand of the SH3 domains provides the interaction with the GK domain, being situated after the variable HOOK region, whose structure was not determined (for review, see [31]). The GK domain interacts with the AID motif and has since been shown to be an important determinant of function for the HVA channels [32, 37, 38].

The primary goal of the present study was to determine the minimal domain(s) of $Ca_V\beta$ subunits that is able to confer voltage dependence on G protein modulation of $Ca_V2.2$ channels.

Materials and methods

Materials

The cDNAs used in this study were $Ca_V2.2$ (D14157), $Ca_V\beta1b$ (X61394), $Ca_V\beta2a$ (M88751), $\alpha_2\delta$-2 [2], and dopamine D_2 receptor (X17458). When used, the green fluorescent protein (GFP-mut3b, U73901) was used to identify transfected cells. All cDNAs were subcloned into pMT_2 vector. Transducin-α was used as described [5].

Construction of truncated β subunit domains

We have been guided by the structure in our choice of truncations and deletions in the present study (Fig. 1). In the case of the GK domains, we have used the exon boundary to determine the C-terminal end, since such boundaries often delimit a stable functional domain, and this marks the end of the second conserved domain, as originally identified (for review, see [7]). It was important that the GK domain constructs were stable since previous studies have examined the properties of several GK domain constructs with varying results, regarding their ability to mimic the functions of intact $Ca_V\beta$ subunits, and it is possible that these constructs have varying stabilities in different cell types [23, 37]. All constructs were made by standard molecular biological techniques and their sequences verified by sequencing both

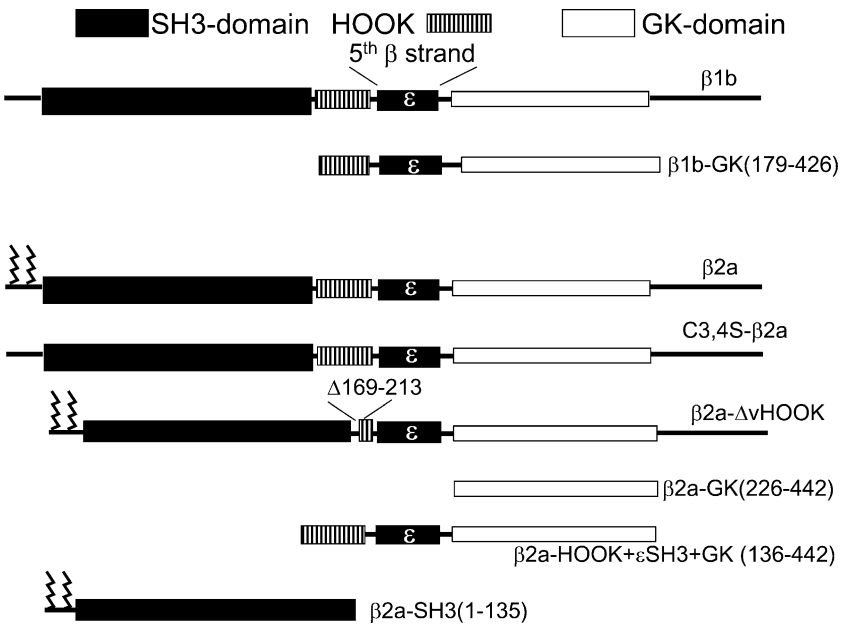

Fig. 1 Diagram of the main constructs used in the electrophysiological experiments in the present study

strands. The truncated constructs used for electrophysiology (with their amino acid residues) were β2a-Δ-vHOOK (Δ169–213), β2a-SH3 (1–135), β2a-(SH3+HOOK+εSH3) (1–225), β2a-GK (226–442), β2a-(HOOK+εSH3+GK) (136–442), β1b-GK (230–426), and β1b-(HOOK+εSH3+GK) (179–426).

Yeast two-hybrid assays

Assays were carried out using the MATCHMAKER GAL4 two-hybrid kit (Clontech). Fragments of Ca_vβ2a (amino acids 5–442, 5–134, 5–224, 135–442, 214–442, or 225–442), the Ca_V2.2 I–II loop (360–483), and Ca_Vβ1b were generated by polymerase chain reaction and subcloned in-frame into the vectors pACT2 and pAS2-1. Plasmids were cotransformed into the yeast strain Y190 and transformants were selected by plating onto minimal selective dropout (SD) *-Leu*, *-Trp* agar. Protein interactions were identified by restreaking colonies onto SD *-Leu*, *-Trp* plates and carrying out colony-lift β-galactosidase assays according to the supplied protocol.

Cell culture, heterologous expression, and whole cell recording

The tsA-201 cells were cultured in a medium consisting of D-MEM, 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cDNAs (all at 1 μg/μl) for Ca_Vα1 subunits, Ca_Vβ, α_2δ-2, and GFP (when used as a reporter of transfected cells) were mixed in a ratio of 3:2:2:0.4. The cells were transfected using Fugene 6 (Roche Diagnostics, Lewes, UK; DNA/Fugene 6 ratio of 2 μg in 3 μl). The tsA-201 cells were replated at low density on 35-mm tissue culture dishes on the day of recording. Whole-cell patch-clamp recordings were performed at room temperature (22–24°C). Only fluorescent cells expressing GFP were used for recording. The single cells were voltage-clamped using an Axopatch 200B patch-clamp amplifier (Molecular Devices). The electrode potential was adjusted to give zero current between pipette and external solution before the cells were attached. The cell capacitance varied from 10 to 40 pF. Patch pipettes were filled with a solution containing (in mM) 140 Cs-aspartate, 5 EGTA, 2 $MgCl_2$, 0.1 $CaCl_2$, 2 K_2ATP, 10 HEPES, titrated to pH 7.2 with CsOH (310 mOsm) with a resistance of 2–4 MΩ. The external solution contained (in mM) 150 tetraethylammonium bromide, 3 KCl, 1.0 $NaHCO_3$, 1.0 $MgCl_2$, 10 HEPES, 4 glucose, 10 $BaCl_2$, pH adjusted to 7.4 with Tris–Base (320 mOsm). The pipette and cell capacitance as well as the series resistance were compensated by 80%. Leak and residual capacitance current were subtracted using a P/4 protocol. All experiments in which quinpirole was applied were carried out in small volume disposable glass chambers (300–500 μl volume) with a perfusion rate of 200–300 μl/s, which were used once only, excluding the possibility that tonic modulation was due to prior quinpirole exposure. Quinpirole was made up as a 10-mM stock solution, and aliquots were diluted as necessary and used once only.

Data were filtered at 2 kHz and digitized at 5–10 kHz. The holding potential was −100 mV, and pulses were delivered every 10 s. Test pulses were normally 40 ms in duration, and in the three pulses, protocol P_1 and P_2 were separated by at least 150 ms, and P_2 was preceded by a 50-ms prepulse to +120 mV. Activation properties were determined from tail current measurements, as previously described [21]. Steady-state inactivation properties were measured by applying a 5- to 20-s pulse (depending on the inactivation properties of the currents) from −120 to +20 mV in 10-mV increments, followed by 11 ms repolarization to −100 mV before the 100-ms test pulse to +20 mV.

Data analysis and curve fitting

Current amplitude was measured 10 ms after the onset of the test pulse, and the average over a 2-ms period was calculated and used for subsequent analysis. The current density–voltage (*I–V*) relationships were fitted with a modified Boltzmann equation as follows: $I = G_{\max} \times (V - V_{\text{rev}}) / \left(1 + \exp\left(-\left(V - V_{50,\text{act}}\right)/k\right)\right)$ where I is the current density (in pA/pF), $G_{\max}$ is the maximum conductance (in nS/pF), V_{rev} is the reversal potential, $V_{50,\ \text{act}}$ is the midpoint voltage for current activation, and k is the slope factor. Activation and steady-state inactivation data were fitted with a single Boltzmann equation of the form: $I = I_{\max}\left((A_1 - A_2)/[1+\exp\left(\left(V - V_{50,\ \text{inact}}\right)/k\right)] + A_2\right.$. where $I_{\max}$ is the maximal current and $V_{50,\ \text{inact}}$ is the half-maximal voltage for current inactivation. For the steady-state inactivation, A_1 and A_2 represent the total and noninactivating current, respectively. Analysis was performed using Pclamp7 and Origin 7.

Data are expressed as the mean±SEM of the number of replicates, *n*. Error bars indicate the standard errors of multiple determinations. Statistical significance was analyzed using Student's paired or unpaired *t* test or by ANOVA with Bonferroni's post hoc test, if multiple comparisons were made.

Results

The isolated GK domains of Ca_Vβ1b and β2a support voltage-dependent G protein modulation

In a previous study, we used a Ca_V2.2 construct with a mutation in the I–II loop (W391A), such that it did not show high-affinity interaction with Ca_Vβ subunits, and observed a lack of voltage dependence of the quinpirole-mediated inhibition in the presence of β1b [21]. From that study, we concluded that the interaction of Ca_Vβ with the I–II linker

was necessary for voltage-dependent G protein modulation. However, it is possible that this interaction with the I–II linker is not sufficient in itself, but is required to bring another region of the $Ca_V\beta$ subunits into proximity with the channel. In particular, we found that for β2a, voltage-dependent G protein modulation was still present, despite the W391A mutation in the I–II linker. Therefore, there remained a question as to whether the palmitoylation of β2a resulted in a sufficiently high concentration of $Ca_V\beta$ subunit being present near to the I–II linker of the channel, such that there was high occupancy by β2a of the mutated I–II linker, despite a very low-affinity interaction or whether the residual interaction was with another domain of β2a on another part of the channel [21].

In order to examine which domain of $Ca_V\beta$ was necessary to promote the voltage dependence of G protein modulation, we compared the ability of full-length β1b or β2a and their isolated GK domains to support voltage-dependent G protein modulation. The constructs used are shown in Fig. 1. We coexpressed all the β subunit constructs with $Ca_V2.2$ and $\alpha_2\delta$-2. For coexpression with full-length $Ca_V\beta1b$, the peak I_{Ba} was −158.2±25.7 pA/pF at +20 mV (n=35). For comparison, in the absence of any $Ca_V\beta$ subunit, the peak I_{Ba} at +30 mV was −8.3±1.0 pA/pF (n=9), in experiments performed over the same time period. For $Ca_V2.2/\alpha_2\delta$-2/β1b currents, application of the dopamine D2 receptor agonist quinpirole (100 nM) produced maximally 63.7±6.6% inhibition at +10 mV (Fig. 2a,b). This inhibition showed a strong voltage dependence, as the P_2/P_1 ratio was 2.97±0.23 at +10 mV (Fig. 2a,c). This is an example of complete voltage dependence, since full reversal of a 64% inhibition predicts a P_2/P_1 ratio of 2.8. In the absence of any coexpressed β subunit, quinpirole (100 nM) application still produced a substantial effect, resulting in 44±13% inhibition at +10 mV (Fig. 2a,b). However, the voltage dependence of this inhibition was very low, the P_2/P_1 ratio being 1.3±0.11 at +10 mV (Fig. 2a,c; P<0.0001 compared to β1b), as we described previously for $Ca_V2.2$W391A, which did not interact with β1b [21].

We then utilized one of the truncated $Ca_V\beta1b$ subunit constructs described previously [32] to examine which domain(s) of $Ca_V\beta1b$ were required to promote the voltage dependence of G protein modulation. We found that a β1b-GK domain construct (β1b-HOOK+εSH3+GK (179–426)), containing both the HOOK region and the fifth β-strand, coexpressed with $Ca_V2.2/\alpha_2\delta$-2, enhanced calcium channel currents to a smaller extent than full-length β1b subunit, the peak current at +20 mV being −79.8±16.2 pA/pF (n=7), as described previously [32]. However, quinpirole (100 nM) produced 67.5±6.9% inhibition of I_{Ba} at +10 mV, and this inhibition could be relieved by a depolarizing prepulse to +120 mV (Fig. 2a,b). The P_2/P_1 ratio was greater than that in the absence of any β subunit at all potentials, being 2.06±0.15 at +10 mV (Fig. 2a,c; P<0.001 compared to no β subunit). It is important to note that for all β1b constructs, the basal facilitation prior to the application of quinpirole was not significantly different from unity (Fig. 2d). This result indicates that the interaction of the $Ca_V2.2$ I–II linker with the GK domain of the $Ca_V\beta1b$ subunit is sufficient to promote voltage dependence of G protein modulation, and the SH3 domain is not required.

For $Ca_V2.2/\alpha_2\delta$-2 together with full-length β2a, the peak I_{Ba} was −145.9 pA/pF at +20 mV (Table 1). Quinpirole (100 nM) inhibited these currents to a smaller extent, producing maximally 38.7±4.9% inhibition at +10 mV (Fig. 3a,b; P=0.0042 compared to β1b). This inhibition showed a P_2/P_1 ratio of 1.88±0.19 at +10 mV (Fig. 3a,c). The low P_2/P_1 ratio was to be expected, in view of the small inhibition by quinpirole.

Based on our previous findings [21], it is likely that there is increased interaction between the β2a subunit and $Ca_V2.2$, as a result of palmitoylation elevating its effective concentration at the plasma membrane and increasing its availability to interact with the channel. We found that removal of the palmitoylation motif on β2a (C3,4S-β2a) resulted in greater inhibition by 100 nM quinpirole (67.6±7.5% inhibition at + 10 mV, Fig. 3a,b; P=0.0047 compared to β2a), and correspondingly increased the P_2/P_1 ratio to 2.93±0.67 at +10 mV (Fig. 3b,c).

Similar to our finding for the β1b-HOOK+εSH3+GK construct, we found that β2a-HOOK+εSH3+GK (136–442) supported voltage-dependent G protein modulation. This construct, coexpressed with $Ca_V2.2/\alpha_2\delta$-2, enhanced calcium channel currents to a smaller extent than full-length β2a subunit, the peak I_{Ba} being −63.2 pA/pF at +20 mV (Table 1). However, the inhibition by 100 nM quinpirole was 56.0±7.2% at +10 mV (Fig. 3b), and the P_2/P_1 ratio was 2.04±0.48 at +10 mV (Fig. 3c).

Similar results were obtained for a minimal GK domain of β2a, β2-GK (226–442), coexpressed with $Ca_V2.2/\alpha_2\delta$-2 where the peak current density at +20 mV was −79.9 pA/pF (Table 1). Although the interaction of this GK domain with the I–II linker of $Ca_V2.2$ was not confirmed in our yeast two-hybrid results (see Fig. 6), this may be a result of misfolding in the yeast system, as in tsA-201 cells, the peak I_{Ba} was significantly greater in the presence of β2a-GK (226–442) than in the absence of any β subunit (P<0.05, Table 1). Furthermore, the steady-state inactivation was also significantly hyperpolarized compared to the absence of any β subunit (P<0.01, Table 1), to a similar extent to full-length β1b [21] or C3,4S-β2a (Table 1) [32]. Both these results indicate that the calcium channel currents are influenced by β2a-GK (226–442) interacting with the I–II linker of $Ca_V2.2$. Moreover, quinpirole (100 nM) produced 62.0±6.7% inhibition at +10 mV, and the inhibition could be relieved by a depolarizing prepulse (Fig. 3a,b). The

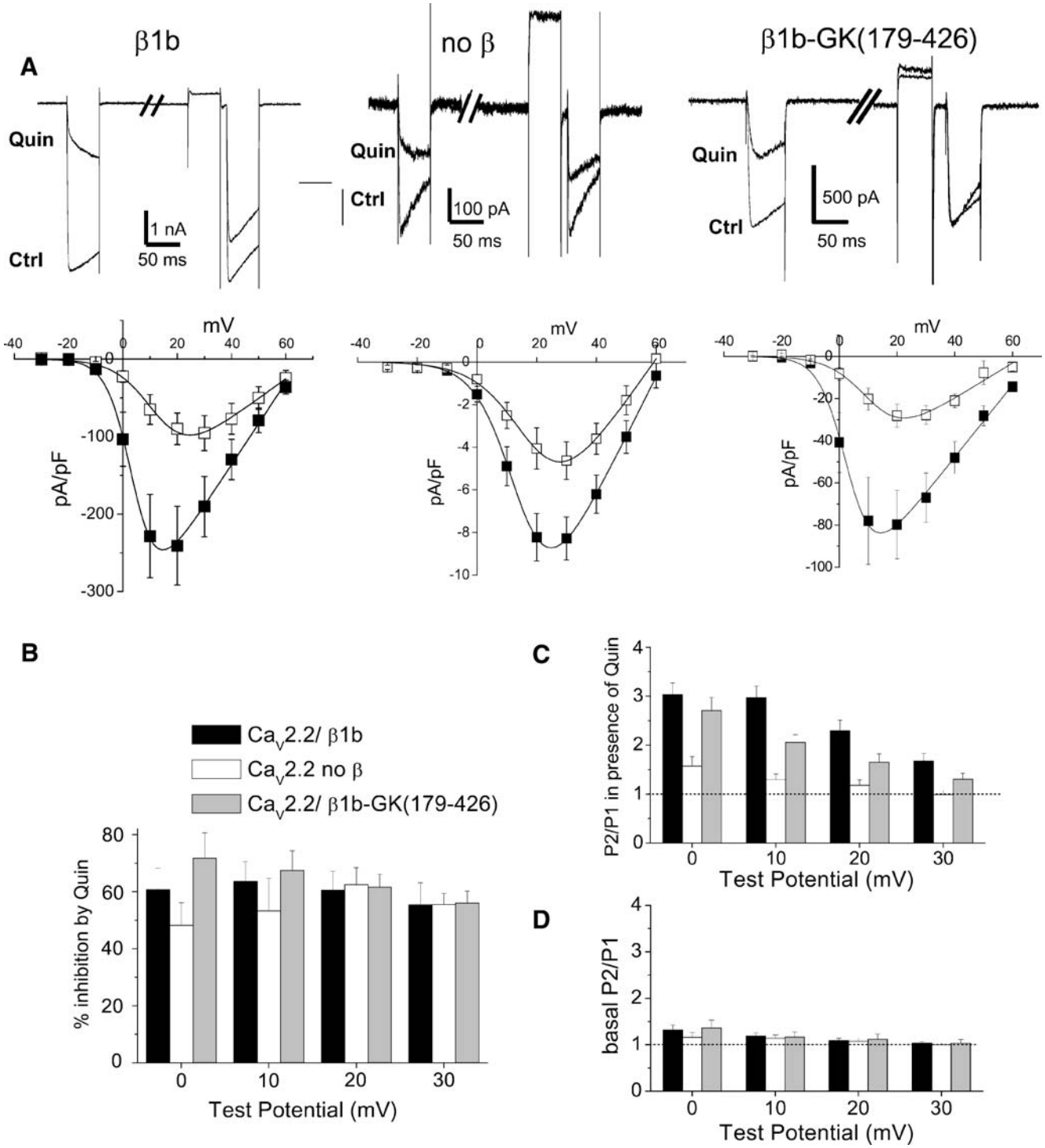

Fig. 2 GK domain of β1b restores voltage dependence to G protein modulation. **a** *Upper panel* example traces showing inhibition of $Ca_V2.2$ currents (*Ctrl*) by quinpirole (*Quin*, 100 nM) for $Ca_V2.2/\alpha_2\delta$-2 coexpressed with β1b (*left*), without β (*center*), and with β1b-GK (179–426) (*right*). Traces are shown for 40 ms depolarizations to +10 mV before and after a depolarizing prepulse to +100 mV. *Lower panel* current–voltage relationships for the same conditions, prior to (*filled squares*) and during quinpirole application (*open squares*) (n= 10, 9, and 7, respectively). **b** Percentage inhibition by quinpirole between 0 and +30 mV for the three conditions depicted in **a**. *Black bars* +β1b (n=10), *white bars* no β (n=7), and *gray bars* +β1b-GK (179–426) (n=7). **c** Facilitation (P_2/P_1) ratio between 0 and +30 mV in the presence of quinpirole for the same experiments as in **b**. The *dotted line* indicates a P_2/P_1 ratio of 1, i.e., no facilitation. **d** Basal facilitation (P_2/P_1) ratio between +10 and +30 mV for the same experiments as in **b**. The *dotted line* indicates a P_2/P_1 ratio of 1

Table 1 The effect of various $Ca_V\beta$ subunit constructs on biophysical parameters of $Ca_V2.2/\alpha_2\delta$-2 calcium channel currents expressed in tsA-201 cells

β species	Peak I_{Ba}, pA/pF (n)	Voltage for peak I_{Ba} (mV)	Steady-state inactivation $V_{50\ inact}$, mV (n)
No β	−8.3±1.0 (9)	+30	−25.4±7.5 (3)
β2a	−145.9±34.9 (14)**	+20	+0.47±2.16 (6)**
C3,4S-β2a	−96.6±12.0 (21)*	+20	−47.6±1.4 (3)** ††
β2-GK (226–442)	−79.9±23.9 (17)*	+20	−54.8±3.0 (5)** ††
β2a-GK (136–442)	−63.2±12.7 (18)†	+20	−45.5±3.0 (5)** ††
β2a-ΔvHOOK	−131.3±26.8 (16)**	+20	−14.2±6.0 (6)
C3,4S-β2a-ΔvHOOK	−101.4±15.4 (20)*	+20	−46.0±1.8 (5)** ††
β2-SH3 (1–135)+β2-GK (226–442)	−28.0±11.1 (9)††	+20	−57.3±1.4 (4)** ††

Statistical significances (one-way analysis of variance and Bonferroni's post hoc test) were determined for differences compared to $Ca_V2.2$ expressed without any β subunit or compared to $Ca_V2.2$ expressed with wild-type β2a subunit
*P<0.05 and **P<0.01, $Ca_V2.2$ expressed without any β subunit; † P<0.05 and †† P<0.01, $Ca_V2.2$ expressed with wild-type β2a subunit

P_2/P_1 ratio was 2.73±0.37 at +10 mV (Fig. 3a,c). This result confirms that the interaction of the $Ca_V2.2$ I–II linker with the GK domain of any $Ca_V\beta$ subunit is sufficient to promote voltage dependence of G protein modulation. For all the $Ca_V\beta2$ constructs depicted in Fig. 3c, their basal P_2/P_1 values were between 1 and 1.5, indicating little basal facilitation was present (Fig. 3d).

Investigation of the roles of the $Ca_V\beta$ SH3 and HOOK domains in voltage-dependent G protein modulation

In order to examine whether the SH3 domain and the HOOK domain of β2a played a role in the reduced G protein modulation shown by β2a, we examined the effect of a β2a construct in which the SH3 domain and the GK domain are present, but which is missing amino acids 169–213, comprising the variable HOOK domain (β2a-δvHOOK) [32]. The basic properties of I_{Ba} resulting from the coexpression of $Ca_V2.2/\alpha_2\delta$-2/β2a-ΔvHOOK are given in Table 1. For β2a-ΔvHOOK, the amount of inhibition by quinpirole was 39.9±6.7% at +10 mV, not significantly different from β2a itself (Fig. 4a,b). Evidence from our previous study [32] indicated that this construct remains palmitoylatable, such that the concentration at the plasma membrane and thus the occupancy by this construct of the I–II linker is likely to remain high. However, unlike full-length β2a, the P_2/P_1 ratio was also high, being 2.85±0.56 at +10 mV (Fig. 4c).

A potential reason for the discrepancy between the low percentage of inhibition and the high P_2/P_1 ratio for β2a-ΔvHOOK, compared to full-length β2a, is that currents in the presence of this construct were also observed to show strong basal facilitation. This is likely to account for the high P_2/P_1 ratio during quinpirole application, as the depolarizing prepulse also removes tonic facilitation. Prior to any agonist application, the P_2/P_1 ratio in the presence of β2a-ΔvHOOK was 2.34±0.22 at +10 mV (Fig. 5a,b). For comparison, wild-type β2a showed only a small degree of basal facilitation, P_2/P_1 under control conditions being 1.43±0.13 at +10 mV (Fig. 3d, P<0.05 compared to β2a-ΔvHOOK), and C3,4S-β2a-GK also showed no tonic facilitation (Fig. 3d). Furthermore, β2-GK (226–442) showed no basal facilitation in the absence of agonist, the P_2/P_1 ratio being 1.2±0.17 at +10 mV (Figs. 3d and 5a,b). For the proximally extended GK domain β2a-GK (136–442), there was also no significant basal facilitation, P_2/P_1 in the absence of agonist being 1.5±0.17 at +10 mV (Fig. 3d).

This basal facilitation in the presence of β2a-ΔvHOOK was due to tonic G protein modulation since it could be removed by coexpression of transducin-α, which acts as a sink for free Gβγ subunits. The basal P_2/P_1 ratio in the presence of transducin-α was 1.14±0.05 at +10 mV (Fig. 5b). Basal facilitation was also absent when guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS, 200 μM) was included in the patch pipette (data not shown). This concentration of GDP-βS also blocked quinpirole-mediated inhibition (data not shown).

In order to examine whether the presence of basal facilitation was favored by the palmitoylation of the β2a-ΔvHOOK, we also examined the properties of C3,4S-β2a-ΔvHOOK. The basic properties of currents in the presence of this construct are given in Table 1. Quinpirole-mediated inhibition was much higher than for the palmitoylatable construct, being 79.2±3.1% at +10 mV, similar to that obtained for C3,4S-β2a (Fig. 4a,b). The P_2/P_1 ratio was correspondingly high, being 3.22±0.74 at +10 mV (Fig. 4a,c). However, this construct showed no basal facilitation, the P_2/P_1 ratio prior to agonist application being 1.02±0.05 at +10 mV (Fig. 5b).

In the presence of β2a-ΔvHOOK, the time constant for dissociation (τ_{dissoc}) of tonically bound Gβγ at +120 mV was 33.6±6.5 ms (n=10, Fig. 5c). In contrast, for the small amount of tonic facilitation shown by wild-type β2a, the τ_{dissoc} at +120 mV was 21.3±4.5 ms (n=10, Fig. 5c), indicating a higher dissociation rate of Gβγ from wild-type β2a at +120 mV of 46.9 s^{-1} compared to 29.7 s^{-1} for β2a-ΔvHOOK, although the difference did not reach statistical

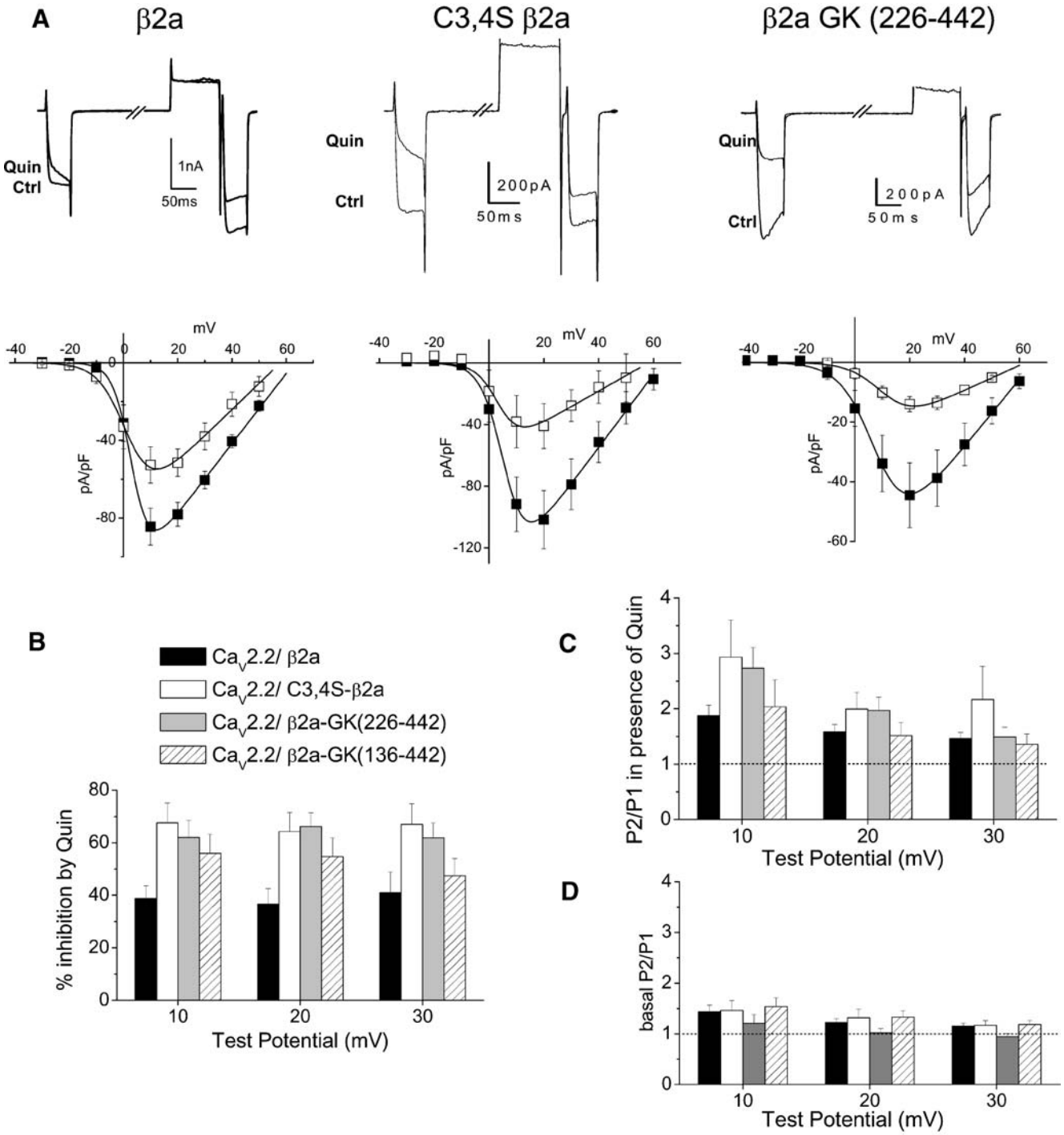

Fig. 3 GK domains of β2a support voltage-dependent G protein modulation. **a** *Upper panel* example traces showing inhibition of $Ca_V2.2$ currents (*Ctrl*) by quinpirole (*Quin*, 100 nM) for $Ca_V2.2/\alpha_2\delta$-2 coexpressed with β2a (*left*), C3,4S-β2a (*center*), and with β2a-GK (226–442) (*right*). Traces are shown for 40 ms depolarizations to +10 mV before and immediately after a depolarizing prepulse to +120 mV. *Lower panel* current–voltage relationships for the same conditions prior to (*filled squares*) and during quinpirole application (*open squares*), (n=6, 6, and 7, respectively). **b** Percentage inhibition by quinpirole between +10 and +30 mV for the three conditions depicted in **a** and the additional GK domain construct β2a-GK (136–442) (n=9, 7, 11, and 11, respectively). **c** Facilitation (P_2/P_1) ratio between +10 and +30 mV in the presence of quinpirole for the three conditions depicted in **a** and β2a-GK (136–442) (n=7, 8, 9, and 6, respectively). The *dotted line* indicates a P_2/P_1 ratio of 1. **d** Basal facilitation (P_2/P_1) ratio between +10 and +30 mV for the three conditions depicted in **a** and β2a-GK (136–442) (n=7, 9, 10, and 9, respectively). The *dotted line* indicates a P_2/P_1 ratio of 1

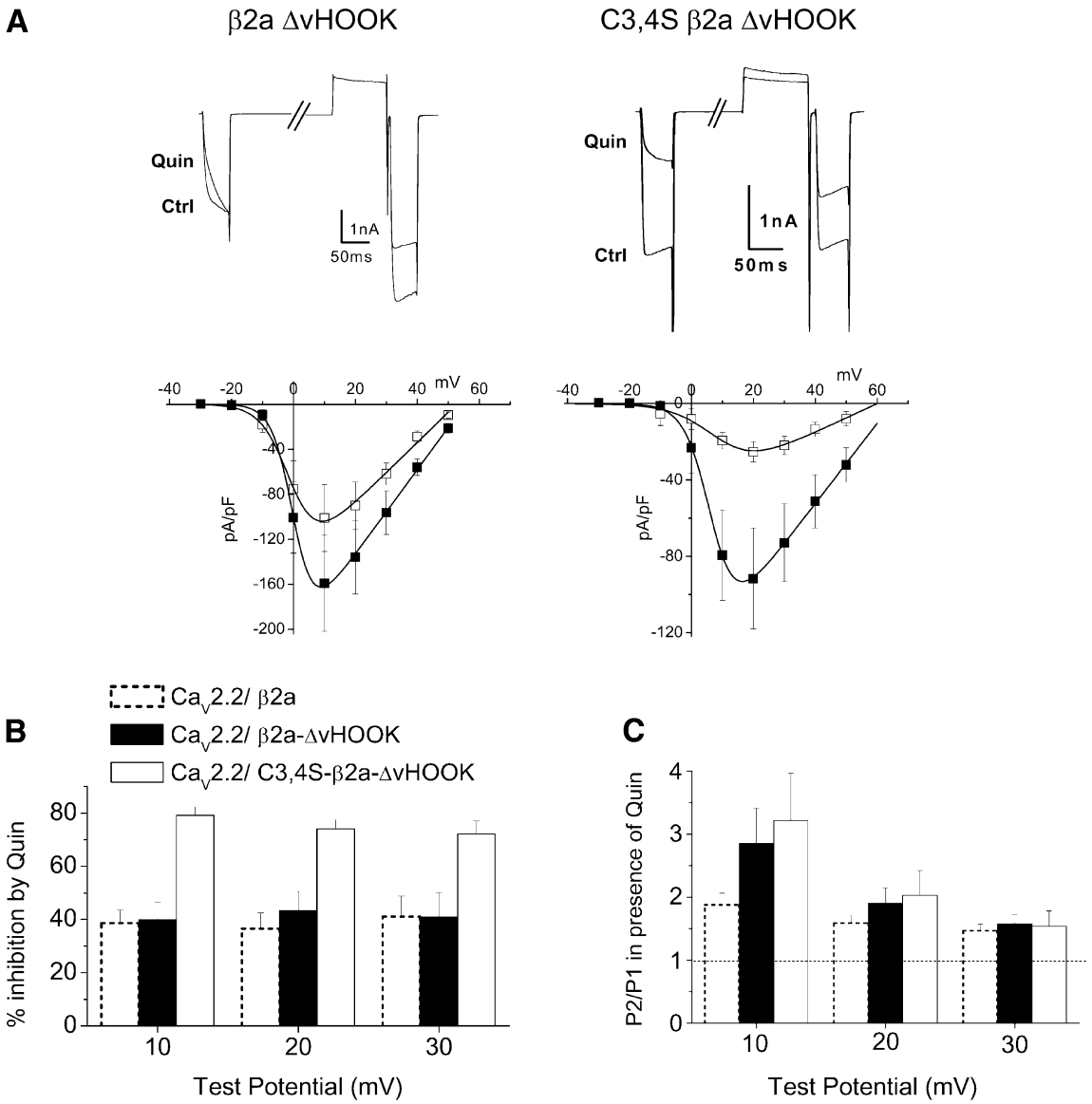

Fig. 4 The determinants for voltage-dependent G protein modulation and facilitation in the presence of β2a constructs. **a** *Upper panel* example traces showing inhibition of $Ca_V2.2$ currents (*Ctrl*) by quinpirole (*Quin*, 100 nM) for $Ca_V2.2/\alpha_2\delta$-2 coexpressed with β2a-ΔvHOOK (*left*) and C3,4S-β2a-ΔvHOOK (*right*). Traces are shown for 40 ms depolarizations to +10 mV before and after a depolarizing prepulse to +120 mV. *Lower panel* current–voltage relationships for the same conditions prior to (*filled squares*) and during quinpirole application (*open squares*) (n=6 for both). **b** Percentage inhibition by quinpirole between +10 and +30 mV for the two conditions depicted in **a** (n=10 and 8, respectively). Data for β2a from Fig. 3 is included for comparison (*dashed bar*). **c** Facilitation (P_2/P_1) ratio between +10 and +30 mV in the presence of quinpirole for the two conditions depicted in **a** (n=9 and 8, respectively). Data for β2a from Fig. 3 is included for comparison (*dashed bar*). The *dotted line* indicates a P_2/P_1 ratio of 1

significance. The time constant for rebinding of Gβγ at −100 mV to wild-type β2a was 247.7±25.9 ms, and for β2a-ΔvHOOK, it was 296.4±24.2 ms (Fig. 5d, P>0.05).

Assuming a basal Gβγ concentration of 50 nM, as estimated previously [34], we can utilize the tonic P_2/P_1 ratio and the rate of Gβγ rebinding after a depolarizing prepulse to determine the K_D for Gβγ at −100 mV, as described previously [34]. For $Ca_V2.2$ coexpressed with wild-type β2a, the k_{off} for Gβγ was calculated to be 2.83 s^{-1} and the k_{on} was 24.2 μM^{-1} s^{-1}, leading to a K_D for Gβγ interaction with the channel of 116.9 nM. We estimated the in vitro affinity of the interaction of the isolated $Ca_V2.2$ I–II linker and Gβγ to be 62 nM [3]. This is likely to be modulated in the intact channel, particularly by the presence of the $Ca_V\beta$ subunit. In contrast, for $Ca_V2.2$ with β2a-ΔvHOOK, the k_{off} was 1.44 s^{-1} and the calculated k_{on} was 38.6 μM^{-1} s^{-1}, leading to a much higher affinity for Gβγ, the K_D being 37.3 nM. It is worth noting that, as expected, the k_{off} was

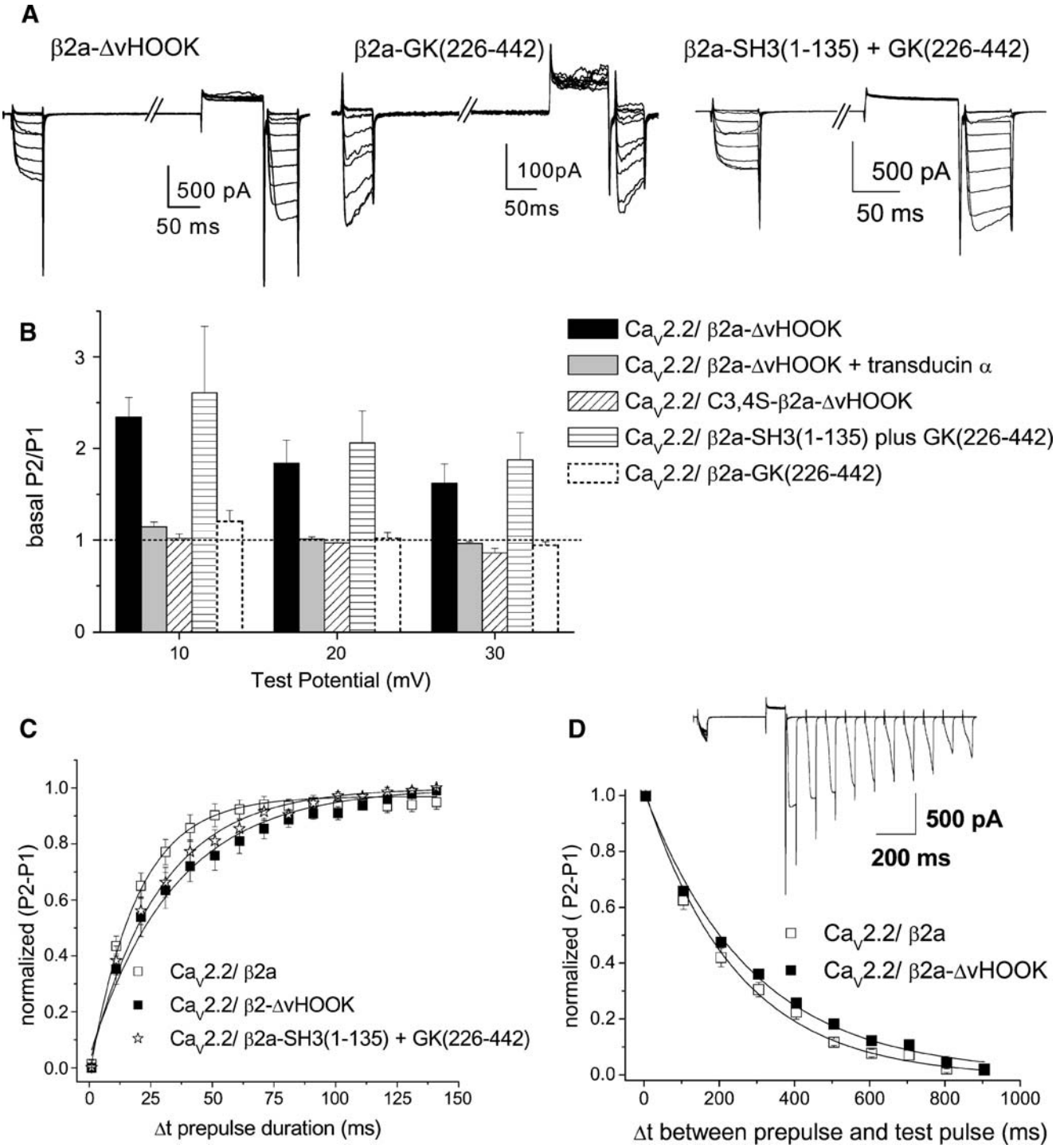

Fig. 5 The determinants for basal facilitation in the presence of β2a constructs. **a** Example traces showing basal facilitation of $Ca_V2.2$ currents for $Ca_V2.2/\alpha_2\delta$-2 coexpressed with β2a-ΔvHOOK (*left*), lack of basal facilitation with β2a-GK (226–442) (*center*), and basal facilitation with β2a-SH3 (1–135)+GK (226–442). Traces are shown for 40 ms depolarizations to +10 mV before and after a depolarizing prepulse to +100 mV. **b** Basal facilitation (P_2/P_1) ratio between +10 and +30 mV for $Ca_V2.2/\alpha_2\delta$-2 coexpressed with β2a-ΔvHOOK (n=9, *black bars*), β2a-ΔvHOOK+transducin-α (n=7, *gray bars*), C3,4S-β2a-ΔvHOOK (n=11, *hatched bars*), β2a-SH3 (1–135)+GK (226–442) (n=9, *horizontal striped bars*), and β2a-GK (226–442) (n=10, *dashed bars*, repeated from Fig. 3d for comparison). The *dotted line* indicates a P_2/P_1 ratio of 1, i.e., no basal facilitation. **c** Time course of facilitation with increasing prepulse duration Δt for β2a (*open squares*, n=10), β2a-ΔvHOOK (*filled squares*, n=10), and β2a-SH3 (1–135) and GK (226–442) (*open stars*, n=9). The *lines* are single exponential fits to the mean data. **d** Time course of reinhibition with increasing duration Δt between prepulse and P_2 test pulse for β2a (*open squares*, n=6) and β2a-ΔvHOOK (*filled squares*, n=6). The *lines* are single exponential fits to the mean data. The *inset* shows an example set of traces showing the increasing inhibition of the P_2 traces with increased Δt

much higher at +120 mV than that estimated at −100 mV, nevertheless, the off-rate of Gβγ from the β2a-ΔvHOOK construct was slower than that for the wild-type β2a at both potentials.

To examine further whether the presence of the SH3 domain played any role in G protein modulation, we finally examined the effect of including the SH3 and GK domains as two separate constructs. We found previously that the β2a-SH3 (1–135) domain, which retains a palmitoylation site and can, therefore, accumulate in the vicinity of the plasma membrane, is able to interact with β2a-GK (136–442) and mimic full-length β2a in its biophysical properties [32]. In contrast, β2a-SH3 (1–135) did not show any interaction with the isolated β2a-GK (226–442), which is lacking the final (ε) β-strand of the SH3 domain that is required for the interaction, but did show interaction with β2a-GK (214–442), which is lacking only the variable HOOK region, but retains the ε-strand that completes the SH3 domain [32]. These results are confirmed by our yeast two-hybrid results (see Fig. 6).

The effect of including together the noninteracting pair of constructs β2a-SH3 (1–135) and β2a-GK (226–442) on the basic current properties was a reduction, rather than an increase, in the peak I_{Ba} compared to β2a-GK (226–442) alone (Table 1) and a depolarization of the voltage for 50% activation of the current to +24.9±2.4 mV (n=5) compared to +32.0±3.6 (n=7) for β2a-GK (226–442) alone. As a possible explanation and in confirmation of the results described above for β2a-ΔvHOOK, we found that the inclusion of β2a-SH3 (1–135) with β2a-GK (226–442) resulted in calcium channel currents that showed strong tonic modulation. In the absence of agonist, the peak I_{Ba} showed a basal facilitation of 2.61±0. 72 at +10 mV (Fig. 5a,b), which is likely to be the reason that the peak I_{Ba} was smaller in the presence than in the absence of β2a-SH3 (1–135) (Table 1). This is in strong contrast to the lack of facilitation shown by either of the β2a-GK domains alone (Figs. 3d and 5b). For this pair of constructs, the time constant for the removal of tonic inhibition (τ_{dissoc}) at +120 mV was 29.1±5.4 ms (Fig. 5c, P>0.05 compared to β2a).

Yeast two-hybrid assays were performed in order to probe any direct interactions between the $Ca_V\beta$ subunit constructs and the I–II linker of $Ca_V2.2$ and whether the interactions between specific β subunit domains matched the results obtained in our electrophysiological experiments (Fig. 6). Our positive control was the interaction between the $Ca_V2.2$ I–II linker and full-length β1b (Fig. 6a, column 1), which has been demonstrated by many different techniques, including surface plasmon resonance [9, 11, 21]. We found a β2a construct missing the extreme N terminus and the C terminus (5–442) also interacted with the I–II linker (Fig. 6a, column 2). The truncated constructs β2a-εSH3+GK (214–442) and β2a-HOOK+εSH3+GK (135–442) also interacted with the I–II linker (Fig. 6a, columns 3 and 4), whereas the shortest construct β2a-GK domain (225–442) did not interact with the I–II linker in this assay (Fig. 6a, column 5). Furthermore, none of the β2a-SH3 domains tested interacted with the I–II of $Ca_V2.2$ (Fig. 6b). These included

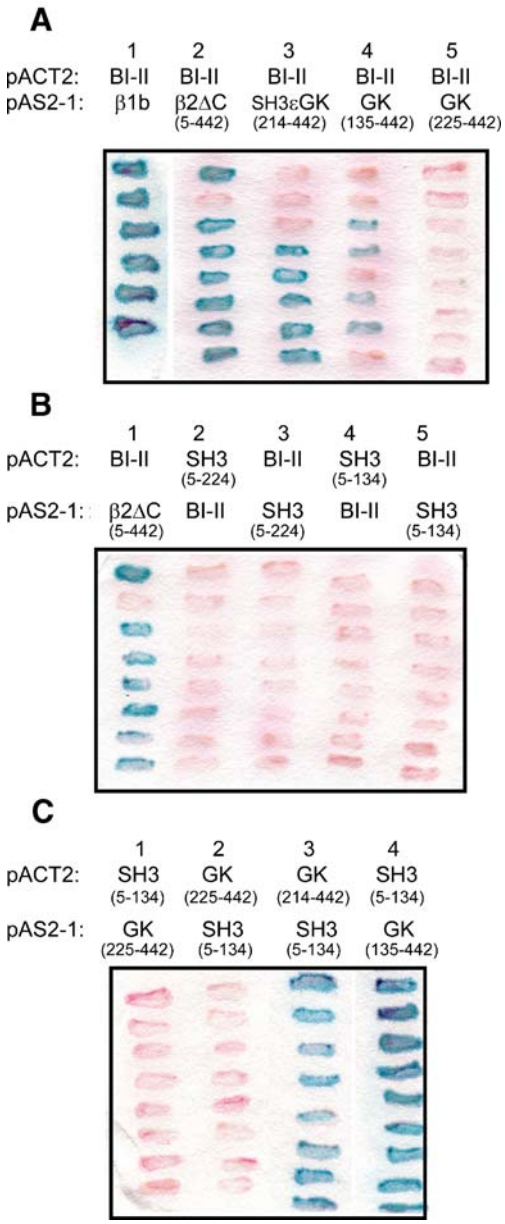

Fig. 6 Protein interactions involving β2a domains. Protein interactions were demonstrated using β-galactosidase assays after cotransformation of plasmids into yeast. *Blue* colonies indicate a positive interaction. **a** Positive control showing $Ca_V2.2$ I–II loop (BI–II) pACT2 and β1b pAS2-1 (*lane 1*). β2a minus the C terminus (5–442), β2a-εSH3+GK (214–442), and β2a-HOOK+εSH3+GK (135–442) interacted with the I–II linker (*lanes 2–4*) whereas β2a-GK (225–442) showed no interaction (*lane 5*). **b** Positive control showing β2a minus the C terminus (5–442) interacting with BI–II (*lane 1*). No interactions were demonstrated between SH3 domains and BI–II (*lanes 2–5*). **c** The shortest GK construct (225–442) was not found to interact with β2a-SH3 (5–134) (*lanes 1* and *2*) whereas the longer GK constructs, β2a-εSH3+GK (214–442) and β2a-HOOK+εSH3+GK (135–442), showed a positive interaction (*lanes 3* and *4*)

β2a-SH3+HOOK (5–224) (Fig. 6b, columns 2 and 3) and β2a-SH3 (5–134) (Fig. 6b, columns 4 and 5).

We also found that β2a-SH3 (5–134) did not interact with β2a-GK (225–442) (Fig. 6c, columns 1 and 2 with the constructs in either vector), whereas it showed strong interactions with β2a-εSH3+GK (214–442) (Fig. 6c, column 3) and with β2a-HOOK+εSH3+GK (135–442) (Fig. 6c, column 4).

Discussion

The core structure of all $Ca_V\beta$ subunits is characterized by a GK and an SH3 domain [13, 18, 27, 39]. The 18-amino-acid AID motif in the I–II loop of HVA Ca_V α_1 subunits is crucial for binding to $Ca_V\beta$ subunits [4, 21, 29]. Recent structural data from three groups have provided detailed information about $Ca_V\beta$ subunits and their interaction with the AID sequence [13, 27, 39]. However, the structural studies provided no insight into the role of the disordered HOOK domain, which intervenes in the split SH3 domain, before its fifth β-strand. In this study, we have examined which $Ca_V\beta$ subunit domains are involved in promoting the voltage dependence of G protein modulation, which is lost in the absence of any $Ca_V\beta$ subunit [9, 21].

Requirement of $Ca_V\beta$ GK domains for plasma membrane expression of HVA calcium channels

One of the main effects of $Ca_V\beta$ subunits on HVA calcium channels is to increase current density. However, the mechanism for this increase remains controversial, either being attributed to increased trafficking [6], increased maximum open probability [26], or both. In agreement with the first hypothesis, we and others have shown biochemically that the proportion of HVA $Ca_V\alpha1$ subunits in the plasma membrane is increased by $Ca_V\beta$ subunit coexpression [1, 9, 14, 21]. This finding was reinforced by the fact that fewer channels were present at the surface when the mutated $Ca_V2.2$W391A channels that did not interact with β subunits were cotransfected with a $Ca_V\beta$ [21]. However, it is clear that $Ca_V\beta$ subunits also increase the open probability for $Ca_V2.2$ as well as other HVA channels [20, 25].

The $Ca_V\beta$ GK domain is sufficient to restore voltage-dependent G protein modulation of $Ca_V2.2$ channels

We showed previously that abrogation of the interaction of $Ca_V2.2$ with a $Ca_V\beta$ subunit, by introduction of the W391A mutation in the AID motif, did not affect the ability of $G\beta\gamma$ to inhibit $Ca_V2.2$, but did prevent the removal of $G\beta\gamma$ by a depolarizing prepulse [21]. In this study, we have obtained similar results when $Ca_V2.2$ was expressed without any $Ca_V\beta$ subunit and we found that coexpression of GK domain constructs from either β2a or β1b is sufficient to restore the voltage dependence of G protein modulation to wild-type $Ca_V2.2$ channels.

It has been found in several studies [3, 15] that the $G\beta\gamma$ subunits, which are responsible for direct G protein modulation of calcium channels, bind to the AID region of the I–II linker, and it has been proposed that they may compete with $Ca_V\beta$ subunits [33]. In contrast, fluorescence resonance energy transfer (FRET) studies have shown that $Ca_V\beta$ and $G\beta\gamma$ are able to bind to calcium channels at the same time [19]. Also in disagreement with a simple competition between $G\beta\gamma$ and $Ca_V\beta$ subunits is the finding that the presence of $Ca_V\beta$ subunits does not reduce the amount of G protein modulation [10, 24]. However, we did find that the presence of $Ca_V\beta$ subunits promoted the voltage-dependent removal of $G\beta\gamma$ by depolarizing prepulses [10, 24]. A similar conclusion was reached by using $Ca_V2.2$ containing the W391A mutation in the I–II linker, such that it did not interact with $Ca_V\beta$ subunits [21].

Involvement of other β subunit domains in G protein modulation of calcium channels

The results described in this study and our previous study [32] indicate that the reduced G protein modulation of the palmitoylatable compared to the nonpalmitoylatable β2 constructs is likely to be related to the fact that palmitoylation maintains an elevated concentration of this $Ca_V\beta$ subunit associated with the inner leaflet of the plasma membrane and, therefore, in the vicinity of the channel. However, as we and others have discussed previously [24], there is unlikely to be a simple competition between $G\beta\gamma$ and $Ca_V\beta$ subunits for binding to the I–II linker. Furthermore, in this study, we found that there is little difference in the amount of G protein modulation in the absence compared to the presence of any of the free GK domains. These findings indicate that the reduced G protein modulation seen with β2a is unlikely to be due solely to the fact that its palmitoylation results in an increased occupancy by its GK domain of the I–II linker. Moreover, the results with the HOOK deletion constructs indicate that the proposed interaction of the HOOK domain of β2a with the channel is also not responsible alone for the reduced modulation observed with palmitoylated β2a. This suggests that the increased interaction of both the SH3 domain and the HOOK domain with the channel, resulting from palmitoylation of β2a, is responsible for this difference in extent of G protein modulation.

Determinants of tonic modulation of $Ca_V2.2$

The strong basal facilitation of the β2a construct lacking the variable part of the HOOK domain requires its palmitoylation

and indicates that the absence of this HOOK domain of β2a promotes, either directly or indirectly, tonic Gβγ binding. It is also possible that removal of the variable HOOK domain constrains the structure of the Ca_Vβ subunit and that other parts of the calcium channel are also involved in Gβγ binding. In support of this, we noted a trend to increased basal facilitation for GK (136–442), containing the HOOK domain, compared to GK (226–442) in which the HOOK domain was absent, although this did not reach statistical significance.

The results from the experiment utilizing the combination of β2a-SH3 (1–135) and β2-GK (226–442) lacking the HOOK domain confirm the results obtained with β2a-ΔvHOOK, since both show strong tonic modulation. We interpret these results as indicating that the presence of the palmitoylatable free SH3 domain, in the absence of the variable part of the HOOK domain of β2a, promotes basal facilitation of $Ca_V2.2$ channels and results in increased tonic Gβγ binding. Our previous evidence [32] indicates that β2a-SH3 (1–135) is palmitoylated, since when it is coexpressed with GK(136–442), it reconstitutes the properties of palmitoylated β2a, in terms of slow inactivation, an effect which has been attributed to palmitoylation [21, 30]. Our present results further indicate that an interaction between the GK domain and the SH3 domain of β2a is not necessary for the demonstration of tonic G protein modulation, since β2a-SH3 (1–135)+β2-GK (226–442) and β2a-ΔvHOOK showed quite similar properties with respect to expression of tonic facilitation.

Although our electrophysiological data indicate that additional interactions are likely to occur between the $Ca_V2.2$ channels and both the SH3 and the HOOK domains of Ca_Vβ subunits, nevertheless, our yeast two-hybrid data do not indicate that there is an interaction between the β2a-SH3 domain and the I–II linker of $Ca_V2.2$, in contrast to a previous study using $Ca_V2.1$ [23]. This was also suggested previously from our binding results for β1b, since it showed the same binding affinity for the full-length $Ca_V2.2$ I–II linker as the I–II linker truncated just after the AID region, indicating that there are no additional binding sites for the β subunit distal to the AID motif [9, 21].

In the present study, we have not addressed the other regions of the $Ca_V2.2$ subunit involved in this interaction, but other studies have shown that Gβγ binds to the C terminus of $Ca_V2.2$ [22] and that the I–II linker itself interacts with other regions of Ca_V2 channels [36]. These results, among others, indicate that Gβγ is likely to bind to a complex state-dependent binding pocket, also including the N terminus of the channel [28].

Our electrophysiological data suggest that the presence of the HOOK domain is important for the voltage-dependent removal of Gβγ. In particular, we calculate that in the absence of the HOOK domain, the affinity of the $Ca_V2.2/Ca_V$β2 complex for Gβγ is increased about threefold. The effect of this change in affinity is manifested particularly at resting Gβγ levels, previously estimated to be about 50 nM [34], when it leads to substantial tonic Gβγ modulation. It is also evidenced by a more rapid rate of rebinding of Gβγ, following its removal by a depolarizing prepulse. These findings are in agreement with the hypothesis proposed previously [9, 11, 21] that the greater the occupancy of the binding site for Ca_Vβ subunit on the channel, the greater is the voltage dependence of G protein inhibition.

In our previous study [32], we provided evidence that the HOOK domain of β2a is involved in modulating voltage-dependent inactivation, since removal of the HOOK domain shifted the steady-state inactivation to more negative potentials and also increased the inactivation kinetics. The contribution of inactivation imposed by different Ca_Vβ subunits on G protein regulation has been investigated previously [25, 41]. We found that the inactivation properties of expressed $Ca_V2.2$ channels depended on the Ca_Vβ subunit species, but only to a minor extent on the presence or absence of Gβγ. Furthermore, the closed times and latency to first opening of the $Ca_V2.2$ channels were increased by Gβγ, but this effect was similar for both β1b and β2a subunits [25]. More recently, the effect of the inactivation on G protein modulation was studied, and an effect was observed on voltage-dependent recovery from G protein modulation because of the opposing effects of different Ca_Vβ subunits on inactivation [41]. In this study, we show directly that removal of the β2a HOOK domain enhances Gβγ binding affinity. In the future, this may help to identify how Gβγ dimers modulate the Ca_V2 channels.

Conclusions

The present results indicate that the interaction of a Ca_Vβ subunit GK domain alone with the $Ca_V2.2$ channel is sufficient to restore voltage dependence to the G protein modulation process. However, these results also suggest that the SH3 and HOOK domains of Ca_Vβ subunits are likely to have a role in preventing tonic binding of Gβγ to the calcium channels.

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Conflict of interests None.

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