Research Paper

The HOOK-Domain Between the SH3 and the GK Domains of Ca\textsubscript{v}\textbeta Subunits Contains Key Determinants Controlling Calcium Channel Inactivation

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ABSTRACT

Ca\textbeta subunits of voltage-gated calcium channels contain two conserved domains, a src-homology-3 (SH3)-domain and a guanylate kinase-like (GK)-domain. 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\textbeta subunits. Here we have been guided by the recent structural studies of Ca\textbeta subunits in the design of specific truncated constructs, with the goal of investigating the role of the HOOK-domain of Ca\textbeta subunits in the modulation of inactivation of N-type calcium channels. We have coexpressed the β subunit constructs with Ca\textsubscript{v}2.2 and α\textgamma2.2, using the N-terminally palmitoylated β\textsubscript{2a} subunit, because it supports very little voltage-dependent inactivation, and made comparisons with β\textsubscript{1b} domains. Deletion of the variable region of the β\textsubscript{2a} HOOK-domain resulted in currents with a rapidly inactivating component, and additional mutation of the β\textsubscript{2a} palmitoylation motif further enhanced inactivation. The isolated GK-domain of β\textsubscript{2a} alone enhanced current amplitude, but the currents were rapidly and completely inactivating. When the β\textsubscript{2a} GK-domain construct was extended proximally, by including the HOOK-domain and the s-strand of the SH3-domain, inactivation was about four-fold slower than in the absence of the HOOK domain. When the SH3-domain of β\textsubscript{2a} truncated prior to the HOOK-domain was coexpressed with (HOOK+eSH3+GK) domain of β\textsubscript{2a}, all the properties of β\textsubscript{2a} were restored, in terms of loss of inactivation. Furthermore, removal of the HOOK sequence from the (HOOK+eSH3+GK)β\textsubscript{2a} construct increased inactivation. Together, these results provide evidence that the HOOK domain is an important determinant of inactivation.

INTRODUCTION

Voltage-gated calcium (Ca\textsubscript{v}) channels play a major role in the physiology of all excitable cells. Three families have been identified, Ca\textsubscript{v}1-3 (reviewed in ref. 1). The high-voltage-activated (HVA) Ca\textsubscript{v}1 and 2 classes are heteromultimers composed of the pore-forming α\textsubscript{1} subunit, associated with auxiliary Ca\textbeta and α\textgammaβ subunits (reviewed in ref. 2). Four Ca\textsubscript{v}1β subunit genes have been cloned, and these subunits are important for HVA channel function (reviewed in ref. 3), since they promote expression of functional channels at the plasma membrane and modulate their biophysical properties.\textsuperscript{4,5} Ca\textbeta subunits bind with high affinity to the α-interaction domain (AID) on the I-II loop of Ca\textsubscript{v}1 and 2 channels,\textsuperscript{6,7} although other α\textsubscript{1} subunit interaction sites are also likely to be important in mediating the actions of Ca\textbeta subunits.\textsuperscript{8,9}

In a previous study we investigated the role of Ca\textbeta subunits in the plasma membrane expression and G-protein modulation of Ca\textsubscript{v}2.2 calcium channels, by mutating the AID tryptophan (W391) in the I-II loop of Ca\textsubscript{v}2.2, and thus completely disrupting the high affinity interaction with Ca\textbeta subunits.\textsuperscript{10} Our main conclusion was that, whereas the Ca\textsubscript{v}2.2W391A mutant channels lost all modulation by Ca\textbeta subunits,\textsuperscript{10} the Ca\textsubscript{v}2.2β\textsubscript{1b} was not true for palmitoylated Ca\textbeta2a. Only expression at the plasma membrane was affected when Ca\textbeta2a was coexpressed with the mutant Ca\textsubscript{v}2.2W391A channel, while all the biophysical properties of the expressed Ca\textsubscript{v}2.2W391A channels remained normally modulated by Ca\textbeta2a, including the marked depolarization of steady-state inactivation, which is typical of palmitoylated Ca\textbeta2a. We concluded that the continuing influence of β\textsubscript{2a} was dependent on its palmitoylation, which increased the local concentration near the plasma membrane sufficiently to allow lower affinity interactions to occur between Ca\textbeta and channel α\textsubscript{1} subunit, which were effective in modulating the channel properties.\textsuperscript{10}

Ca\textbeta subunits were predicted by structural modelling to contain an SH3-domain followed by a guanylate kinase (GK) domain.\textsuperscript{11} X-ray crystallographic studies have
produced detailed information on the domain structure.\textsuperscript{12-14} The N-terminal SH3-domain is divided, with its final (5\textsuperscript{th}) β-strand, providing the interaction with the GK-domain, being situated after the variable HOOK region, whose structure was not determined (reviewed in ref. 15). We have been guided by the structure in our choice of truncations and deletions in the present study (Fig. 1A and B). In the case of the GK domains, we have used the excision 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 (reviewed in ref. 16). 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, according to their ability to mimic the functions of intact Ca\textsubscript{2+}β subunits, which might have indicated varying stabilities in different cell types.\textsuperscript{17,18} We have now dissected the role of the variable HOOK-domain, between the conserved SH3 and GK domains, and our results indicate that this region contains key determinants of both closed and open state calcium channel inactivation. Furthermore, it is likely that the extent of interaction of the channel with this HOOK domain is determined by the palmitoylation state of the SH3-domain.

**MATERIALS AND METHODS**

**Materials.** The cDNAs used in this study were Ca\textsubscript{2,2} (D14157), Ca\textsubscript{β\textsubscript{1b}} (X61394), Ca\textsubscript{β\textsubscript{2a}} (M88751) and α\textsubscript{δ}-2.19 When used, the green fluorescent protein (GFP-mut3b, U73901) was used to identify transfected cells. All cDNAs were subcloned into pMT\textsubscript{2}.

**Construction of truncated β subunit domains.** All constructs were made by standard molecular biological techniques and their sequences verified by sequencing both strands. The constructs used (with their amino acid residues) were β\textsubscript{2a-core} (1–442), β\textsubscript{2a-D-vo-HOOK} (Δ169–213), β\textsubscript{2a} truncated SH3 (1–135), β\textsubscript{2a-(SH3+HOOK+eSH3)} (1–225), β\textsubscript{2a-GK} (226–442), β\textsubscript{2a-(HOOK+eSH3+GK)} (136–442), β\textsubscript{2a-(ΔHOOK+eSH3+GK)} (214–442), β\textsubscript{1b-truncated-SH3} (82–178), β\textsubscript{1b-GK} (230–426), β\textsubscript{1b-(HOOK+eSH3+GK)} (179–426) and β\textsubscript{1b-(ΔHOOK+eSH3+GK)} (217–426).

Two electrode voltage-clamp. Xenopus oocytes were prepared, injected and utilized for two electrode voltage clamp electrophysiology as previously described.\textsuperscript{20} Briefly, plasmid cDNAs for the different VDCC subunits Ca\textsubscript{2,2}, α\textsubscript{δ}-2 and β subunit were mixed in weight ratios of 2:1:2 at 1 μg/μl.\textsuperscript{21} When separate GK and SH3-domains were used, these cDNAs were mixed in a ratio of 2:1. The final DNA mix was diluted 3-fold and 9 nl injected intranuclearly into stage V or VI oocytes. Injected oocytes were incubated at 18°C for 3–4 days in ND96 saline plus 1.8 mM CaCl\textsubscript{2} supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Paisley, UK). Recordings from oocytes were made in the two-electrode voltage clamp configuration, using a Geneclamp 500B and Digidata 1200 (Molecular Devices, Foster City, CA), with an extracellular chloride-free solution containing (in mM): Ba(OH)\textsubscript{2} 10; NaOH 70; CaO\textsubscript{2} 2; Hepes 5 (pH 7.4 with methanesulfonic acid). Oocytes were injected with 30–40 nl of a 100 mM solution of K\textsubscript{1},1,2,3-bis (amino-phenoxo) ethane-N,N,N’,N’-tetra-acetic acid (BAPTA) in order to suppress endogenous Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents, three hours prior to recording. Electrodes contained 3M KCl, and had resistances of 0.3–1 MΩ. The holding potential (V\textsubscript{H}) was -100 mV.

**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\textsubscript{α\textsubscript{1}}, Ca\textsubscript{β\textsubscript{1b}} subunits, Ca\textsubscript{β\textsubscript{2a}}, α\textsubscript{δ}-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

![Figure 1](https://example.com/figure1.png)
Role of the HOOK-Domain of β Subunits in Ca Channel Inactivation

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 10–40 pF. Patch pipettes were filled with a solution containing (in mM) 140 Cs-aspartate, 5 EGTA, 2 MgCl₂, 0.1 CaCl₂, 2 K₂ATP, 10 HEPES, titrated to pH 7.2 with CsOH (310 mM), with a resistance of 2–3 MΩ. The external solution contained (in mM) 150 tetraethylammonium (TEA) bromide, 3 KCl, 1.0 NaHCO₃, 1.0 MgCl₂, 10 HEPES, 4 glucose, 10 BaCl₂, pH adjusted to 7.4 with Tris-Base (320 mM). 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. Data were filtered at 2 kHz and digitized at 5–10 kHz. The holding potential was -100 mV, and pulses were delivered every 10 seconds.

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:

\[ I = \frac{G_{max} * (V-V_{rev})}{(1+exp(-(V-V_{50,act})/k))} \]

where \( I \) is the current density (in pA/pF), \( G_{max} \) is the maximum conductance (in nS/pF), \( V_{rev} \) is the reversal potential, \( V_{50,act} \) is the midpoint voltage for current activation, and \( k \) is the slope factor. Steady-state inactivation properties were measured by applying a 5–20 second pulse (depending on 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. Steady-state inactivation and activation data were fitted with a single Boltzmann equation of the form:

\[ I/I_{max} = \frac{1}{1+exp((V-V_{50,inact})/k))} + A2 \]

where \( I_{max} \) is the maximal current, \( V_{50,inact} \) is the half-maximal voltage for current inactivation. For the steady-state inactivation, \( A1 \) and \( A2 \) represent the proportion of inactivating and non inactivating current, respectively. Inactivation kinetics of the currents were estimated by fitting the decaying part of the current traces with the equation:

\[ I(t) = C + A1*exp(-(t-t_0)/\tau_{inact}) \]

where \( t_0 \) is zero time, \( C \) the fraction of noninactivating current, \( A \) the relative amplitude of the exponential, and \( \tau_{inact} \) its time constant. Analysis was performed using Pclamp6 or 7 and Origin 7.

Data are expressed as mean ± s.e.m. of the number of replicates: \( n \). Error bars indicate standard errors of multiple determinations. Statistical significance was analyzed using Student’s paired or unpaired t-test, or by ANOVA, with Tukey’s post hoc test, if multiple comparisons were made.

RESULTS

The relative roles of N-terminal palmitoylation and the variable HOOK-domain in the retardation of inactivation by Caβ2a. Caβ subunits are members of the membrane-associated guanylate-kinase-like protein family (MAGUKs).11,21 The SH3-domain in Caβ subunits is split, in that the variable loop or HOOK region (so-named in other MAGUK proteins) is intercalated before the final (5th or 6th) β-strand of the SH3-domain (Fig. 1A).12-14 In this study we wished to examine the role of this variable HOOK-domain of both β2a and β1b (Fig. 1B and C). We took advantage of the fact that rat β2a is N-terminally palmitoylated, endowing it with the ability almost completely to remove inactivation. We were then able to examine the relative importance of mutation of the N-terminal palmitoylation motif and deletion of the HOOK region (Fig. 2). The effects of the various Caβ subunit mutations were examined on Caβ2.2/αδ-2 currents. As expected, mutation of the dicysteine palmitoylation motif to serine (C3,4S-β2a) markedly increased the inactivation rate compared to wild-type β2a (Fig. 2A and B). It also hyperpolarized the midpoint of the steady-state inactivation (V50,inact) compared to wild-type β2a by 42.6 mV, from -7.9 ± 1.4 mV (n = 17) to -50.5 ± 0.6 mV (n = 18, Fig. 2C). Deletion of the variable part of the HOOK region of β2a (between AIDID and FFKK, called β2a Δ-vHOOK, Fig. 1C) resulted in a different behavior, inducing two current components, about 50% being rapidly inactivating, with the remaining current being completely noninactivating (Fig. 2A and B).

For this reason, only a partial steady-state inactivation was obtained for this construct, even when using a 20-second conditioning step, with a V50,inact of -47.5 ± 1.5 mV (n = 22, Fig. 2C). We then combined the two mutations in the C3,4S-β2a Δ-vHOOK construct, and observed a rapidly and completely inactivating current (Fig. 2A and B). This current exhibited a strongly hyperpolarized steady-state inactivation, with a V50,inact of -62.4 ± 0.6 mV (n = 13, Fig. 2C).

The GK-domain constructs of β2a reconstitute β-subunit mediated trafficking behavior in Xenopus oocytes, both with and without the associated HOOK domain. In order to examine further the role of the HOOK domain, we made two pairs of Caβ2a-GK-domain constructs by cutting the β2a subunit at two different points, either before or after the HOOK plus the εSH3 domain (Fig. 1A–C). We took advantage of the fact that rat β2a oocytes, there is an additional effect of the truncated SH3-domain protein, when fifth domain or BID, which was originally described as the shortest motif to serine (C3,4S-β2a) and deleted the HOOK region (Fig. 2). The effects of the combined the two mutations in the C3,4S-β2a Δ-vHOOK construct, and observed a rapidly and completely inactivating current (Fig. 2A and B). This current exhibited a strongly hyperpolarized steady-state inactivation, with a V50,inact of -62.4 ± 0.6 mV (n = 13, Fig. 2C).

Both the GK-domains contain the β-binding pocket, which binds the AID region (Fig. 1B),12,13 and would therefore be expected to traffic the channels. However, the minimal GK-domain did not contain the entire region formerly designated as the β-interaction domain or BID, which was originally described as the shortest region able to enhance calcium currents.22 It is now clear from the Caβ subunit structure that whilst this region does not actually bind the AID sequence,12-14 it must be important for the structural integrity of the GK-domain. The variable C-terminal region after the conserved GK-domain was removed from both β2a-GK constructs, while retaining 10–20 amino acids after the end of the minimal GK constructs used for structural determinations, taking the GK sequence up to the exon boundary.

In Xenopus oocytes it was found previously that the isolated GK-domain of Caβ3 commencing after the final SH3 β-strand is able to reconstitute trafficking,14 whereas this was reported not to be the case in mammalian cells.18 Maltez et al17 found that although protein formed from a long β2a-GK-domain, containing the SH3 fifth β-strand, enhanced Caβ2.1 currents in Xenopus oocytes, there was an additional effect of the truncated SH3-domain protein, when co-injected with a sub-maximal amount of the GK-domain protein.

Our initial results showed that in Xenopus oocytes both the β2a-GK-domain and β2a-(HOOK+εSH3+GK)-domain were able to fulfill the role of Caα1, trafficking to a similar extent, promoting the formation of functional channels. The peak current size was increased 8–10-fold over the currents formed by the Caα2.2/αδ-2 combination.
in the absence of expressed Caβ subunits. The current enhancement was similar to that observed with full-length β2a (Fig. 3A).

In contrast, and as previously indicated in mammalian cells, we found that in tsA201 cells, both the β2a-GK-domain and the β2a-(HOOK+eSH3+GK)-domain were much less effective than full-length β2a in enhancing current amplitude (Table 1), indicating that mammalian cells may have more stringent endoplasmic reticulum (ER) retention signals for the Ca2.2/α1 subunit, or that the isolated GK-domain proteins are less stable in tsA201 cells than in oocytes. For this reason most of the subsequent studies described in this paper were performed in Xenopus oocytes, although comparisons were made with tsA201 cells in several instances, and these are stated in the text.

Distinctive inactivation properties of the β2a GK domain when associated with the HOOK domain. In Xenopus oocytes, both the β2a-GK-domain constructs caused a hyperpolarizing shift in the voltage-dependence of activation compared to the Ca2.2/α2β-2 currents in the absence of Caβ subunits (Fig. 3B). In contrast to Caβ β2a, which depolarized the steady-state inactivation compared to the absence of coexpressed Caβ subunits, both the free GK-domains of β2a hyperpolarized the steady-state inactivation. This hyperpolarization was significantly greater for the β2a-GK-domain (V0.5 inact of -55.0 mV) than for the β2a-(HOOK+eSH3+GK)-domain (V0.5 inact of -45.6 mV, p < 0.001; Fig. 3C and D), and was similar to the effect of the nonpalmitoylated β subunits, such as β1b (Fig. 3C and D). As a control that the lack of a C-terminus was not responsible for any differences between the isolated GK-domains and full length β2a, we expressed the β2a core domain, which spans both these two domains, but is lacking the C-terminus (Fig. 1A). We found that this construct behaves identically to β2a with regard to the parameters measured here (Fig. 3A and B, and data not shown).

In addition to its steady-state inactivation being more depolarized, the kinetics of inactivation of the β2a-(HOOK+eSH3+GK)-domain were 4.3-fold slower than those of the β2a-GK-domain at 0 mV (Fig. 4A and B), and similar to those of C3,4S-β2a (Fig. 2A and B), again implicating the HOOK region in retarding the process of inactivation. The effect on inactivation kinetics was much more marked than the effect on closed-state inactivation. We therefore removed the HOOK-sequence to form a β2a-(ΔHOOK+eSH3+GK)-domain construct (Fig. 1A). The removal of the HOOK-domain resulted in a β2a-GK construct with properties that were identical to the β2a-GK-domain. The t 0.5 inact for this construct was significantly smaller than that of the β2a-(HOOK+eSH3+GK)-domain at all potentials examined (Fig. 4A and B; p < 0.01). Furthermore, the steady-state inactivation was also significantly hyperpolarized compared to the β2a-(HOOK+eSH3+GK)-domain (Fig. 3D, p < 0.001), being similar to the β2a-GK-domain. This confirms that it is the HOOK sequence itself that is responsible for retarding inactivation.

Very similar results were obtained in tsA201 cells, where, despite exerting a relatively small effect on trafficking, the β2a-GK-domain strongly hyperpolarized the steady-state inactivation, whereas the β2a-(HOOK+eSH3+GK)-domain produced less hyperpolarization (Table 1). Similarly, in tsA201 cells the kinetics of inactivation

Figure 2. Relative roles of palmitoylation and the variable part of the HOOK (vHOOK) region in the retardation of inactivation mediated by β2a. (A) Overlaid current traces during 2 second step depolarizations from -100 mV to -20, -10, 0 and +10 mV for β2a (top), C3,4S-β2a (upper middle), β2a ΔvHOOK (lower middle) and C3,4S-β2a ΔvHOOK (bottom). (B) Bar chart of t 0.5 values (upper panel) and % of inactivating current (lower panel), for the four conditions shown in (A): β2a (black bars, n = 17), C3,4S-β2a (white bars, n = 13), β2a ΔvHOOK (gray bars, n = 17) and C3,4S-β2a ΔvHOOK (hatched bars, n = 16). Statistical significance of the differences between the t 0.5 for C3,4S-β2a and C3,4S-β2a ΔvHOOK are given *** p < 0.001. (C) Normalized steady-state inactivation for Ca2.2/α2β-2 with β2a (open circles, n = 17), C3,4S-β2a (open triangles, n = 18), β2a ΔvHOOK (closed diamonds, n = 22) and C3,4S-β2a ΔvHOOK (closed squares, n = 13). Data were fit by a Boltzmann function, and the mean parameters for the fits are given in the text and Figure 3C.
Figure 3. Current amplitude, normalized current-voltage relationships and steady-state inactivation for β2a-GK-domains, compared to full length β2a. (A) Current voltage-relationships were obtained for CaV2.2/α2δ-2, without β, or together with the truncated constructs shown, and the peak current at +5 mV was normalized compared to the value for β2a in each experiment. Data are without β (vertical stripes), + β2a (SH3+HOOK+εSH3) (hatched), + β2a (black), + β2a core (white), + β2a (HOOK+εSH3+GK) (cross-hatched) or + β2a (grey). The numbers of determinations are shown in parentheses. (B) Current voltage-relationships were obtained for CaV2.2/α2δ-2, without β (open stars, n = 17; V50,act = +10.49 ± 0.96 mV), or together with the various β subunit constructs shown in the legend, and the peak current was normalized to one. β2a (open circles, n = 16; V50,act = +0.41 ± 0.62 mV), β2a core (open diamonds, n = 7; V50,act = -0.41 ± 0.97 mV), β2a-GK (open triangles, n = 5; V50,act = +1.60 ± 0.93 mV), β2a (HOOK+εSH3+GK) (closed triangles, n = 21; V50,act = -2.41 ± 0.59 mV) and β1b (closed squares, n = 13; V50,act = -2.65 ± 1.09 mV). Data were fit by a combined Boltzmann and straight line function. (C) Normalized steady-state inactivation for CaV2.2/α2δ-2, without β (open stars, n = 12), or together with the various constructs shown: β2a (dashed line, repeated from Fig. 2C), β2a-GK-domain (closed triangles, n = 25), β2a (HOOK+εSH3+GK)-domain (open triangles, n = 14), and β1b (closed squares, n = 18). Data were fit by a Boltzmann function, and the mean parameters for the fits are given in Figure 3D. (D) Bar chart of mean ± s.e.m. values for V50,inact for CaV2.2/α2δ-2 expressed together with the β subunit constructs whose steady state inactivation is shown in Figures 2C and 3B, and also for the β2a-(HOOK+εSH3+GK)-domain. The number of determinations is shown on the bars. The statistical significance of the difference between the β2a-(HOOK+εSH3+GK)-domain and either the β2a-GK-domain alone or the β2a-(HOOK+εSH3+GK)-domain is indicated by *** p < 0.0001.
The effect of various Ca\(^{2+}\) subunit constructs on biophysical parameters of Ca\(_{2.2}/\alpha_\delta\)-2 calcium channel currents in tsA 201 cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Peak amplitude (pA/pF/10 ms)</th>
<th>$I_{\text{inact}}(\text{peak})/I_{\text{ba}}$ (600 ms)</th>
<th>$V_{50,\text{inh}}$ (mV)</th>
<th>$V_{50,\text{out}}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (\beta)</td>
<td>-10.3 ± 2.1 (n=7)</td>
<td>0.24 ± 0.07 (n=10)</td>
<td>15.5 ± 0.1 (n=7)</td>
<td>-26.9 ± 0.4 (n=3)</td>
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<tr>
<td>(\beta_{2a})</td>
<td>-227.6 ± 69.0 (n=8)†</td>
<td>0.71 ± 0.09 (n=18)†</td>
<td>8.5 ± 0.5 (n=8)†</td>
<td>-5.4 ± 1.9 (n=8)†</td>
</tr>
<tr>
<td>(\beta_{2a}^{\text{GK}})</td>
<td>-46.4 ± 21.3 (n=7)*</td>
<td>0.17 ± 0.02 (n=15)*</td>
<td>11.1 ± 0.4 (n=7)*</td>
<td>-46.9 ± 0.6 (n=8)*</td>
</tr>
<tr>
<td>(\beta_{2a}(\text{HOOK+SH3+GK}))</td>
<td>-47.2 ± 12.9 (n=7)**</td>
<td>0.30 ± 0.04 (n=14)**</td>
<td>9.9 ± 0.3 (n=7)**</td>
<td>-37.5 ± 0.4 (n=10)**</td>
</tr>
<tr>
<td>(\beta_{1b})</td>
<td>-240.8 ± 50.8 (n=15)††</td>
<td>0.27 ± 0.06 (n=15)††</td>
<td>4.8 ± 0.4 (n=15)††</td>
<td>-41.9 ± 1.2 (n=9)††</td>
</tr>
<tr>
<td>(\beta_{1b}^{\text{SH3+HOOK+SH3}})</td>
<td>-8.7 ± 3.5 (n=8)*</td>
<td>0.38 ± 0.03 (n=14)*</td>
<td>12.5 ± 0.3 (n=8)*</td>
<td>-28.3 ± 0.8 (n=11)*</td>
</tr>
<tr>
<td>(\beta_{1b}^{\text{trunc SH3}})</td>
<td>-7.5 ± 0.8 (n=6)*</td>
<td>0.30 ± 0.07 (n=6)*</td>
<td>9.5 ± 0.9 (n=6)*</td>
<td>-31.5 ± 0.9 (n=3)*</td>
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<tr>
<td>(\beta_{1b}^{\text{GK}})</td>
<td>-13.2 ± 5.3 (n=5)*</td>
<td>0.28 ± 0.04 (n=11)*</td>
<td>8.4 ± 2.4 (n=5)*</td>
<td>-32.8 ± 1.3 (n=5)*</td>
</tr>
<tr>
<td>(\beta_{1b}(\text{HOOK+SH3+GK}))</td>
<td>-79.8 ± 16.2 (n=10)**</td>
<td>0.08 ± 0.01 (n=10)**</td>
<td>3.5 ± 0.6 (n=10)**</td>
<td>-48.9 ± 1.3 (n=7)**</td>
</tr>
<tr>
<td>(\beta_{1b}^{\text{SH3+HOOK+SH3}}) plus (\beta_{1b}^{\text{GK}})</td>
<td>-19.0 ± 5.3 (n=12)**</td>
<td>0.48 ± 0.03 (n=15)**</td>
<td>12.5 ± 0.5 (n=12)**</td>
<td>-32.6 ± 1.1 (n=5)**</td>
</tr>
<tr>
<td>(\beta_{1b}^{\text{trunc SH3} + \beta_{1b}^{\text{HOOK+SH3+GK}}})</td>
<td>-128.2 ± 33.4 (n=11)**</td>
<td>0.08 ± 0.01 (n=14)**</td>
<td>3.6 ± 0.4 (n=11)**</td>
<td>-56.3 ± 1.6 (n=11)**</td>
</tr>
</tbody>
</table>

Statistical significances were determined for differences compared to Ca\(_{2.2}\) expressed without any \(\beta\) subunit, where† indicates $p \leq 0.05$; or compared to Ca\(_{2.2}\) expressed with the relevant wild-type \(\beta\) subunit counterpart, where* indicates $p \leq 0.05$.

Figure 4. The kinetics of inactivation of \(\beta_{2a}^{\text{GK}}\)-domains provides evidence for a role of the HOOK-domain. (A) Families of current traces following 2 s step depolarizations from -100 mV to -10, 0 and +10 mV for \(\beta_{2a}^{\text{GK}}\)-domain alone (left), \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (center) and \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (right). (B) Bar chart of $t_{\text{inact}}$ values (± s.e.m.) for the three conditions shown in (A). \(\beta_{2a}^{\text{GK}}\)-domain alone (black bars, \(n = 5\)), \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (grey bars, \(n = 17\)) and \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (white bars, \(n = 5\)). Statistical significance of the difference between \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain and \(\beta_{2a}^{\text{GK}}\)-domain is ** $p < 0.01$.

Role of the HOOK-Domain of \(\beta\) Subunits in Ca Channel Inactivation

For \(\beta_{1b}^{\text{GK}}\)-domain were accelerated compared to those of \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (Table 1).

Demonstration of interaction between \(\beta_{1b}^{\text{trunc SH3}}\)-dominated SH3 and \(\beta_{2a}(\text{HOOK+SH3+GK})\). To examine further the role of the HOOK-domain of \(\beta_{2a}\), we used the split domain approach, which has previously been shown to be successful in restoring \(\beta\) subunit function. In order to confirm the results of prior split domain studies, before further studying the role of the HOOK-domain, we therefore examined the coexpression of the \(\beta_{2a}^{\text{GK}}\)-domain constructs with their respective \(\beta_{2a}^{\text{GK}}\)-SH3 domains (pairs I and II, Fig. 1A). Both of the \(\beta_{2a}^{\text{GK}}\)-SH3-domain constructs retained the N-terminal palmitoylation motif, which has previously been shown to be responsible for the complete removal of inactivation exhibited by those \(\beta\) splice variants, including \(\beta_{2a}\) in which it is present. We were therefore able to use this as an assay of whether the pairs of \(\beta_{2a}^{\text{GK}}\) and GKD domain constructs would reconstitute this property, as this would provide evidence of interaction between the two domains.

As expected, neither of the \(\beta_{2a}^{\text{SH3}}\)-domains complimentary to the respective GKD domains (constructs Ia and Ila, Fig. 1A) increased the current amplitude alone, compared to the small currents observed in the absence of Ca\(_{\beta}\) subunits (Fig. 3A and data not shown). For the \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain coexpressed with the \(\beta_{2a}^{\text{trunc SH3}}\)-dominated SH3-domain, the \(\epsilon\)-strand of the SH3-domain is expressed as part of the GKD-domain construct, potentially promoting interaction between the two domains. The data in Figure 5 show that this was indeed the case, in that a combination of these two domains resulted in removal of inactivation, almost to the same extent as wild-type \(\beta_{2a}\) (Fig. 5A and B). Furthermore, the steady-state inactivation was very substantially depolarized compared to the \(\beta_{2a}^{\text{GK}}\)-dominated SH3+GKD-domain alone (Fig. 6A and B). The steady-state inactivation curve was best fit by a combination of two Boltzmann functions, with the larger component (~82%) having a $V_{50,\text{inh}}$ similar to wild-type \(\beta_{2a}\), and a minor component with a $V_{50,\text{inh}}$ similar to the \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain (Fig. 6B), suggesting either that not all the \(\beta_{2a}(\text{HOOK+SH3+GK})\)-domain is associated with palmitoylated \(\beta_{2a}^{\text{trunc SH3}}\)-dominated SH3-domain, or that a minor fraction of this SH3-domain is not palmitoylated.

In contrast, coexpression of the \(\beta_{2a}(\text{SH3+HOOK+SH3})\)-domain with the corresponding \(\beta_{2a}^{\text{GK}}\)-domain had no effect on the kinetic (Fig. 5A and B) or voltage-dependent (Fig. 6A and B) properties of the current formed by the combination of Ca\(_{2.2}/\alpha_\delta\)-2 with the \(\beta_{2a}^{\text{GK}}\)-domain alone, providing no evidence of interaction between these two intact Ca\(_{\beta}\) domains. In agreement with this conclusion, the kinetics of inactivation for \(\beta_{2a}(\text{SH3+HOOK+SH3})\)-domain plus \(\beta_{2a}^{\text{GK}}\)-domain were not significantly slower than those of \(\beta_{2a}^{\text{GK}}\)-domain alone (Fig. 5B, compared to Fig. 4B), and both were much more rapid than those of \(\beta_{2a}\) itself.

www.landesbioscience.com Channels 97
The relative roles of the β2a palmitoylated SH3-domain and the β2a GK-domain in removal of inactivation involving the β2a GK-domain. There are three nonexclusive mechanisms that may be responsible for the removal of inactivation when the β2a (HOOK+eSH3+GK)-domain and the β2a truncated SH3-domain are coexpressed. Firstly, it is likely that the presence of the palmitoylation on the SH3-domain reduces inactivation by anchoring the GK-domain and its associated I-I linker. This requires that the two domains interact. Secondly, it may be that their interaction then promotes a change in conformation of the GK-domain. Thirdly, there appears to be a role for a specific motif in the β2a HOOK-domain in retarding inactivation. The involvement of the β2a HOOK region is suggested by the fact that the β2a (HOOK+eSH3+GK)-domain shows a four-fold slowing of inactivation kinetics, and a steady-state inactivation that is significantly more depolarized than the short β2a GK-domain (Figs. 3C and D and 4A and B), and by the finding that deletion of the variable region of the HOOK-domain of β2a results in a rapidly inactivating current component (Fig. 2).

We then wished to determine whether the demonstrated interaction between the β2a truncated SH3-domain and the β2a (HOOK+eSH3+GK)-domain was important, for example to present the HOOK region in a correct orientation, by using the truncated SH3-domain from β1b, which is not palmitoylated. We first examined the effect of the β1b truncated SH3-domain on the β1b (HOOK+eSH3+GK)-domain. Their coexpression resulted in currents with a steady-state inactivation that was hyperpolarized to a similar extent to β1b itself, and was also similar to either of the β1b GK-domains alone (Fig. 7A and B). In tsA201 cells, similar results were observed for the combination of the β1b truncated SH3-domain and β1b (HOOK+eSH3+GK)-domain (Table 1). In contrast, in this expression system, there was little evidence that the short β1b GK-domain, either alone or in combination, had any influence to enhance current amplitude, or on other parameters of activation or inactivation (Table 1).

In Xenopus oocytes, we observed comparable hyperpolarization of the steady-state inactivation with the combination of the β1b truncated SH3-domain and either β1b (HOOK+eSH3+GK)-domain or β1b (ΔHOOK+eSH3+GK)-domain (Fig. 7B). In contrast, when the β1b truncated SH3-domain was coexpressed with the β2a (HOOK+eSH3+GK)-domain, this combination significantly shifted the steady-state inactivation to more positive potentials (Fig. 7A and B). This effect was lost if the HOOK sequence was removed to form the β2a (ΔHOOK+eSH3+GK)-domain. Using this construct together with β1b truncated SH3 domain, the steady-state inactivation that is significantly more depolarized than the short β2a GK-domain (Figs. 3C and D and 4A and B), and by the finding that deletion of the variable region of the HOOK-domain of β2a results in a rapidly inactivating current component (Fig. 2).
inactivation was strongly hyperpolarized, to a similar extent to a combination with any of the β1b GK-domain constructs (Fig. 7A and B). Similarly, the kinetics of inactivation were much more rapid for the combination of the β1b truncated SH3-domain with the β2a-(ΔHOOK+eSH3+GK)-domain (open circles, n = 28) and β1b truncated SH3-domain plus β2a-(ΔHOOK+eSH3+GK)-domain (open triangles, n = 10). Data were fit by single Boltzmann function, and the mean parameters for individual fitted data curves are given in Figure 7.B. (B) Bar chart of \( V_{50,\text{inact}} \) for CaA.2.2/αβ2-2 expressed together with the β subunit constructs stated. The number of determinations is shown on the bars. The statistical significances of the differences between the \( V_{50,\text{inact}} \) values for +\( \beta_{2a} \) +\( \beta_{1b} \) and without \( \beta \) are indicated for comparison by dashed vertical lines.

**DISCUSSION**

Requirement of CaAβ GK-domains for plasma membrane expression of HVA calcium channels. One of the main effects of CaAβ subunits on HVA calcium channels is to increase current density. However, the mechanism for this increase remains controversial, either being attributed to increased trafficking, increased maximum open probability or both. In agreement with the first hypothesis, we and others have shown biochemically that the amount of CaAα subunits in the plasma membrane is increased by CaAβ subunits. This finding was reinforced by the fact that fewer channels were present at the surface when the mutated CaA.2.2.W391A channels, that did not interact with \( \beta \) subunits, were cotransfected with a CaA.β subunit.

It is evident that any combination containing the β2a-(HOOK+eSH3+GK)-domain results in a slower inactivation rate and a decrease in the extent to which the voltage-dependence of inactivation is hyperpolarized. This specifically requires the presence of the HoOk-domain of β2a. Thus, despite the absence of the palmitoylation motif on the β1b truncated SH3-domain, the presence of other attributes, in particular the unique HoOk sequence contributed by the β2a-(HOOK+eSH3+GK)-domain contributes to the removal of inactivation. The combination of the nonpalmitoylatable C3,4S-β2a-SH3-domain with the β2a-(HOOK+eSH3+GK)-domain resulted in a \( V_{50,\text{inact}} \) for steady-state inactivation similar to that for the β1b truncated SH3-domain and β2a-(HOOK+eSH3+GK)-domain (Fig. 7B). It also showed similar inactivation kinetics, which were much slower than in the absence of the HoOk domain (Fig. 8A and B)

![Figure 7](image_url)

**Figure 7.** Steady-state inactivation for combinations of β-subunit constructs, showing the role of the β2a HoOk-domain. (A) Normalized steady-state inactivation curves for CaA.2.2/αβ2-2, together with the constructs shown. No β (dashed line), repeated from Fig. 3C, β1b (dotted line, repeated from Fig. 3C), β1b-(HOOK+eSH3+GK)-domain (closed circles, n = 9), β1b truncated SH3-domain plus β1b-(HOOK+eSH3+GK)-domain (closed triangles, n = 8), β1b truncated SH3-domain plus β2a-(HOOK+eSH3+GK)-domain (open circles, n = 28) and β1b truncated SH3-domain plus β2a-(ΔHOOK+eSH3+GK)-domain (open triangles, n = 10). Data were fit by single Boltzmann function, and the mean parameters for individual fitted data curves are given in Figure 7.B. (B) Bar chart of \( V_{50,\text{inact}} \) for CaA.2.2/αβ2-2 expressed together with the β subunit constructs stated. The number of determinations is shown on the bars. The statistical significances of the differences between the \( V_{50,\text{inact}} \) values for +\( \beta_{2a} \) +\( \beta_{1b} \) and without \( \beta \) are indicated for comparison by dashed vertical lines.

![Figure 8](image_url)

**Figure 8.** Kinetics of inactivation for combinations of β2a and β1b truncated SH3-domain with the β2a-(HOOK+eSH3+GK)-domain in the presence or absence of the HoOk-domain or palmitoylation motif. (A) Example traces showing inactivation kinetics for 2 s depolarizing steps to -20, -10, 0 and +10 mV from -100 mV, for β1b truncated SH3-domain together with β2a-(HOOK+eSH3+GK)-domain (left), β1b truncated SH3-domain together with β2a-(ΔHOOK+eSH3+GK)-domain (centre) and C3,4S-β2a truncated SH3-domain together with β2a-(HOOK+eSH3+GK)-domain (right). (B) Bar chart of time constants of inactivation obtained by fitting a single exponential to the inactivating phase of the 2-second steps, for β2a truncated SH3-domain together with β2a-(HOOK+eSH3+GK)-domain (black bars, n = 12) and C3,4S-β2a truncated SH3-domain together with β2a-(ΔHOOK+eSH3+GK)-domain (grey bars, n = 9). Statistical significance of the differences between β1b truncated SH3-domain together with β2a-(ΔHOOK+eSH3+GK)-domain and the other conditions are given by *** p < 0.001, and between β1b truncated SH3-domain together with β2a-(HOOK+eSH3+GK)-domain and C3,4S-β2a truncated SH3-domain together with β2a-(ΔHOOK+eSH3+GK)-domain at +10 mV1 p < 0.05.
produced an enhancement of current density in *Xenopus* oocytes by a manner largely indistinguishable from full-length Ca\(_{\beta}\) subunits, whereas in tsA201 cells the effect on current amplitude was reduced, compared to full length \(\beta_{2a}\) or \(\beta_{1b}\) particularly for the minimal GK domains. This suggests that the processes limiting plasma membrane expression of Ca\(_{\alpha}\), subunits are to some extent dependent on the expression system used, or they may depend on differences in the stability or folding of the expressed domains in the different systems. Oocytes are incubated at 17–18°C, whereas mammalian cells are maintained at 37°C, and lower temperatures are known to reduce protein mis-folding. The GK domains used in the present study terminated at the exon boundary, marking the end of the second conserved domain, as this was most likely to delineate a domain that would be stable to proteolysis.

**Determinants of inactivation: Role of the Ca\(_{\beta}\)\_\(\alpha_{\theta}\) I-II linker.** In the absence of any expressed Ca\(_{\beta}\) subunits, the steady-state inactivation of Ca\(_{2.2}\) was relatively depolarized, with a mid-point at about -15 mV in *Xenopus* oocytes in 10 mM Ba\(^{2+}\). The \(V_{50}\_\text{inact}\) was hyperpolarized by all Ca\(_{\beta}\) subunits, except palmitoylated \(\beta_{2a}\). In the present study \(\beta_{1b}\) produced a negative shift (of -27 mV) compared to the absence of coexpressed Ca\(_{\beta}\) subunits. Here we have shown that steady-state inactivation was hyperpolarized by the presence of any of the free GK-domains of \(\beta_{1b}\) and \(\beta_{2a}\). This suggests that the binding of the GK-domain itself to the AID initiates the processes involved in inactivation. This is in agreement with the finding that mutation of the AID amino acids is also able to modify inactivation.\(^{10,32}\) It has been suggested that binding of the GK-domain to the AID sequence may induce the residues in the I-II linker proximal to the AID to form a rigid \(\alpha\)-helix, and that this is involved in initiating inactivation.

**Determinants of inactivation: Role of the HOOK-domain and potential interaction with \(\beta_{2a}\) palmitoylation.** This study provides several main pieces of evidence that the HOOK-domain is involved in regulating the process of inactivation. Firstly, the difference between \(\beta_{2a}\)- (HOOK+\(\epsilon\)SH3+GK)-domain compared to \(\beta_{2a}\)-GK alone is a depolarisation of the steady state inactivation by 10 mV and -4-fold slowing of inactivation kinetics. Secondly, the difference between C3,4S-\(\beta_{2a}\) compared to C3,4S-\(\beta_{2a}\)_\(\Delta\_\text{vHOOK}\) is a 12 mV shift of steady-state inactivation and -7-fold slowing of the inactivation kinetics. Thirdly, the difference between \(\beta_{2a}\_\text{truncSH3},\text{GK}\) and \(\beta_{1b}\_\text{truncSH3},\text{GK}\) to \(\beta_{2a}\_\text{truncSH3+GK}\) or \(\beta_{1b}\_\text{truncSH3}\) or C3,4S-\(\beta_{2a}\_\text{truncSH3}\) is -20 mV shift of the steady-state inactivation and -15-fold slowing of the inactivation kinetics. Further removal of inactivation is promoted by coexpressing the palmitoylated \(\beta_{2a}\)_\(\Delta\_\text{vHOOK}\) truncated SH3-domain with the \(\beta_{2a}\_\text{truncSH3},\text{GK}\)-domain, to a value approaching that for \(\beta_{2a}\)-GK itself. It is possible that this interaction allows presentation of the \(\beta_{2a}\)_\(\Delta\_\text{vHOOK}\) domain in the correct orientation to slow the kinetics of inactivation and depolarize steady-state inactivation.

Taken together, these results all point to the importance of the HOOK region of \(\beta_{2a}\) in the retarding inactivation. They also point to the possibility that palmitoylation of \(\beta_{2a}\) resulting in a much higher concentration beneath the plasma membrane, may allow stable docking of the \(\beta_{2a}\)_\(\Delta\_\text{vHOOK}\)-domain with a potential binding site on the \(\alpha_{\theta}\) subunit.

**Role of specific residues in the HOOK-domain.** The HOOK-domain of all \(\beta\) subunits is involved in alternative splicing and contains either a long exon (AIDATGLDAEENDIPANHREHOOK-domain of all subunits is to some extent dependent on the expression system used, or they may depend on differences in the stability or folding of the expressed domains in the different systems. Oocytes are incubated at 17–18°C, whereas mammalian cells are maintained at 37°C, and lower temperatures are known to reduce protein mis-folding. The GK domains used in the present study terminated at the exon boundary, marking the end of the second conserved domain, as this was most likely to delineate a domain that would be stable to proteolysis.


**CONCLUSION**

Our results indicate that two processes are occurring to retard inactivation. Firstly if palmitoylation is present, it tethers the Ca\(_{\beta}\) subunit to the membrane and enhances its local concentration, ensuring the AID region is always occupied by a \(\beta\) subunit. Potentially this palmitoylation also restricts the movement of the I-II linker via its binding to the Ca\(_{\beta}\_\text{SH3}\)-GK-domain, and prevents it from instigating inactivation.\(^{33,36}\) Secondly, residues in the HOOK-domain, particularly of \(\beta_2\), in a concerted manner to retard the inactivation processes. It was shown previously using mutated \(\beta_2\) subunits, where the SH3 and GK domains were separately mutated so that they did not interact intramolecularly, that it was possible to obtain interaction in trans, restoring the very slow inactivation seen with intact palmitoylated \(\beta_2\).\(^{23}\) Our results suggest that palmitoylation of the SH3-domain is likely to ensure that the HOOK sequence is present at high concentration and able to associate with its binding site on the \(\alpha_{\theta}\) subunit. Whilst it is possible that the HOOK-domain enhances the interaction between the \(\beta_{2a}\_\text{SH3}\) and GK-domains or interacts with part of the GK domain, the fact that the \(\beta_{2a}\_\text{HOOK+eSH3+GK}\)-domain showed a significantly more depolarized steady-state inactivation and much slower inactivation kinetics than the \(\beta_{2a}\_\text{GK}\)-domain suggests the HOOK domain has a direct role in retarding inactivation possibly by interaction with the calcium channel \(\alpha_{\theta}\) subunit itself.

**References**


