Functional Modularity of the β-Subunit of Voltage-Gated Ca^{2+} Channels

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ABSTRACT The β-subunit of voltage-gated Ca^{2+} channels plays a dual role in chaperoning the channels to the plasma membrane and modulating their gating. It contains five distinct modular domains/regions, including the variable N- and C-terminus, a conserved Src homology 3 (SH3) domain, a conserved guanylate kinase (GK) domain, and a connecting variable and flexible HOOK region. Recent crystallographic studies revealed a highly conserved interaction between the GK domain and α interaction domain (AID), the high-affinity binding site in the pore-forming α_1 subunit. Here we show that the AID-GK domain interaction is necessary for β-subunit-stimulated Ca^{2+} channel surface expression and that the GK domain alone can carry out this function. We also examined the role of each region of all four β-subunit subfamilies in modulating P/Q-type Ca^{2+} channel gating and demonstrate that the β-subunit functions modularly. Our results support a model that the conserved AID-GK domain interaction anchors the β-subunit to the α_1 subunit, enabling α_1-β pair-specific low-affinity interactions involving the N-terminus and the HOOK region, which confer on each of the four β-subunit subfamilies its distinctive modulatory properties.

INTRODUCTION

High voltage-activated (HVA) Ca^{2+} channels are multimeric complexes of four subunits: α_1, $\alpha_2\delta$, and β (some channels have an additional γ-subunit) (1,2). Although the α_1 subunit contains the essential elements for ion permeation, gating, and pharmacology, it expresses poorly on the plasma membrane by itself. Rather, cell surface expression of HVA Ca^{2+} channels requires the association of the β-subunit. The molecular mechanism underlying this β-subunit chaperon function is unclear, although it has been proposed that the β-subunit masks one or more endoplasmic reticulum (ER) retention signal on the α_1 subunit (3,4). The β-subunit also modulates a host of channel biophysical properties. However, the modulatory effects of the four β subfamilies are markedly different (5,6). All four types of β-subunits shift the activation voltage to more negative potentials, making the channels easier to open, and speed up the rate of channel opening. They also greatly alter the voltage dependence and kinetics of voltage-dependent inactivation. In general, the β_1, β_3, and β_4 subunits hyperpolarize the voltage dependence of inactivation, making the channels inactivate at more negative potentials, and speed up the inactivation kinetics. On the other hand, partly due to palmitoylation of two cysteines in its N-terminus, the rat and human β_{2a} subunit depolarizes the voltage dependence of inactivation, making the channels inactivate at more positive potentials, and dramatically slow down the kinetics of inactivation (7–11).

Biochemical and functional studies indicate that the β-subunit interacts directly with the α_1 subunit (12,13). The most robust and strongest interaction is with the α_1 I-II loop, with an affinity in the range of 2–20 nM (13–15). An 18-amino acid motif in the I-II loop, called the α interaction domain or AID, has been shown to bind directly to all four types of β-subunits in vitro (12).

Previous studies further show that the β-subunit contains five regions (Fig. 1 *A* and Fig. S1), with the second and fourth being highly conserved (68%–92% identity) and the others highly variable among the four subfamilies (5,13, 16–18). The three middle regions form a core that is able to confer some of the functional properties of the full-length β-subunit (13,18). Recently, the three-dimensional structures of the core region of three different β-subunits (β_{2a}, β_3, and β_4), alone or in complex with the AID, have been solved (19–21). These structures show that the β core contains a conserved Src homology 3 (SH3) domain, a conserved guanylate kinase (GK) domain, and a variable and flexible HOOK region that connects them. The structures also show that the AID binds to a hydrophobic pocket in the GK domain through extensive hydrophobic interactions and hydrogen bonds (Fig. 1 *B*). The amino acids involved in these interactions are highly conserved among different α_1 and β-subunits, suggesting a critical and conserved function. Indeed, mutations of several key residues in the AID have been shown to severely reduce or abolish β-subunit-stimulated Ca^{2+} channel current (12,15,22–25). On the other hand, two recent studies reported that certain essential properties of the β-subunit were independent of the AID-GK domain interaction (26,27). Thus, the functional importance of this interaction needs to be further examined.

In this study we investigated the molecular mechanism and determinants of the two essential functions of the β-subunit in trafficking the P/Q-type Ca^{2+} channels to the plasma membrane and modulating their biophysical properties, utilizing the β-subunit crystal structures as the blueprint to make point mutations, deletions, and chimeras with high precision. The goal was to determine the functional importance of the

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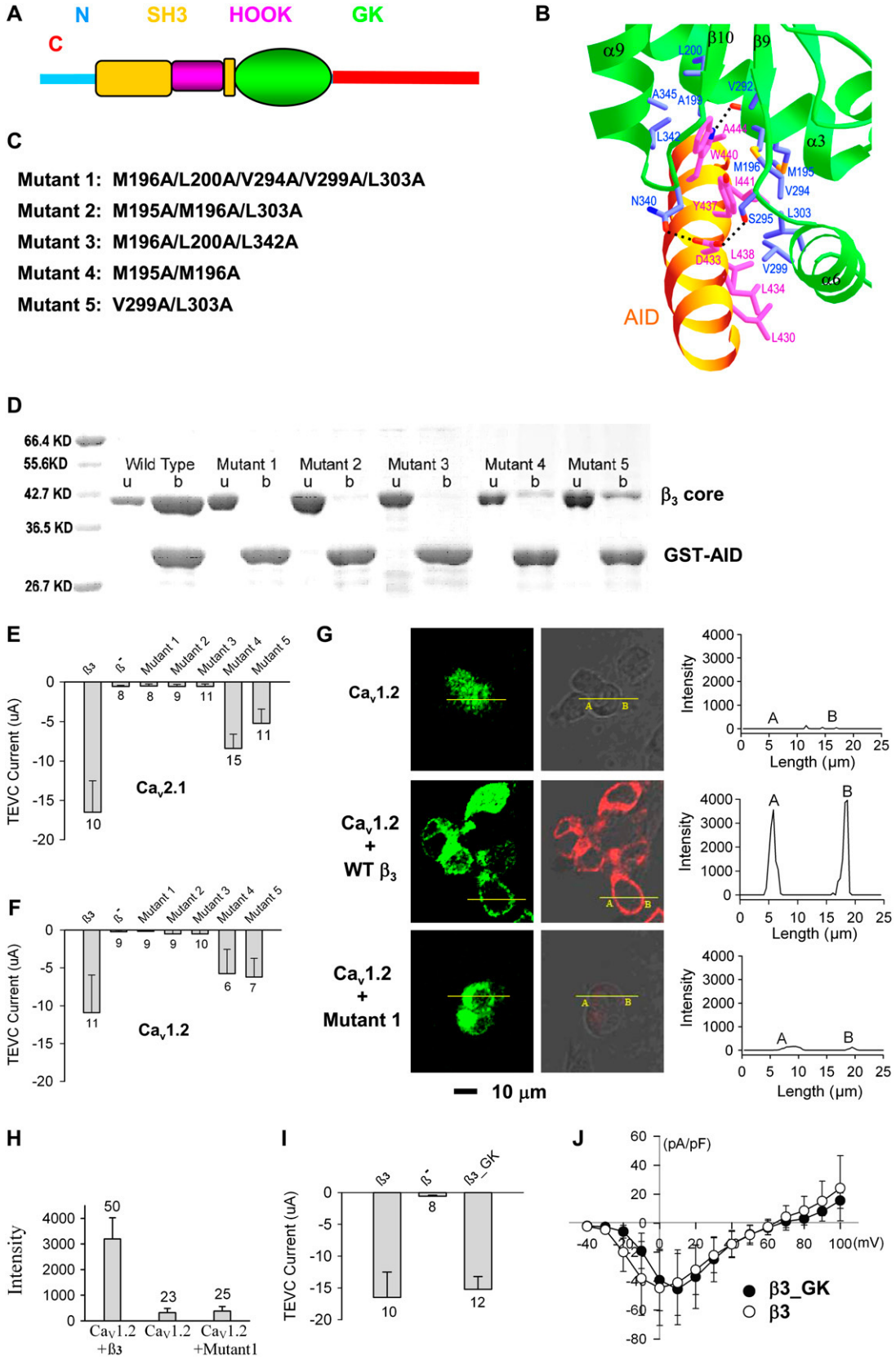

FIGURE 1 The AID-GK domain interaction is essential and sufficient for Ca^{2+} channel surface expression. (*A*) Schematic domain organization of Ca^{2+} channel β-subunits. (*B*) Close-up of the AID-GK domain interaction interface of the β3 subunit. (*C*) List of mutations in the β_3 subunit. (*D*) Coomassie blue

AID-GK domain interaction and the specific modulatory functions of each of the five domains/regions of the β-subunit. Our results indicate that the conserved AID-GK domain interaction is necessary for β-stimulated Ca^{2+} channel surface expression and that the GK domain alone is sufficient to perform this function. Our results further show that the variable N-terminus and HOOK region, but not the C-terminus, are critical for conferring on each of the four β-subunit subfamilies its distinctive modulatory functions on P/Q-type Ca^{2+} channels.

MATERIALS AND METHODS

Boundaries of the five domains/regions for the β-subunits

The boundaries of the five domains/regions for the β-subunits were defined according to the crystal structure and are indicated in the amino acid sequence alignment in Fig. S1. For the β_3 subunit, the boundaries for each domain/region are N-terminus: M-1-P-59; SH3 domain: V-60-S-120 and P-170-P-175 (the β-subunit SH3 domain has a split configuration consisting of the two indicated regions; see Chen et al. (19) Opatowsky et al. (20), and Van Petegem et al. (21)); HOOK region: P-121-P-169; GK domain: S-176-T-360; C-terminus: H-361-Y-484; β_3 core: G-16-G-366. The β-subunit domains, deletion mutants, and chimeras were constructed by polymerase chain reaction and were sequenced to check for errors. The SH3 domain and the HOOK region chimeras were constructed strictly according to the boundaries indicated above, but the GK domain and the C-terminal deletion constructs were extended to G-366.

Binding of the AID and β-subunits

The wild-type (WT) and mutant β_3 cores were subcloned into the pET-26b vector for expression of His-6-tagged proteins in BL21(DE3) bacteria. A glutathione S-transferase (GST)-tagged 44-amino-acid segment of the rabbit brain P/Q-type Ca^{2+} channel α_1 subunit $Ca_v2.1$ (residues R-369–Q-413) containing the AID was subcloned into a modified pGEX 4T-1 and expressed separately in the same strain of bacteria. These proteins were purified independently, and the GST-AID was immobilized in a GST column and was used to pull down the WT or mutant β_3 core. The β_3 core bound to the AID was eluted from the GST column with glutathione and was detected by Coomassie blue staining on sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Determination of Ca^{2+} channel surface expression

Rat brain $Ca_v1.2$ (rbC-II) α_1 subunit, β_3, and β_3_Mut1 (Fig. 1) coding sequences were subcloned into a mammalian expression vector (pcDNA3 or pcDNA4). The $Ca_v1.2$ subunit contained green fluorescence protein (GFP) at the N-terminus and the hemagglutinin (HA) epitope (YPYDVPDYA) between G-1135 and P-1136. The GFP and HA-tagged $Ca_v1.2$ subunit cDNA was transfected into human embryonic kidney (HEK) 293 cells either alone or together with WT β_3 or β_3 mutant 1 subunit DNA. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One to two days after transfection, cells were washed in phosphate buffered saline (PBS) and fixed for 20 min with a solution containing 4% paraformaldehyde at room temperature. Cells were then incubated in blocking buffer containing 2.5% goat serum for 1 h at room temperature, followed by incubation with 10 μg/ml anti-HA monoclonal antibody at 4°C overnight. The next day, cells were incubated with 10 μg/ml secondary antibody (Alexa 594 goat anti-mouse) for 1 h at room temperature. Subsequently, the cells were washed several times with PBS and mounted on glass slides and were imaged using an Olympus FluoView 500 confocal microscope (Olympus America Inc., Center Valley, PA). Surface Ca^{2+} channels were detected by red fluorescence. We used $Ca_v1.2$ rather than $Ca_v2.1$ for these experiments because, for reasons yet to be determined, all the HA-tagged $Ca_v2.1$ subunits we tested failed to produce clear and robust surface HA staining under nonpermeablized conditions. Since the AID is nearly identical between $Ca_v1.2$ and $Ca_v2.1$ and the GK-AID interaction is highly conserved, the results we obtained with $Ca_v1.2$ are likely applicable to $Ca_v2.1$.

Oocyte expression

All WT (including rabbit brain $Ca_v2.1$, rat brain $Ca_v1.2$, human β_{1b}, rat brain β_{2a}, β_3, and β_4, and rabbit skeletal muscle $\alpha_2\delta$) and mutant constructs for oocyte expression were subcloned into variants of the oocyte expression vector pGEMHE. cRNAs were synthesized in vitro and were injected in various combinations into *Xenopus* oocytes. The amount of α_1, $\alpha_2\delta$, and β cRNA injected was 7.5 ng, 7.5 ng, and 6.0 ng, respectively (in some cases, only α_1 and $\alpha_2\delta$ cRNAs were injected). Because the extent of β-subunit effects has been reported to depend on its concentration (14,25,28), we always injected an excess amount of β-subunit cRNA (the molar ratio of β/α_1 was >3.5). Thus, most of the α_1 subunit on the plasma membrane is likely associated with an exogenous β-subunit. This notion was further supported by the observation that application of purified β-subunit core does not further change channel gating in inside-out membrane patches (Y. Zhang and J. Yang, unpublished observations).

Ovarian lobes were obtained from adult *Xenopus laevis* (*Xenopus* I) under anesthesia. Stages V–VI oocytes were prepared by treatment with 2.5 mg/ml collagenase A (Boehringer Mannheim, Basel, Switzerland) for 1.5–2.5 h under 200 rpm shaking in a solution containing 82.4 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.6), and then rinsed twice (15 min each) with ND96 solution containing 96 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, 100 units/ml penicillin, and 100 μg/ml streptomycin (pH 7.6). Single, defoliculated oocytes were individually selected, injected with various combinations of cRNA, maintained in ND96 solution in an 18°C incubator, and used for recordings 3–7 days after injection.

FIGURE 1 (Continued)
staining illustrating the interaction between the indicated WT or mutant β_3 cores and the AID. GST-AID was immobilized in a GST column and was used to pull down the various β_3 cores. b: bound; u: unbound. (*E, F*) Whole-oocyte Ca^{2+} channel peak current from oocytes expressing $Ca_v2.1$ (*E*) or $Ca_v1.2$ (*F*), $\alpha_2\delta$ and the indicated WT or mutant β_3 (β^-: no β-subunit was injected). (*G*) Confocal images of nonpermeabilized HEK 293 cells transfected with $Ca_v1.2$ subunit tagged with GFP and HA, either alone or with WT β_3 or a mutant β_3 subunit (mutant 1). Left column: GFP fluorescence illustrating the distribution of $Ca_v1.2$. Middle column: HA staining under nonpermeablized conditions, illustrating $Ca_v1.2$ on the plasma membrane, superimposed with the brightfield image of the cells. Right column: intensity of HA staining (in artificial unit) at the plasma membrane at points indicated in the middle column. We used $Ca_v1.2$ instead of $Ca_v2.1$ for technical reasons (see Materials and Methods). (*H*) Average of HA staining intensity (in artificial unit) of $Ca_v1.2$ at the plasma membrane in the above three groups of HEK 293 cells. (*I*) Whole-oocyte Ca^{2+} channel peak current from oocytes expressing $Ca_v2.1$, $\alpha_2\delta$, and either WT β_3 or β_3 GK domain (β_3_GK). (*J*) Current-voltage relationship of whole-cell Ca^{2+} channel currents from HEK 293T cells transfected with $Ca_v1.2$, $\alpha_2\delta$, and either WT β_3 or β_3_GK ($n = 7$–9). Untransfected cells and cells transfected with $Ca_v1.2$ and $\alpha_2\delta$ only had no currents.

Electrophysiology

For two-electrode voltage clamp, the electrodes were filled with 3 M KCl and had resistances of ~0.5–1 MΩ. The bath solution contained (in mM) 10 $BaCl_2$, 5 KCl, 60 tetraethylammonium (TEA)-OH, 20 NaOH, and 5 HEPES (pH = 7.4 with HCl). Currents were evoked every 6 s by 40-ms depolarizations to various levels from a holding potential of −80 mV. For inside-out macropatch recordings, the pipettes had a diameter of 15–30 μm and were filled with a solution containing (in mM) 85 $BaCl_2$ and 10 HEPES (pH = 7.3 with KOH). The bath solution contained (in mM) 125 KCl, 4 NaCl, 10 HEPES, 10 EGTA (pH = 7.3 with KOH), and 2 Mg-ATP. In some cases when rundown was severe in inside-out patch-clamp recordings, cell-attached macropatch recordings were used instead. In those experiments, the pipette was filled with a solution containing (in mM) 45 $BaCl_2$, 80 KCl, and 10 HEPES (pH = 7.3 with KOH), and to abolish Cl^- current mediated by the endogenous Ca^{2+}-activated Cl^- channels, 45 nl of 10 mM BAPTA was injected into oocytes 10–30 min before recording.

For whole-cell recording, a GFP-tagged $Ca_v1.2$ subunit cDNA was transfected into HEK 293T cells with $\alpha_2\delta$ cDNA and WT β_3 or β_3_GK cDNA using Lipofectamine 2000 (Invitrogen). These cells were gifts from Dr. Hiroaki Matsunami at Duke University and seem to produce better expression of HVA Ca^{2+} channels. Recordings were performed 24–48 h after transfection. The electrodes had a diameter of ~1 μm, were filled with a solution containing (in mM) 135 $CsMeSO_3$, 5 CsCl, 5 EGTA, 1 $MgCl_2$, 3 Mg-ATP, 10 HEPES (pH = 7.3 with CsOH), and had a resistance of ~1.5 MΩ. The bath solution contained (in mM) 140 TEA-OH, 5 $BaCl_2$, 10 HEPES (pH = 7.3 with $MeSO_3$).

Currents were sampled at 10 kHz and filtered at 2.5 kHz (for deactivation kinetics, currents were sampled at 25 kHz and filtered at 10 kHz). Experiments were performed at 22°C. Macroscopic currents recorded from oocytes were evoked by 20-ms depolarizations ranging from −40 mV to +100 mV in 10 mV increments from a holding potential of −80 mV at a 6-s interval. Tail currents were recorded by repolarization to −20 mV after the various depolarization pulses. Steady-state inactivation was determined by a three-pulse protocol in which a 20-ms normalizing pulse to +30 mV (pulse A) was followed sequentially by a 25-s conditioning pulse (ranging from −60 mV to +40 mV) and a 20-ms test pulse to +30 mV (pulse B). The holding potential was −80 mV, and the interval between each protocol was 2 min. Peak current evoked by pulse B was normalized by that evoked by pulse A and was plotted against the conditioning potentials to obtain the voltage dependence of inactivation. Whole-cell currents recorded from HEK 293T cells were evoked every 6 s by 30-ms depolarizations ranging from −40 mV to +100 mV in 10 mV increments, from a holding potential of −90 mV.

Data analysis

The voltage dependence of activation and inactivation was fitted with the Boltzmann function of the form $1/(1 + \exp[-(V - V_{1/2})/k])$, where $V_{1/2}$ and k are the midpoint of activation or inactivation and slope factor, respectively. Unless indicated otherwise, data are presented as mean ± SD (number of observations). Statistical comparisons were always made between only two groups and were performed using the two-tail Mann-Whitney U test.

RESULTS

The GK domain is necessary and sufficient for Ca^{2+} channel surface expression

The crystal structures show that the AID interacts with the GK domain through a network of predominantly hydrophobic interactions and hydrogen bonding (19–21). To investigate the functional importance of this interaction, we mutated some key residues in the β_3 subunit that appear most critical for binding the AID, including M-195, M-196, L-200, V-294, V-299, L-303, and L-342 (Fig. 1 *B*), to alanine in groups of two, three, or five (Fig. 1 *C*) and tested the ability of the mutant β-subunits to stimulate Ca^{2+} channel current. We also made the same mutations in the core region of the β_3 subunit (β_3 core), which was amicable for expression in bacteria and for purification, and tested the binding between the mutant β_3 cores and the AID by pull-down experiments (Fig. 1 *D*).

Each mutant β-subunit was coexpressed with either $Ca_v2.1$ (the α_1 subunit of P/Q-type channels) or $Ca_v1.2$ (the α_1 subunit of L-type channels) and $\alpha_2\delta$ subunits in oocytes, and whole-oocyte current was measured with two-electrode voltage clamp (TEVC). Owing to the presence of two endogenous β-subunits (5,29), oocytes expressing only $Ca_v2.1$ or $Ca_v1.2$ and $\alpha_2\delta$ subunits (dubbed β^- oocytes) still showed variable levels of Ba^{2+} current, although in most cases the peak current was <0.3 μA. Coexpression of any of the four types of WT β-subunits greatly increased Ca^{2+} channel current (for example, Fig. 1 *E*), as has been universally reported.

The mutant β-subunits containing double mutations could still bind the AID, but with a much lower affinity (Fig. 1 *D*, mutants 4 and 5). Accordingly, they produced less Ca^{2+} channel current than the WT β_3 subunit did, with either $Ca_v2.1$ or $Ca_v1.2$ (Fig. 1, *E* and *F*). On the other hand, all mutant β-subunits bearing three or five mutations lost the ability to bind the AID (Fig. 1 *D*, mutants 1–3). Correspondingly, they were defective in stimulating Ca^{2+} channel current, which was indistinguishable from that recorded from the β^- oocytes (Fig. 1, *E* and *F*). This current abolishment was obviously the result of abolished surface expression of Ca^{2+} channels (Fig. 1, *G* and *H*). These results indicate that the AID-GK domain interaction is necessary for trafficking Ca^{2+} channels to the plasma membrane.

We next examined whether the GK domain by itself is capable of trafficking Ca^{2+} channels to the cell surface. We constructed the GK domain from each of the four β-subunit subfamilies, using the β-subunit crystal structures as the blueprint (Supplementary Fig. 1). In both oocyte and a mammalian cell line, HEK 293T cells, the β_3 GK domain stimulated Ca^{2+} channel current to a similar extent as its WT counterpart did (Fig. 1, *I* and *J*). Thus, the GK domain alone is sufficient for promoting Ca^{2+} channel surface expression.

Functional properties of four WT β-subunits

To fully understand how β-subunits modulate various channel properties, it is preferable to examine the modulatory effects of all four subfamilies of WT β-subunits in the same expression system and under the same experimental conditions. In this study, we systematically examined the voltage dependence (including the midpoint $V_{1/2}$ and slope factor K) of activation and steady-state inactivation and the kinetics of inactivation of P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes containing $Ca_v2.1$, $\alpha_2\delta$, and either β_{1b}, β_{2a}, β_3, or β_4

subunits, using the cell-attached or inside-out patch-clamp recordings with Ba^{2+} as the charge carrier. Although such comparative studies have been done before (11,16,30,31), we needed to perform these experiments to establish a set of reference data under our experimental conditions.

Relative to the Ca^{2+} channels recorded from the β^- oocytes (which are referred to as β^- channels because they are most likely devoid of a β-subunit, see Discussion), the voltage dependence of activation of channels with a β-subunit was shifted to more hyperpolarized potentials and the activation kinetics became faster (Table 1 and Fig. S2 *A*). These effects were similar among different β-subunits. In contrast to the similar effect on activation, the four β-subunits exerted drastically different effects on inactivation (Table 1 and Fig. S2, *B* and *C*). Thus, β_{1b}, β_3, and β_4 subunits greatly increased the inactivation kinetics and shifted the voltage dependence of inactivation toward more hyperpolarized potentials. On the other hand, the β_{2a} subunit dramatically slowed down the inactivation kinetics and shifted the voltage dependence of inactivation toward more depolarized potentials. These β-subunit-specific modulatory effects are in qualitative agreement with those reported previously (11,16,30,31) (for reviews, see Birnbaumer et al. (5) and Dolphin (6)). Quantitative differences from previous studies may arise from differences in the heterologous expression system, the α_1 subunit, and the recording conditions, including the ionic concentrations.

Functional effects of the GK domain

We next examined the effect of each of the five regions of the β-subunit on various channel properties, starting with the GK domain. As shown above, the GK domain alone is sufficient for trafficking Ca^{2+} channels to the cell surface, but can it recapitulate the modulatory properties of the β-subunit? Compared with the β^- channels, all four GK domains produced a slight (3–5 mV) hyperpolarizing shift in the voltage dependence of activation (Fig. 2 *A* and Table 1) and a stronger (~10 mV) hyperpolarizing shift in the voltage dependence of inactivation (Fig. 2 *B* and Table 1) and increased the speed of inactivation (Fig. 2 *C* and Table 1). Thus, the GK domain by itself can exert some modulatory effects on channel biophysical properties. Furthermore, the effects of all four GK domains were the same (Fig. 2, *A–C*, and Table 1). This is not surprising considering that the four GK domains have very high amino acid identity and that the three GK domains whose structure is available have the same structure (19–21) (Fig. S3 *A*). However, the effect of the GK domain on the voltage dependence of activation and inactivation and on the inactivation kinetics is markedly different from that of the corresponding WT β-subunit (Fig. 2, *D–K*, and Table 1), indicating that the GK domain alone cannot fully reconstitute the modulatory effects of the WT β-subunit and that the other four domains/regions of the β-subunit are critical in modulating channel biophysical properties. These results are not unexpected considering that the N-terminus has long been known to modulate inactivation properties (see Discussion).

Lack of functional effect of the C-terminus

We next studied the role of the C-terminus. This region is highly diverse in amino acid sequence among the four β-subunit subfamilies and constitutes about one-third to one-fourth of the β-subunit in length (Fig. S1). It is thus quite natural to hypothesize that the C-terminus is critically involved in modulating gating. Surprisingly, deleting the C-terminus had no effect on the voltage dependence of activation and inactivation and on the speed of inactivation (Fig. 3). Furthermore, the gating properties of Ca^{2+} channels

TABLE 1 Gating properties of Ca^{2+} channels containing the indicated WT β-subunit or the β-subunit core region or GK domain

	Activation			Inactivation		
Construct	$V_{1/2}$ (mV)	K(mV)	τ_{act} (ms) (30 mV)	$V_{1/2}$ (mV)	K(mV)	$T_{1/2}$ (s) (30 mV)
β^-	31.0 ± 3.0(6)	9.2 ± 0.5(6)	4.9 ± 0.9(6)	1.3 ± 0.8(5)	−5.5 ± 0.6(5)	0.99 ± 0.2(5)
β_{1b}	19.4 ± 2.1(6)	9.2 ± 0.5(6)	2.1 ± 0.9(5)	−15.6 ± 2.8(6)	−6.4 ± 1.0(6)	0.11 ± 0.03(5)
β_{2a}	22.9 ± 3.0(7)	8.4 ± 2.0(7)	2.5 ± 0.8(7)	11.0 ± 3.2(6)	−6.8 ± 1.9(6)	8.7 ± 1.0(6)
β_3	24.8 ± 2.2(11)	8.2 ± 1.5(11)	2.1 ± 0.2(6)	−12.2 ± 3.6(6)	−6.6 ± 0.8(6)	0.14 ± 0.03(6)
β_4	22.5 ± 3.0(6)	8.0 ± 1.0(6)	2.5 ± 0.4(8)	−4.8 ± 2.8(5)	−7.0 ± 1.4(5)	0.5 ± 0.2(5)
β_{1b}_core	22.3 ± 4.0(5)*	7.4 ± 2.0(5)	2.5 ± 0.4(7)	−10.5 ± 1.9(5)*	−5.4 ± 0.5(5)	0.7 ± 0.4(5)†
β_{2a}_core	23.6 ± 1.7(7)	8.6 ± 1.2(7)	2.5 ± 0.7(6)	3.6 ± 1.0(5)†	−5.6 ± 0.5(5)	2.9 ± 0.7(5)†
β_3_core	24.8 ± 3.0(9)	7.4 ± 1.5(9)	2.4 ± 0.8(10)	−3.6 ± 2.0(7)†	−7.7 ± 0.3(7)*	0.97 ± 0.2(7)†
β_4_core	22.1 ± 2.0(6)	7.7 ± 1.0(6)	2.3 ± 0.3(6)	0.0 ± 0.80(4)*	−6.2 ± 0.3(4)	1.99 ± 0.3(4)†
β_{1b}_GK	26.9 ± 3.0(14)†	9.2 ± 1.8(14)	2.2 ± 0.7(8)	−9.6 ± 4.0(5)*	−5.7 ± 0.9(5)	0.23 ± 0.04(5)*
β_{2a}_GK	27.1 ± 2.3(11)*	9.3 ± 1.0(11)	2.2 ± 0.4(6)	−10 ± 3.0(5)†	−5.9 ± 0.9(5)	0.26 ± 0.09(5)†
β_3_GK	27.5 ± 3.0(5)	8.9 ± 1.3(5)	2.2 ± 0.5(5)	−9.2 ± 4.0(7)	−5.8 ± 0.7(7)	0.23 ± 0.05(5)*
β_4_GK	26.3 ± 1.9(11)*	9.2 ± 1.0(11)	2.3 ± 0.7(8)	−10.8 ± 2.3(5)*	−6.0 ± 1.1(5)	0.24 ± 0.02(7)†

The parameters include the midpoint ($V_{1/2}$) and slope factor (K) of current activation and inactivation, time constant of current activation (τ) at +30 mV and the time for the current to decay to 50% of the peak value at +30 mV ($T_{1/2}$ of inactivation at +30 mV). Currents were recorded in inside-out patches. Data represent mean ± SD (number of patches). β^-: no β-subunit was injected. Significance tests were performed between a given mutant and its WT counterpart. *$p < 0.05$; †$p < 0.01$.

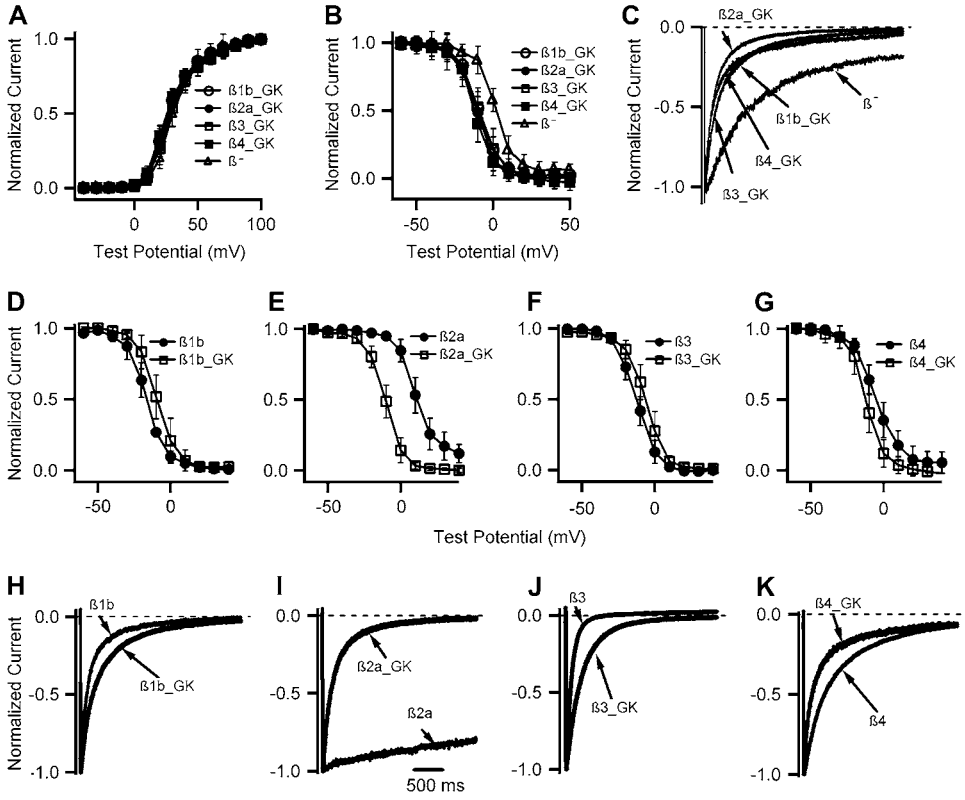

FIGURE 2 Functional effects of the GK domain. (*A*) Voltage dependence of activation of β^- channels and channels containing the indicated GK domain. In this and the following figures, data points represent normalized tail currents recorded at −20 mV after depolarization to a given test potential. $N = 5–14$. (*B*) Voltage dependence of steady-state inactivation of channels containing the indicated GK domain. In this and the following figures, steady-state inactivation was determined by a three-pulse protocol in which a 20-ms normalizing pulse to +30 mV (pulse A) was followed sequentially by a 25-s conditioning pulse (ranging from −60 mV to +40 mV) and a 20-ms test pulse to +30 mV (pulse B). The holding potential was −80 mV, and the interval between each protocol was 2 min. Peak current evoked by pulse B was normalized by that evoked by pulse A and was plotted against the conditioning potentials. $N = 5–7$. (*C*) Comparison of the kinetics of inactivation of channels containing the indicated GK domain. In this and the following figures, current was evoked by the 25-s conditioning depolarization to +30 mV from above and was normalized by the peak amplitude. (*D–G*) Comparison of the voltage dependence of steady-state inactivation of channels containing the indicated WT or mutant β-subunit. $N = 5–11$. (*H–K*) Comparison of the kinetics of inactivation of channels containing the indicated WT β-subunit or GK domain. Currents were recorded in inside-out patches.

containing a truncated β-subunit consisting of the GK domain and the C-terminus (named GKC) were essentially the same as those of channels containing only the GK domain (Fig. S4). These results suggest that the C-terminus is not involved in modulating P/Q-type Ca^{2+} channel gating and that the β-subunit-specific modulatory properties are defined by the N-terminus, the SH3 domain, and/or the HOOK region.

Functional effects of β-subunit core

The β-subunit core (for β_3, amino acids G-16–G-366) defined in this study contains the interacting SH3 and GK domains and the connecting HOOK region. In addition, it contains the highly conserved proximal portion (~43 amino acids) of the N-terminus (Fig. S1). All four β cores produced a similar hyperpolarization shift of the activation curve (Fig. 4, *A–D*, and Table1). Strikingly, the voltage dependence of activation was essentially identical for three β-subunits (β_{2a}, β_3, or β_4) and their core region (Fig. 4, *B–D*, and Table 1). Thus, this activation property appears to be regulated largely by the conserved core region. In contrast, for each pair of β-subunits and its core, the effect on the voltage dependence and speed of inactivation is markedly divergent (Fig. 4, *E–L*, and Table 1). Since the C-terminus does not appear to play any role in modulating channel properties (Fig. 3), this result indicates that the variable distal portion of the N-terminus, which ranges from ~30 to ~60 amino acids (Fig. S1), is partly involved in fine-tuning the inactivation properties.

Interestingly, the four β cores also produced highly dissimilar effects on the voltage dependence and speed of inactivation among themselves (Fig. 4 *M* and Table 1). Since the proximal N-terminus is highly conserved (Fig. S1) and the four GK domains behaved the same way (Fig. 2), these results indicate that the SH3 domain and/or the HOOK region play a key role in modulating these properties.

The HOOK region modulates inactivation properties

To examine the role of the SH3 domain and HOOK region, we swapped them individually between β_{2a} and β_{1b} cores, which differed the most in their effects on the voltage dependence and speed of inactivation among the four β cores (Table 1). The reason for using the core region instead of the full-length β-subunit is to eliminate the contribution of the variable distal N-terminus, especially the effect of palmitoylation of the N-terminus of the β_{2a} subunit (7–11). Exchanging the SH3 domain did not alter the modulatory effects of either resulting chimeric β core. Thus, the β_{1b} core

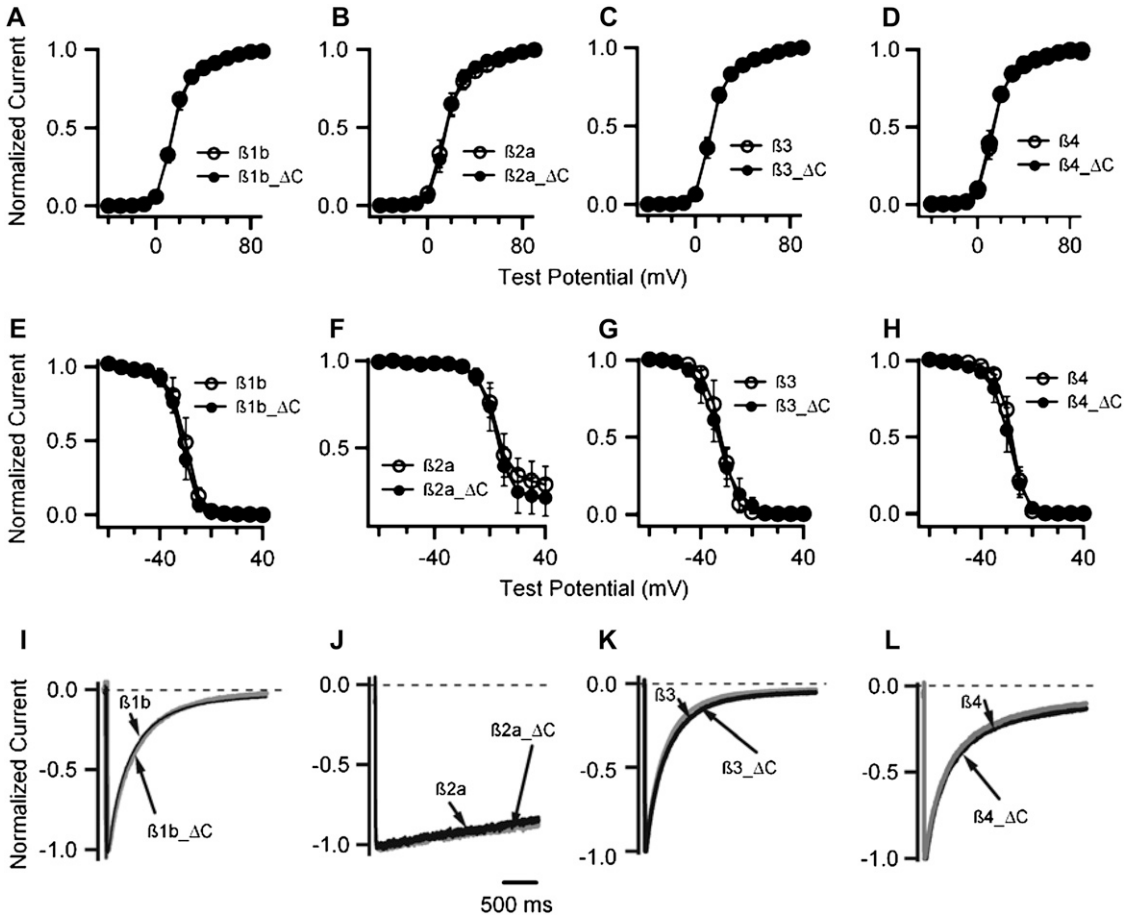

FIGURE 3 Deletion of the β-subunit C-terminus has no functional effects. (*A–D*) Comparison of the voltage dependence of activation of channels containing the indicated WT or mutant β-subunit. $N = 5–10$. (*E–H*) Comparison of the voltage dependence of steady-state inactivation of channels containing the indicated WT or mutant β-subunit. $N = 5–9$. (*I–L*) Comparison of the kinetics of inactivation of channels containing the indicated WT or mutant β-subunit. Black and gray lines represent WT and mutant β-subunits, respectively. These experiments were performed in cell-attached macropatch recordings with 45 mM $BaCl_2$, 80 mM KCl, and 10 mM HEPES (pH = 7.3 with KOH) in the pipette.

containing the β_{2a} SH3 domain behaved just like the β_{1b} core did, and the β_{2a} core containing the β_{1b} SH3 domain behaved just like the β_{2a} core did (Fig. 5, *A–C*). This result is not surprising given the high amino acid and structural conservation of the SH3 domain (Fig. S1 and S3 *B*). On the other hand, swapping the HOOK region produced a complete and reciprocal change of the functional effect of the resulting β core on the voltage dependence and speed of inactivation. Thus, the β_{1b} core containing the β_{2a} HOOK region behaved as the β_{2a} core did, and the β_{2a} core containing the β1b HOOK region behaved as the β_{1b} core did (Fig. 5, *D* and *E*). These results indicate that the HOOK region is critical for modulating the inactivation properties, consistent with the fact that it has a variable amino acid sequence among the four β-subunit subfamilies.

DISCUSSION

It has long been recognized that the β-subunit has a modular structure consisting of five domains/regions (5,13,16–18). The fact that the β-subunit contains an SH3-GK module was also recognized several years ago by homology modeling (17), although some structural details from this modeling did not match those shown later by the β-subunit crystal structures. Subsequent functional studies show that the SH3 and GK domains and their interactions are important for β-subunit functions (27,32–34). In this study we took advantage of the newly obtained β-subunit crystal structures to construct point mutants, deletion mutants, and individual domains/regions and examined their functional effects on Ca^{2+} channel membrane trafficking and/or gating. Our results support an emerging general model (6,35) in which the conserved high-affinity binding of the β-subunit to the AID anchors the β-subunit to the α_1 subunit and facilitates low-affinity interactions of other β-subunit domains/regions with different parts of the α_1 subunit, which in turn are responsible for the modulation of gating. Our work also leads us to the following specific conclusions regarding β-subunit effects on P/Q-type Ca^{2+} channels: 1), The AID-GK domain interaction is necessary and the GK domain alone is

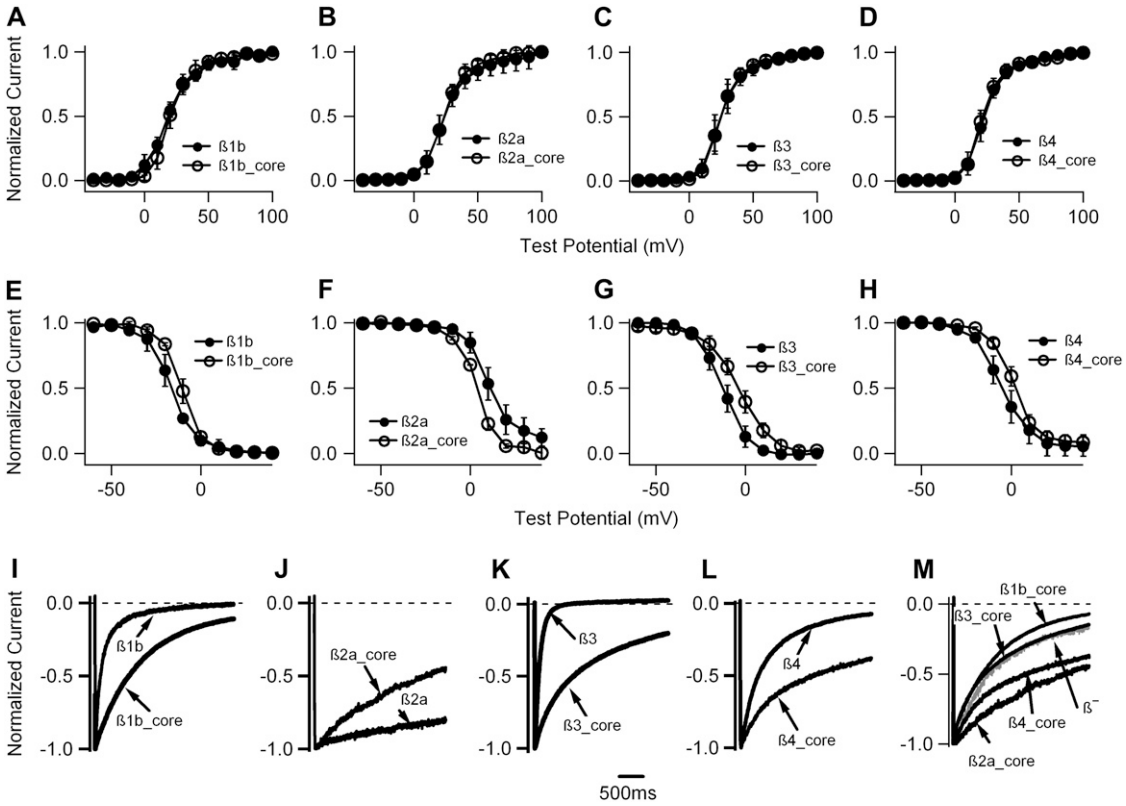

FIGURE 4 Functional effects of the β core domain. (*A–D*) Comparison of the voltage dependence of activation of channels containing the indicated WT β-subunit or β core. $N = 5–11$. (*E–H*) Comparison of the voltage dependence of steady-state inactivation of channels containing the indicated WT β-subunit or β core. $N = 4–7$. (*I–L*) Comparison of the kinetics of inactivation of channels containing the indicated WT β-subunit or β core. (*M*) Comparison of the kinetics of inactivation of channels containing the indicated β core. Gray line represents β^- channels. Currents were recorded in inside-out patches.

sufficient for the chaperone function of the β-subunit. 2), The β-subunit C-terminus is not involved in modulating gating of P/Q-type channels. 3), The β core containing the SH3-HOOK-GK module governs the modulatory effects on activation. 4), The HOOK region and the N-terminus (especially the distal variable region) are critical for modulating inactivation. Some of these results have been reported for specific combinations of α_1 and β-subunits (see below). In this study, we systematically examined the effect of all four β subfamilies in the same expression system.

Essential role of the GK domain in Ca^{2+} channel surface expression

The AID is the sole region in the α_1 subunit that has been found to interact with the β-subunit with high affinity (12,36). The strict conservation of the AID-GK domain interaction interface, as revealed by the crystal structures (19–21), suggests that this interaction is essential for β-subunit functions. This notion is strongly supported by the observation that mutations of several conserved residues in the AID greatly reduce binding with the β-subunit as well as β-subunit-induced current enhancement (12,15,22–25). Our finding that mutating three or more AID-binding residues in the GK domain leads to diminished Ca^{2+} channel surface expression and current (Fig. 1) also supports this prediction. These mutations are unlikely to change the folding or structure of the protein, since the mutated residues are exposed to the binding pocket rather than being involved in structural maintenance (Fig. 1 *B*). Thus, the lack of function of the mutants is most likely due to their inability or much weakened ability to bind the α_1 subunit.

Our finding that mutations in the AID-binding pocket in the GK domain can abolish Ca^{2+} channel surface expression and subsequent functional modulation stands in contrast with two recent studies stating that this interaction is not necessary for certain essential β-subunit functions (26,27). One study found that single or double mutations in the AID-binding pocket of the β_{2a} subunit did not affect its ability to stimulate Ca^{2+} channel expression and modulate their properties (27). This observation, however, does not exclude the essential roles of the AID-GK domain interaction since our results indicate that three or more AID-binding residues need to be mutated to abolish these β-subunit functions (Fig. 1). The same study also found that the β_{2a} subunit could not stimulate the current of Ca^{2+} channels formed by a $Ca_v2.1$ subunit lacking the AID (ΔAID) but was still able to modulate their biophysical properties (27). The former observation

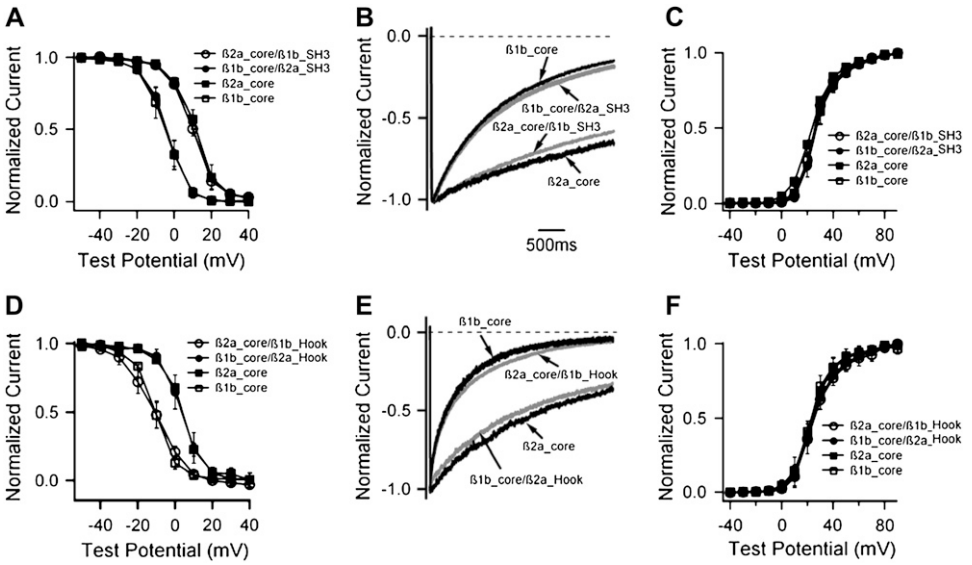

FIGURE 5 Functional effects of the SH3 domain and HOOK region. (*A*) Comparison of the voltage dependence of steady-state inactivation of channels containing the indicated β-subunit mutant. β_{2a}_core/β_{1b}_SH3 represents β_{2a} core with the β_{1b} SH3 domain, and β_{1b}_core/β_{2a}_SH3 represents β_{1b} core with the β_{2a} SH3 domain. $N = 5$–6. (*B*) Comparison of the kinetics of inactivation of channels containing the indicated β-subunit mutant. (*C*) Comparison of the voltage dependence of activation of channels containing the indicated β-subunit mutant. $N = 5$–8. (*D*) Comparison of the voltage dependence of steady-state inactivation of channels containing the indicated β-subunit mutant. β_{2a}_core/β_{1b}_HOOK presents β_{2a} core with the β_{1b} HOOK region, and β_{1b}_core/β_{2a}_HOOK represents β_{1b} core with the β_{2a} HOOK region. $N =$ 4–6. (*E*) Comparison of the kinetics of inactivation of channels containing the indicated β-subunit mutant. (*F*) Comparison of the voltage dependence of activation of channels containing the indicated β-subunit mutant. $N = 6$–7. Currents were recorded in inside-out patches.

in fact supports the notion that the AID-GK domain interaction is essential for Ca^{2+} channel surface expression. The latter does not actually contradict this notion but may have an alternative explanation: through palmitoylation the β_{2a} subunit can be anchored to the membrane and thus probably near the ΔAID Ca^{2+} channels even without the AID-GK domain interaction and this proximity allows the β_{2a} subunit to modulate these channels. It would be interesting to test whether nonpalmitoylated (or otherwise tethered to the membrane) β-subunits that are predominantly located in the cytoplasm can still modulate the properties of ΔAID Ca^{2+} channels.

In another study, Harry et al. (26) found that two short splice variants of the β_2 subunit lacking the majority or all of the GK domain were still able to enhance $Ca_v1.2$ channel currents and weakly affect their gating. However, the current enhancement was much less than that produced by full-length β_2 subunits. It is worth noting that there are two instances of nonspecific enhancement of Ca^{2+} channel currents by short polypeptides. First, a 35-amino acid β-subunit peptide containing the so-called β-interaction domain or BID was able to stimulate Ca^{2+} channel current (13,15), but the β-subunit crystal structures show that this peptide is largely buried in the protein and cannot possibly be involved in binding the AID or other regions of the α_1 subunit. Second, a 43-amino-acid peptide that has a random amino acid sequence was also found to be able to enhance Ca^{2+} channel current (19).

A surprising finding of our study is that the GK domain alone is sufficient to chaperone Ca^{2+} channels to the plasma membrane in both *Xenopus* oocytes and HEK 293T cells. However, it is unclear whether the GK domain alone functions as efficiently as the WT β-subunit in this regard since quantitative comparisons are difficult owing to the large variations in the peak current amplitude or current density in different oocytes or cells. Curiously, the GKC construct used by Takahashi et al. (33,34), which contains the GK domain, the C-terminus, and a portion of the HOOK region, performed poorly in trafficking Ca^{2+} channels to the cell surface in HEK 293 cells. The reasons for this discrepancy are unclear. It is possible that the GKC fragment used by Takahashi and colleagues does not fold properly alone in the HEK 293 cells they used or the ER and/or Golgi complex in their cells have additional check points for membrane protein trafficking. In any event, our finding that the GK domain alone possesses the chaperon function has interesting implications for the molecular mechanism of Ca^{2+} channel trafficking. It has been suggested that the chaperon function of the β-subunit arises, at least in part, by masking one or more ER retention signals on the α_1 subunit (3,4). However, the AID, which is the sole site where the GK domain binds with high affinity, does not contain any known ER retention signals. The observation by Maltez et al. (27) that elimination of the AID does not increase Ca^{2+} channel current further indicates that the AID does not represent the ER retention signal where the β-subunit binds to promote Ca^{2+} channel trafficking to the plasma membrane. It is possible that the GK domain masks yet unrecognized ER retention signals on the α_1 subunit or carries yet unrecognized ER export signals.

Role of the N-terminus and HOOK region

Our results reinforce previous findings that the N-terminus and HOOK region are critically involved in modulating inactivation. The role of the N-terminus is evident from

chimeric and deletion studies (37–39), from comparisons of various β_2 and β_4 splice variants (40–42), and from the effect of palmitoylation of cysteines in the N-terminus of the rat and human β_{2a} subunit (7–11). The recently obtained NMR solution structure of the N-terminus of the β_{4a} subunit shows that it folds into a compact domain. Combining this structure with that of the β core indicates that the N-terminus tilts away from the AID and other parts of the β-subunit (28), making it available to interact with other regions of the α_1 subunit or with other proteins.

Although previous chimeric studies also implicated the HOOK region (38,39), our results (Fig. 5, *D* and *E*) provide definitive evidence that this region is a major determinant for modulation of inactivation. It is unclear where in the α_1 subunit the N-terminus and HOOK region interact to exert their modulatory effects on inactivation, but candidate regions include the N-terminus, the III-IV loop, and the C-terminus, which have been shown to be involved in either regulating inactivation (11,43) or interacting with β-subunits, at least in vitro (29,44,45).

Role of the C-terminus

It is surprising that in our study deletion of the β-subunit C-terminus has no effect on activation and inactivation of $Ca_v2.1$ Ca^{2+} channels (Fig. 3), given that it constitutes a large portion of the β-subunits and that the C-terminus of β_4 has been shown to interact with both the N- and C-termini of $Ca_v2.1$ in vitro (35,45). A previous study reported that deletion of the β_4 C-terminus increased the inactivation kinetics of $Ca_v2.1$ Ca^{2+} channels (45), although this increase was very small. The discrepancy between our work and this work could be due to the different types of recordings used (TEVC versus macropatch). Our observation, however, is in agreement with that by Stotz et al. (39), who found that exchanging the C-terminus between β_3 and β_4 had little effect on inactivation of $Ca_v2.2$ Ca^{2+} channels. Whether the C-terminus contributes to the modulation of biophysical properties of other types of HVA Ca^{2+} channels remains to be determined. So, what are the functions of the β-subunit C-terminus? One possible function is to interact with other proteins. It has been shown that the C-terminus of β_{1a}, which is skeletal muscle specific and constitutes part of the skeletal muscle L-type Ca^{2+} channel, interacts directly with the ryanodine receptor and that this interaction strengthens excitation-contraction coupling (46). It is also interesting to note that a deletion mutation in β_4 that removes the last 38 C-terminal residues has been identified in a patient with juvenile myoclonic epilepsy (47).

The β^- channels are probably devoid of a β-subunit

In our study, the properties of channels containing an exogenously expressed β-subunit (or mutant) are compared with those of the so-called β^- channels recorded from oocytes injected with the cRNA of only $Ca_v2.1$ and $\alpha_2\delta$ subunits. Most of these oocytes exhibited little or no Ca^{2+} channel currents in macropatch or even in TEVC recordings, but a very small fraction showed small currents (0.3–1 nA) in macropatches. The reason these oocytes are able to express some surface Ca^{2+} channels is because *Xenopus* oocytes have two endogenous β_3-like β-subunits (29), which are apparently of sufficient concentration in the ER to chaperon some Ca^{2+} channels to the plasma membrane. It has been shown that β_3 antisense oligonucleotides greatly decrease or even abolish Ca^{2+} channel currents in oocytes expressing an α_1 subunit alone (14,29), suggesting an obligatory role of the endogenous β-subunits in the expression of Ca^{2+} channels in these oocytes. However, it appears that, due to their low concentration in the oocyte cytoplasm, the endogenous β-subunits eventually dissociate from the surface channels such that at steady state most of the surface Ca^{2+} channels are devoid of a β-subunit (i.e., β^- channels). This notion is supported by numerous observations that the activation and inactivation properties of the β^- channels differ greatly from those of channels containing an exogenously expressed β-subunit (for review, see Birnbaumer et al. (5) and Dolphin (6)) and by the finding that injection of β_3 protein into *Xenopus* oocytes expressing $Ca_v1.2$ subunits alone produced acute effects on their biophysical properties (48).

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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