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Calcium Channel Function Regulated by the SH3-GK Module in β Subunits

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Summary

β subunits of voltage-gated calcium channels (VGCCs) regulate channel trafficking and function, thereby shaping the intensity and duration of intracellular changes in calcium. β subunits share limited sequence homology with the Src homology 3-guanylate kinase (SH3-GK) module of membrane-associated guanylate kinases (MAGUKs). Here, we show biochemical similarities between β subunits and MAGUKs, revealing important aspects of β subunit structure and function. Similar to MAGUKs, an SH3-GK interaction within β subunits can occur both intramolecularly and intermolecularly. Mutations that disrupt the SH3-GK interaction in β subunits alter channel inactivation and can inhibit binding between the α_1 and β subunits. Coexpression of β subunits with complementary mutations in their SH3 and GK domains rescues these deficits through intermolecular β subunit assembly. In MAGUKs, the SH3-GK module controls protein scaffolding. In β subunits, this module regulates the inactivation of VGCCs and provides an additional mechanism for tuning calcium responsiveness.

Introduction

Voltage-gated calcium channels (VGCCs) increase the permeability of cell membranes to calcium in response to depolarization. This calcium influx mediates a variety of functions, including muscle excitation-contraction coupling, endocrine secretion, and synaptic transmission (Catterall, 2000). Accordingly, electrophysiological studies have characterized a number of different voltage-dependent calcium currents designated L- (Ca_v1.x), P/Q- (Ca_v 2.1), N- (Ca_v 2.2), R- (Ca_v 2.3), and T-type (Ca_v 3.x), each of which contribute to a subset of these cellular activities (Tsien et al., 1995).

VGCCs are composed of an α_1 pore-forming subunit, a disulfide-linked complex of $\alpha_2\delta$, and a cytosolic β ($Ca_v\beta$) subunit (Takahashi et al., 1987). Molecular cloning has identified ten α_1, four $\alpha_2\delta$, and four β subunits (Catterall, 2000). The α_1 subunits form the pore of the channel and contain four repeated domains (I–IV) of six transmembrane segments (S1–S6) and a membrane-associated loop between S5 and S6 (Tanabe et al., 1987). Expression of the α_1 subunit alone produces a voltage-dependent calcium current, but these "α_1 only" channels express poorly and display abnormal kinetics of channel activation and inactivation as compared to endogenous calcium currents (Perez-Reyes et al., 1989). Coexpression of an α_1 subunit with an $\alpha_2\delta$ subunit and a β subunit improves surface expression of the channel and yields voltage dependence and inactivation kinetics similar to native calcium currents (Walker and De Waard, 1998).

The β subunits contain two conserved regions, C1 and C2, of ~130 amino acids and ~150 amino acids in length, respectively, that are flanked by three variable regions of low sequence similarity. The variable regions V1 and V3, which flank the C1 and C2 domains, influence subunit localization (Bogdanov et al., 2000; Wittemann et al., 2000) and are targets for posttranslational modification such as phosphorylation (Gerhardstein et al., 1999). One β subunit, β_{2a}, is palmitoylated at its N terminus (Chien et al., 1995), and this modification is required for the slow inactivation characteristic of channels containing β_{2a} (Qin et al., 1998). Experiments with β_1/β_{2a} chimeras reveal that regulation and activation of VGCCs by β subunits occur through a common mechanism, as the modulatory properties associated with the N termini of β_1 and β_{2a} exert their effects in the context of all β subunits tested (Olcese et al., 1994).

The putative binding sites between the α_1 and β subunits are the AID, a cluster of 18 amino acids within the intracellular loop connecting the first and second repeats of the α_1 subunit (I-II loop) (Pragnell et al., 1994), and the BID, about 30 amino acids located within the C2 domain of β subunits (De Waard et al., 1994). How these small regions mediate the binding of α_1 and β subunits in the context of intact proteins has not been elucidated. Interaction with the β subunit masks an ER retention motif in the α_1 subunit that restricts surface expression of the channel (Bichet et al., 2000). However, the BID alone does not mediate the changes of inactivation kinetics that accompany incorporation of β subunits into calcium channels (De Waard et al., 1994; Walker and De Waard, 1998).

Structural modeling of β subunits suggests that C1 shares homology with Src homology 3 domains (SH3), whereas C2 has minor but detectable similarity to guanylate kinase (GK) domains (Hanlon et al., 1999). Despite these homologies for C1 and C2, the functional significance of these domains has not been explored experimentally. The apposition of SH3 and GK domains is characteristic of a large family of membrane-associated guanylate kinases (MAGUKs). Genetic screens have identified several mutations in the SH3 and GK domains of MAGUKs in invertebrates, indicating that these regions are critical for gene function (Hoskins et al., 1996; Woods et al., 1996). SH3 domains typically bind to short

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proline-rich sequences (Mayer, 2001; Mayer and Eck, 1995), and the GK domain shares homology with yeast guanylate kinase. However, in MAGUKs, the SH3 domain is predicted not to bind canonical proline-rich ligands (McGee et al., 2001), and the GK domain is not catalytically active (Olsen and Bredt, 2003). Instead, the GK domain is a protein binding domain in MAGUKs (Sheng and Sala, 2001).

In addition to binding other proteins, the GK domain in MAGUKs binds the SH3 domain (Masuko et al., 1999; McGee and Bredt, 1999; Nix et al., 2000; Shin et al., 2000; Wu et al., 2000). This binding can occur in an intermolecular fashion to form MAGUK oligomers (Nix et al., 2000); however, intramolecular binding between tandem SH3 and GK domains generally predominates. This binding is atypical for an SH3 domain interaction, as the GK domain lacks a polyproline sequence that normally binds to SH3 domains (Lim and Richards, 1994; McGee and Bredt, 1999). Nevertheless, this interaction is critical for MAGUK function, as all mutations in either the SH3 or GK domains corresponding to lethal alleles of the *Drosophila discs large* (*dlg*) and *C. elegans lin-2* (Hoskins et al., 1996; Woods et al., 1996) disrupt SH3-GK binding.

The crystal structure of the SH3-GK region of the prototypical MAGUK, postsynaptic density 95 (PSD-95), reveals the structural basis for this interaction. Unexpectedly, the SH3 domain comprises β strands that are not contiguous in the primary amino acid sequence. In fact, one of the β strands follows the GK domain (McGee et al., 2001). The β strands compose two subdomains that can assemble in either an intra- or intermolecular fashion to complete the SH3 fold. Thus, the SH3-GK interaction does not involve the binding between two independently folded domains but rather is the assembly of an SH3 fold from separable structural components. Although this "split" SH3 fold is conserved among MAGUKs, the function of this motif has not been determined or identified in any other proteins.

Here, we demonstrate that the C1 and C2 regions of the $Ca_V\beta_{2a}$ interact and assemble much like the SH3-GK module of MAGUKs. Mutations that disrupt the assembly of the SH3 fold (the SH3-GK interaction) in β_{2a} interfere with the modulation of the VGCC by this β subunit. Furthermore, a functional β subunit requires intramolecular or intermolecular SH3-GK assembly. The SH3-GK module therefore appears to transduce much of the regulation of VGCC activity by β subunits. Given the structural homology between MAGUKs and β subunits, we propose that the SH3-GK module in MAGUKs performs similar functions to regulate properties of associated channels.

Results

Identification of a Split SH3 Fold in Calcium Channel β Subunits

The split SH3 fold in MAGUK PSD-95 includes a core of four β strands (A–D), a fifth β strand (βE) that follows the "hinge" ("HOOK") region, and a sixth β strand (βF′) that follows the GK domain (Figure 1A). To identify whether these features might be shared with $Ca_V\beta$ subunits (Hanlon et al., 1999; Hanlon and Wallace, 2002), we aligned β_{1b} and β_{2a} with the SH3-GK module of PSD-95 (Figure 1B). The SH3 core (βA–βD) appears to be wholly contained within the C1 domain of $Ca_V\beta$ subunits. We then examined this alignment to ascertain if, like PSD-95, the fifth β strand (βE) of the SH3 fold might be displaced toward the C terminus relative to its location in canonical SH3 domains. Our search was guided by the signature characteristic of βE sequences in MAGUKs: conserved tyrosine and valine residues that are separated by an acidic amino acid and an additional residue, conforming to the consensus sequence Y-D/E-X-V. The C2 domain of $Ca_V\beta$ subunits contains the sequence Y-D-V-V immediately N-terminal to the predicted first β strand of the GK-like domain, suggesting that this region may contain βE.

The SH3-GK interaction in PSD-95 requires a pair of β strands, βE and βF′, that flank the GK domain (Figure 1A) (McGee et al., 2001), so we sought the βF′ strand in VGCC β subunits. The alignment of PSD-95 and the VGCC β subunits does not pinpoint the location of a βF′ strand in the $Ca_V\beta$ subunits, as amino acid residues conserved among MAGUKs, W-V-P, are not present within C2. However, one study of the β_4 subunit demonstrates that constructs containing only the first through second conserved domains (C1V2C2) are functionally indistinguishable from an intact β_4 subunit (Wittemann et al., 2000). Because the βF′ strand in PSD-95 is located C-terminal to the guanylate kinase domain, we predicted that the βF′ strand occurs near the end of the C2 domain (Figure 1B). To emphasize the conserved domain structure between PSD-95 and $Ca_V\beta$ subunits predicted by alignment, we subsequently refer to the β subunit C1 domain as β-SH3 and the β subunit C2 domain as β-GK.

Identification of an SH3-GK Interaction in a Calcium Channel β Subunit

To explore for possible interaction between β-SH3 and β-GK regions, we bacterially expressed and purified these polypeptides. By gel filtration chromatography (Figure 2A), β-GK eluted in a single peak of ~50 kDa, consistent with it being monomeric. β-SH3 eluted in peaks of ~25 and 50 kDa, suggesting some dimerization of the isolated β-SH3 domain. Importantly, when β-GK and β-SH3 were mixed together, they eluted from the gel filtration column in a single peak of ~75 kDa, which indicates stoichiometric heterodimerization of these purified proteins. A recent study (Opatowsky et al., 2003) also found that β subunits comprise a core of two interacting domains that correspond closely to the SH3 and GK modules we identify here.

To better define the molecular requirements for interaction between the β-SH3 and β-GK regions, we used the yeast two-hybrid assay. Much like the binding of the SH3 to the GK regions of PSD-95 (Figure 2B), we found that the β-SH3 region of β_{2a} binds to the β-GK region (Figure 2B). This interaction requires only amino acids composing the β-GK domain, as both a short construct (β-GK) and a longer construct (β-GKV3) bind β-SH3.

The intramolecular assembly of the SH3 fold in MAGUKs occludes competing intermolecular SH3-GK interactions (McGee and Bredt, 1999; Nix et al., 2000; Shin et al., 2000; Wu et al., 2000). Similarly, a β_{2a} construct containing both the β-SH3 and β-GK regions (β2a

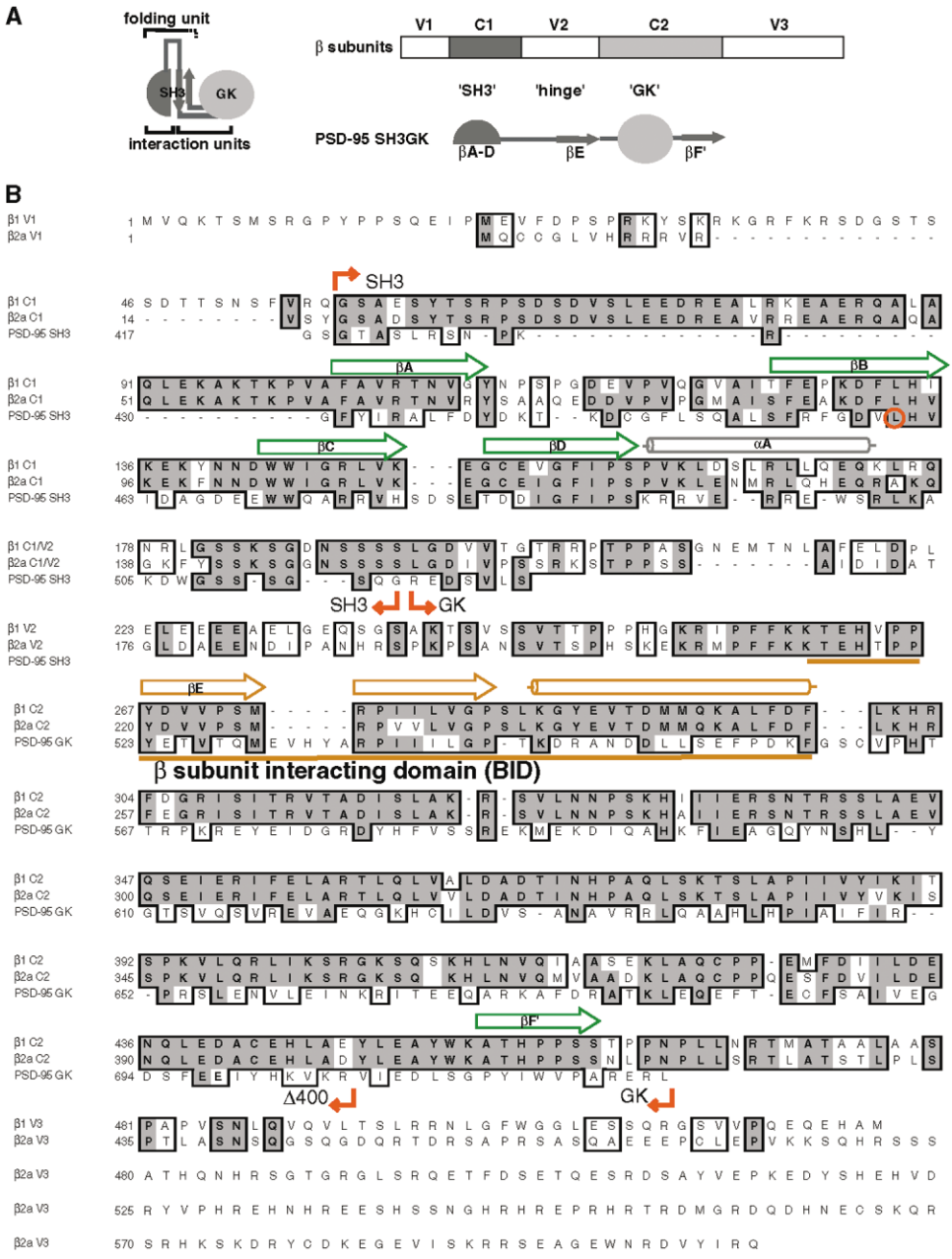

Figure 1. Comparison of Calcium Channel β Subunits with the SH3-GK Module of PSD-95

(A) The diagram at top schematizes the structure of the SH3-GK module of PSD-95. The folding unit comprises a core of four β strands (βA–βD), a fifth β strand (βE) that follows the hinge region, and the sixth β strand (βF′) that follows the GK domain. As indicated, the SH3 core interacts with the GK domain flanked by both β strands. Our predicted placement of SH3-GK elements within β subunits is shown.

(B) The conserved regions of the family of calcium channel β subunits C1 and C2 are similar in sequence to the SH3 and GK domains of MAGUKs, respectively. The calcium channel subunits β_{1b} and β_{2a} were aligned with the SH3 and the GK fragments of PSD-95. Amino acid residues that contribute to the β strands of the SH3 domain are conserved within β subunits. The labeled arrows above the associated sequences represent the position of the β strands in PSD-95. The βE strand as well as the first β strand and α helix (no labels) of the GK domain are components of the β interaction domain (BID) and are colored brown. The red circle highlights the leucine residue analogous to the one mutated to proline in the *dlg*^{m30} mutant. The brown line indicates the conserved region of the β subunits (BID) that interacts with the α subunit's AID region. The red arrows demarcate the boundaries of the SH3, GK, and SH3GKΔ400 constructs used for the binding assays.

SH3V2GK) does not interact with either a β-SH3 or β-GK construct (Figure 2C). The intramolecular assembly of the PSD-95 SH3 fold can be disrupted by mutating a leucine in the βB strand to proline (L460P in PSD-95) or by truncating the GK domain to remove the βF′ strand. These mutations of the PSD-95 SH3-GK module permit their interaction with isolated SH3 or GK regions, respectively (Figure 2A). Furthermore, SH3-GK modules containing reciprocal mutations interact (McGee and Bredt, 1999). Similarly, truncating β-GK at amino acid 400 facili-

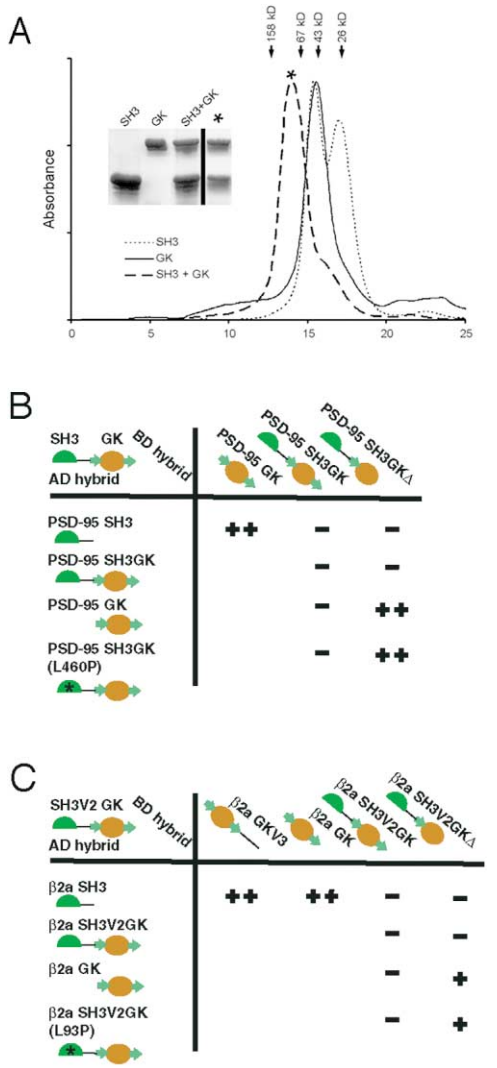

Figure 2. Identification of an SH3-GK Interaction in VGCC β_{2a}

(A) The β-SH3 (amino acids 18–137) and β-GK domains (amino acids 203–411) of VGCC β_{2a} were expressed in bacteria and purified to homogeneity. On gel filtration chromatography, β-GK eluted in a single peak corresponding to ~50 kDa, whereas β-SH3 eluted in two peaks of ~25 and ~50 kDa. An equimolar mixture of SH3 and GK eluted as a single peak of ~75 kDa, indicating formation of a stoichiometric heterodimer. The inset shows a Coomassie-stained gel with the inputs for each of the gel filtration experiments. The lane marked with an asterisk (*) shows the fraction collected from the peak (also marked with an asterisk) of the gel filtration experiment performed with the mixture of SH3 and GK.

(B) The binding of fragments of PSD-95 and VGCC β_{2a} was also analyzed by a yeast two-hybrid assay. As previously reported, the SH3 (amino acids 417–523) and GK (amino acids 523−724) domains of PSD-95 interact robustly, whereas intact PSD-95 SH3-GK (amino acids 417–724) does not bind to either the SH3 or GK fragments or to another intact SH3-GK protein. Truncation of the last β strand of the PSD-95 SH3 domain (amino acids 712–724; SH3GKΔ) from the SH3-GK construct promotes intermolecular interactions with a GK domain flanked by the pair of β strands and with an SH3-GK protein in which the SH3 domain has been mutated (L460P).

(C) Similarly, protein fragments from the β_{2a}-SH3 (C1) (amino acids 16–151) and β_{2a}-GK (C2) (amino acids 151–410) bind to one another. Likewise, the β_{2a}-SH3GK fragment (C1V2C2) (amino acids 16–410) does not bind to either the β_{2a}-SH3 or β_{2a}-GK protein fragment or to another β_{2a}-SH3GK. However, in a manner similar to that observed with PSD-95, truncating ten residues from the GK-like region of C2 (amino acids 400–410; SH3GKΔ) promotes interactions with both the GK fragment and an SH3-GK construct containing the critical SH3 domain point mutation (L93P).

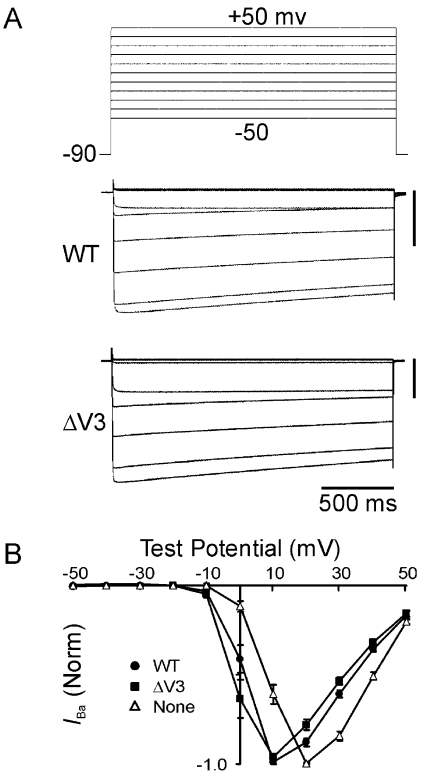

Figure 3. Currents from Channels in Oocytes Expressing α_{1A} and $\alpha_2\delta$ Together with Either β_{2a} or β_{2a} ΔV3

(A) Representative scaled current traces demonstrate that deletion of the V3 domain has little effect upon β subunit-induced inactivation of I_{Ba}. Current scale bar, 1 μA. Shown above are representative voltage recordings that indicate the voltage protocol used.

(B) Normalized current-voltage relationships (mean ± SEM) from $V_h = -90$ mV of wt (n = 8) and β_{2a} ΔV3 (n = 8) show that the β_{2a} ΔV3 activates at similar voltages, as does wt. The I-V relationship for α_{1A} expressed alone (n = 18) is shown for comparison.

tates the binding of β-SH3V2GKΔ to an isolated β-GK construct or a construct containing the β-SH3 point mutation β-SH3(L93P)V2GK but not to β-SH3V2GK (Figure 2B). Thus, the conserved domains of VGCC β subunits display the same binding characteristics as the SH3-GK module of MAGUKs and likely also have a split SH3 fold.

Regulation of Calcium Channel Function by the β Subunit SH3-GK Module

To evaluate the contribution of the β-SH3GK module to VGCC function, Ca^{2+} channel currents were examined in *Xenopus* oocytes expressing either wild-type (wt) or mutant β_{2a} subunits together with the pore-forming α_{1A} subunit (α_1 2.1) and $\alpha_2\delta$. $Ca_V\beta$ subunits promote the surface expression of the calcium channel (Bichet et al., 2000), induce hyperpolarization of the I-V relationships, and modulate inactivation kinetics (Walker and De Waard, 1998). Since the β_{2a} subunit imparts particularly

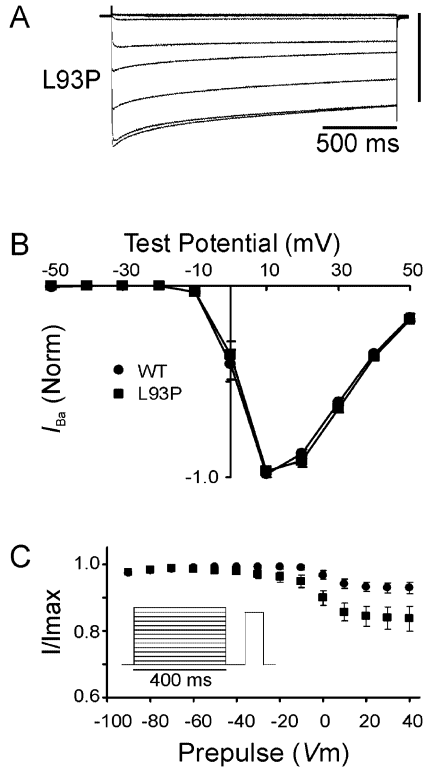

Figure 4. Functional Effects of a Point Mutation in β_{2a}(L93P) that Disrupts the β-SH3 GK Fold

(A) Representative scaled current traces of channels expressing L93P β_{2a} subunit show accelerated inactivation. Current scale bar, 1 μA.

(B) Normalized I-V relationships (mean ± SEM) for wt (n = 11) and L93P (n = 9).

(C) Plot of I/I_{max} for wt (n = 7) and L93P (n = 5) from a +30 mV test pulse from V_h = −90 mV after a 400 ms prepulse to the indicated potentials and a 50 ms interpulse return to V_h (protocol shown in inset). The L93P mutant shows abnormal inactivation. Data are mean ± SEM.

Table 1. Kinetics of I_{Ba} Inactivation at +10 mV

β Subunit(s)	τ_1 (ms)	τ_2 (ms)	n
None	147 ± 4.58	585 ± 42.3	8
β_{2a} wt	>2000	–	10
β_{2a} ΔV3	>2000	–	5
β_{2a} L93P	155 ± 7.08	≥1890 ± 231	9
β_{2a} ΔF′	134 ± 11.0	778 ± 26.5	9
β_1 wt	173 ± 24.5	505 ± 36.4	8
β_1 L134P	125 ± 21.4	462 ± 37.6	4
β_1 ΔF′	128 ± 12.7	664 ± 35.5	5
β_{2a} L93P + β_{2a} ΔF′	>2000	–	6
β_{2a} L93P + β_1 ΔF′	118 ± 4.33	696 ± 47.4	10
β_1 L134P + β_{2a} ΔF′	>2000	–	8

During a 2000 ms test pulse to +10 mV (+20 mV for ΔF′ constructs and "None"), inactivation time constants were estimated by fitting the inactivating component of the current trace to the following equation: $I = I_o + I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2)$. I_o is the residual current amplitude at equilibrium. I_1 and I_2 are the amplitudes of the current components. I_{Ba} of wt, β_{2a} ΔV3, β_{2a} L93P + β_{2a} ΔF′, and β_1 L134P + β_{2a} ΔF′ were best fit by a single-exponential equation. Values are means ± SEM. n, number of tested oocytes.

slow inactivation kinetics, this feature is an excellent candidate for study. The β_{2a} subunit also stands apart in having a particularly large V3 region, so we first assessed whether this V3 region contributed to the β modulatory effects. We found that a β_{2a} construct containing the V1SH3V2GK regions but lacking the V3 region (ΔV3) still produced a hyperpolarizing shift in the I-V relationship and modulation of inactivation kinetics of $Ca_V2.1$ currents that were similar to the intact β_{2a} subunit (Figure 3). This result allowed us to focus our attention on the β-SH3GK module.

We designed experiments to test whether mutations that disrupt the β-SH3GK interaction influence the modulatory effects of β subunits on Ca^{2+} channel currents. Genetic studies in invertebrates indicate that the SH3-GK module is a critical component of MAGUKs, as a leucine to proline substitution in the SH3 (allele m30 in *dlg*) and various truncations of the GK domain are lethal mutations (Woods et al., 1996), but how the SH3-GK interactions function within this module has not been determined. Therefore, analogous mutations within the β subunits might offer a first glimpse into uncovering functional implications of SH3-GK interactions. Currents from calcium channels in which β_{2a} contained the point mutation L93P, analogous to the m30 allele in *dlg*, displayed abnormal inactivation kinetics when compared to wt (Figure 4A). This effect upon inactivation cannot be attributed to a defect in the mutant β subunit's ability to chaperone the α_1 subunit to the membrane or to an effect upon the mutant's binding to α_1, as currents from calcium channels in which β_{2a} contained the point mutation L93P were of similar amplitude to wt, and the L93P mutation did not affect the ability of a purified β_{2a} protein to interact with the I-II loop (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/42/1/89/DC1). Although the I-V relationship of the L93P mutant is identical to wt (Figure 4B), the decay phase of currents from channels containing this mutant was best fit with two exponentials rather than the single exponential required for ΔV3 or wt (see Table 1). The two decay phases in the mutant reflect an initial faster component of inactivation not seen with wt. To assess this further, we utilized a two-pulse protocol in which the oocytes were first prepulsed to a range of potentials for 400 ms to permit the fast component of inactivation to develop without triggering the slow component and, after a brief interpulse interval, depolarized to a test potential of +30 mV. Shown in Figure 4C is a plot of I/I_{max} of the test pulse versus the prepulse potential. Prepulse potentials more positive than −50 mV induced more inactivation in the mutant than in the wt. Thus, in the context of β_{2a}, the β-SH3 fold is required to mask or inhibit the faster inactivation component.

We then examined currents from calcium channels containing a truncated β_{2a} ΔV3 subunit that also lacks the presumed βF′ strand (ΔF′), a mutation that also disrupts SH3-GK interactions (Figure 5). These currents differ from wt currents in several measures: inactivation was accelerated, peak current amplitude was dramatically reduced, and the I-V relationship was shifted ~10 mV in the depolarizing direction. The acceleration in inactivation for currents from the ΔF′ mutant was even more pronounced than in currents from the L93P mutation.

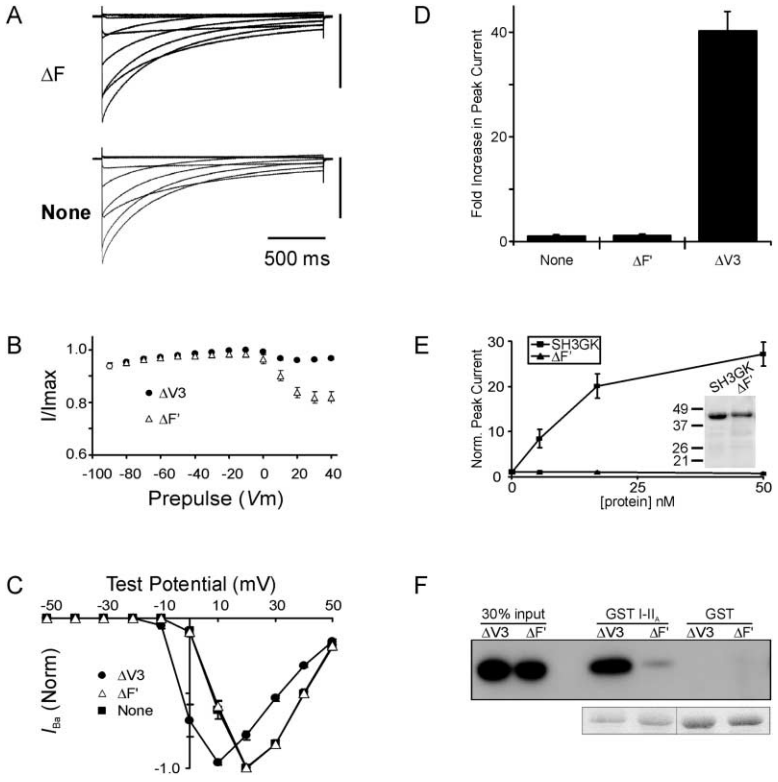

Figure 5. Functional Effects of the ΔF′ Deletion

(A) Scaled current traces show that currents from channels with the β_{2a} ΔF′ subunit resemble currents from channels without a β subunit. Current scale bar, 1 μA.

(B) Two-pulse protocol (as in Figure 4) shows abnormal inactivation of channels coexpressed with β_{2a}ΔF′.

(C) The normalized current-voltage relationships (mean ± SEM) demonstrate that channels with the β_{2a} ΔF′ (n = 18) subunit, like channels without a β subunit (n = 8), fail to show the hyperpolarizing shift seen with an intact β-SH3GK module (data for β_{2a}ΔV3 repeated from Figure 3 for comparison purposes).

(D) Bar graph showing normalized currents (mean ± SEM) from oocytes expressing either β_{2a}ΔV3, β_{2a}ΔF′, or no β subunit (n = 5, 6, and 6, respectively). The β_{2a}ΔF′ subunit fails to induce the increased channel surface expression seen with wt. All currents were recorded on the same day and from oocytes harvested from a single frog.

(E) Normalized peak current amplitudes of oocytes injected with α_{1A} mRNA and β_{2a} SH3-GK or β_{2a} SH3GKΔF′ (ΔF′) protein. The inset shows an SDS-PAGE analysis of the purified ΔV3 and ΔF′ proteins used for injection.

(F) GST pull-down experiment showing that the 6xHis-SH3GKΔF′ protein fails to interact with the α_{1A} I-II loop. Shown is an immunoblot-probed with an anti-6xHis antibody. Ponceau staining of the membrane before immunoblotting confirmed equal loading of all GST fusion proteins (below).

This is shown by the parameters in Table 1 and by the plot of I/I_{max} from the two-pulse protocol in Figure 5B. These currents resemble those from calcium channel α_1 subunits expressed in the absence of β subunits (Perez-Reyes et al., 1989). Indeed, currents from oocytes expressing only α_1 2.1 and $\alpha_2\delta$ show accelerated inactivation, an almost identical reduction in peak current amplitude, and a rightward shift in the I-V relationship (Figures 5A–5D). To confirm that our electrophysiological results with β_{2a} ΔF′ were not due to lack of its expression, we injected the β_{2a} SH3-GK or β_{2a} SH3GKΔF′ protein together with mRNA for the α_{1A} subunit and recorded the resultant currents. Whereas the β_{2a} SH3-GK protein increased current amplitude in a dose-dependent fashion, the β_{2a} SH3GKΔF′ protein had no effect (Figure 5E). To ensure that the ΔV3-induced shift was independent of I_{Ba} amplitude, we performed additional experiments in which peak currents from α_{1A} + ΔV3 (−0.54 ± 0.10 μA, n = 6) were comparable to the peak currents from α_{1A} alone or α_{1A} + β_{2a} ΔF′ (the peak current amplitudes for α_{1A} alone and α_{1A} + β_{2a} ΔF′ in Figure 5C were −0.46 ± 0.18 μA and −0.52 ± 0.10 μA, respectively) and observed a similar shift. Under these conditions, the $V_{1/2}$ for activation for α_{1A} + ΔV3 was 2.7 ± 0.42 mV, similar to the 1.6 ± 0.69 mV $V_{1/2}$ for activation of the set with higher peak current amplitudes shown in Figure 5C. Thus, the shift in the I-V relationship shown in Figure 5C cannot derive from the differences in the level of expression or I_{Ba} amplitude.

This lack of modulatory effect by β_{2a} ΔF′ suggested that it may not interact with the α_1 subunit. To address this, we performed in vitro binding between the I-II loop of α_{1A} and either β_{2a} SH3-GK or a truncated β_{2a} SH3GKΔF′. Whereas the GST-I-II loop bound robustly to β_{2a} SH3-GK, it bound minimally to β_{2a} SH3GKΔF′ (Figure 5F).

Intermolecular Assembly of Functional Calcium Channel β Subunits

To determine whether reciprocally mutant β subunits can reconstitute a functional β-SH3GK module from separate polypeptides, we coexpressed α_1 2.1 and $\alpha_2\delta$ with both the L93P and the ΔF′ mutant β_{2a} subunits. Indeed, current inactivation in oocytes expressing both mutant β_{2a} subunits together was similar to that in channels containing the intact subunit (Figures 6A and 6B). The decay phase of currents was best fit with a single exponential, and the I/I_{max} from the two-pulse protocol was indistinguishable from wt (Table 1).

To test whether heteromeric β subunits can be reconstituted functionally from different β subunits, we engineered β_1 constructs containing either the appropriate L-to-P mutation in the β-SH3 domain β_1(L134P) or the ΔF′ truncation (β_1 ΔF′). When β_1(L134P) was coexpressed with α_1 2.1 and $\alpha_2\delta$, we observed rapidly inactivating calcium channel currents (Figure 6C). However, reconstitution of an intact SH3-GK fold by addition of β_{2a} ΔF′ to β_1(L134P) yielded currents with inactivation

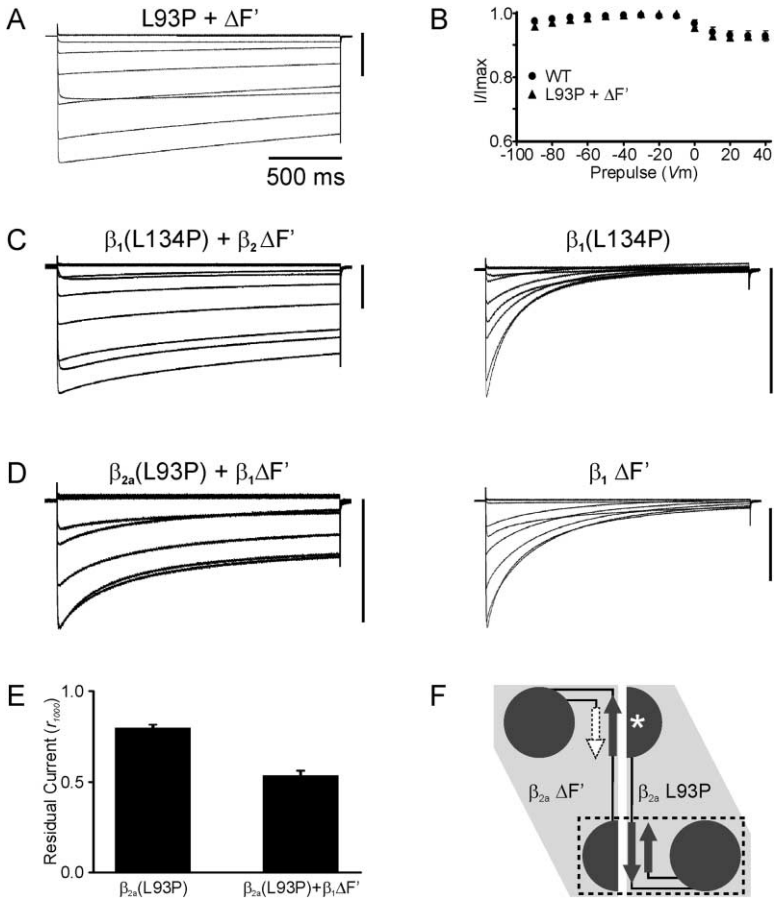

Figure 6. Reconstitution of Calcium Channel Function with Reciprocally Mutant β Subunits

(A) Current traces from channels coexpressing both β_{2a}(L93P) and β_{2a} ΔF′. Current scale bar, 1 μA throughout the figure.

(B) Two-pulse protocol (as in Figure 4) shows that coexpression of β_{2a}(L93P) with β_{2a}ΔF′ restores inactivation to resemble that of wild-type (wt).

(C) Scaled current traces from channels coexpressing β_1(L134P) and β_{2a}ΔF′ or β_1(L134P) alone.

(D) Scaled current traces from channels coexpressed with β_{2a}(L93P) and β_1ΔF′ or β_1ΔF′ alone.

(E) Analysis of residual current at 1000 ms shows that the current from channels coexpressed with β_{2a}(L93P) and β_1ΔF′ inactivates more rapidly than does the current from channels coexpressed with β_{2a}(L93P) alone.

(F) Schematic of the intermolecular assembly between β_{2a}(L93P) with β_{2a}ΔF′ to form a functional wt β subunit (in box). The asterisk (*) represents the L93P mutation.

kinetics similar to those produced by intact β_{2a} (Figure 6C). The decay phase of currents with this reconstitution is slower than the decay phase with either of the two mutants expressed singly (compare Figures 5A and 6C). This second observation confirms that the currents cannot derive from an average of two distinct populations of calcium channels but rather suggests assembly of a functional β subunit from the two mutant proteins.

We next performed the complementary reconstitution by coexpressing β_{2a} (L93P) with β_1 ΔF′. Currents from channels expressing only β_1 ΔF′ displayed all the hallmarks of currents from calcium channel α_1 subunits expressed in the absence of β subunits (Figure 6D, see Supplemental Figures S2E and S3C at http://www.neuron.org/cgi/content/full/42/1/89/DC1). However, coexpression of β_{2a} (L93P) with β_1 ΔF′ reconstituted β subunit function. Currents from channels coexpressing β_1 ΔF′ with β_{2a} (L93P) inactivated much more rapidly and to a greater extent than did currents from channels expressing β_{2a} (L93P) alone (compare Figures 4A and 6D). The smaller residual current at 1000 ms (r_{1000}) shown in Figure 6E quantifies this augmented inactivation.

The stimulation of peak current amplitude, compared to α_1 2.1 expressed in the absence of a β subunit, was tested for each individual, and each combination of β subunits was tested in these reconstitution experiments (see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/42/1/89/DC1). For β_1 ΔF′, there was no increase (see Supplemental Figure S2E), consistent with the result for β_{2a} ΔF′ and the interpretation that the ΔF′ mutation renders β subunits unable to interact with α_1. Expression of the β_1(L134P) stimulated an increase in peak current similar to the β_{2a}(L93P), as did each of the rescue combinations (see Supplemental Figure S2).

Discussion

This study utilized the crystal structure of the SH3-GK module from the MAGUK PSD-95 to examine the structure/function relationship of the distantly related VGCC β subunits. Although the regulatory functions of β subunits are well-described (Walker and De Waard, 1998), the mechanisms by which the β subunits modulate VGCC activity have not been determined. Our study suggests that β subunits, like MAGUKs, contain a split SH3 fold that can assemble from subdomains composed of the C1 (β-SH3) and C2 (β-GK) regions in either an intra- or intermolecular fashion. An empiric assessment of the biochemical properties of β subunit domains supports our hypothesis that the C1 and C2 assemble in an intramolecular fashion to form the β subunit core, although neither the presence of the split SH3 fold nor its role in forming this interaction was discussed (Opatowsky et al., 2003). Analysis of the calcium currents associated with mutant β_{2a} subunits reveals that this fold is required for the channel inactivation characteristics of β subunits.

Our data predict that the SH3 domain from β subunits

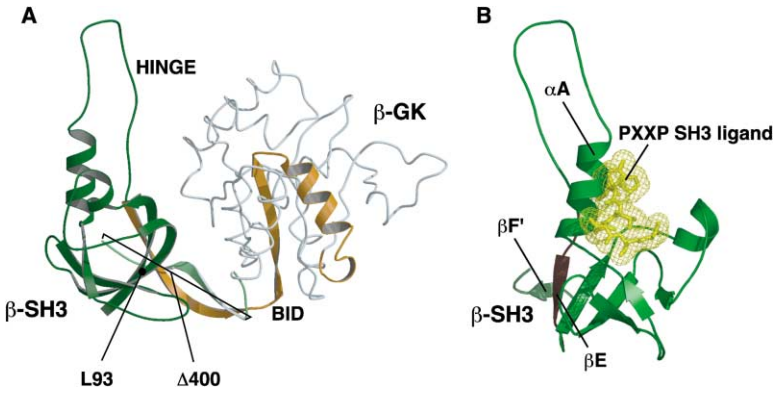

Figure 7. Model of the SH3-GK Module of Calcium Channel β Subunits

(A) A schematic illustration of the β subunit SH3-GK module is based on the structure of PSD-95 and the functional studies described here for β subunits. The BID (brown) comprises structural components of both the β-SH3 (green) and β-GK. The BID extends from the fifth β strand of the SH3 fold through the first α helix of the β-GK. The remainder of the β-GK shares less homology with other MAGUKs and is shown in gray. Truncation of β_{2a} at amino acid 400 promotes intermolecular assembly of the SH3 fold, suggesting that this region (amino acids 400–410) reflects the final β strand of the SH3 fold. The position of the critical leucine residue (L93) within βB is also shown.

(B) The binding site in canonical SH3 domains for polyproline ligands is likely occluded by the αA helix present within the hinge region of the SH3 fold of β subunits. A space-filling representation (yellow wire) of a polyproline peptide ligand is superimposed onto the canonical binding surface on β-SH3 (green). This ligand overlaps in space with the α carbons of residues within helix αA. The positions of βE (brown) and βF′ (light green) are shown relative to the core βA–βD (green).

comprises a core of four β strands, followed by an α helix and a pair of β strands that flank the GK domain. Figure 7 presents a model of this structure, which includes several important features. First, a large helix in the β subunit SH3 domain is predicted to occlude the hydrophobic surface where polyproline ligands interact with canonical SH3 domains (Figure 7B). This implies that the β subunit SH3 domain cannot mediate such polyproline interactions or that these ligands displace the α helix, potentially altering the conformation of the SH3 fold. Instead, our data (Figures 4 and 6) demonstrate that the SH3 fold modulates channel inactivation.

Also unexpected is the location of the BID, an ~35 amino acid segment which supposedly can interact with α_1 subunits and impart some features of β subunit modulation on channel activity (De Waard et al., 1994). Previous studies suggested that β-GK wholly contains the BID (Hanlon et al., 1999); however, our model indicates that the BID includes elements from both β-SH3 and β-GK (Figure 7A). This may explain why we find that a β_{2a} ΔF′ construct, which leaves the BID intact, does not modulate channel activity and does not even bind to the α_1 subunit. Our model suggests that the βF′ strand orients the βE strand within the BID for interaction with the AID, and this likely explains why SH3GKΔF′ does not interact with the channel.

We find that β subunits can be reconstituted functionally through an intermolecular assembly. This intermolecular assembly of a β subunit is reminiscent of three-dimensional strand swapping that generates a functional protein (Liu and Eisenberg, 2002). Multimerization of PSD-95 and other MAGUKs has been proposed to occur through a regulated intermolecular interaction controlled by putative factors that bind to the hinge region, or HOOK domain, situated between the two subdomains of the SH3 fold (Hough et al., 1997; McGee et al., 2001). This hinge region is occupied by V2 in VGCC β subunits and is a site of alternative splicing (Colecraft et al., 2002). Similar to the PSD-95 hinge, this region in β subunits is predicted to start with an α helix (αA in Figure 1B) and contains several evenly spaced charged residues followed by a glycine/serine-rich stretch. Interestingly, the BID immediately follows the predicted hinge and overlaps with the fifth β strand of the SH3 fold, βE, and the first β strand of the GK domain (Figure 7A). These two β strands are more closely apposed in VGCC β subunits than MAGUKs, which may affect their relative orientation. Whether interaction with the α_1 subunit influences the stability of the SH3 fold and promotes intermolecular β subunit assembly, analogous to a model proposed for MAGUKs, has not been addressed.

We find that β subunits can be reconstituted from two distinct isoforms. Coexpression of β_1 and β_{2a} constructs with complementary mutations in the SH3 and GK domains functionally restores channels whose properties differ from those derived from coexpression with either subunit alone. Interestingly, the inactivation properties of these reconstituted channels reflect those associated with the intact SH3 core. Thus, channel inactivation with the β_1(L134P) and β_{2a}ΔF′ reconstitution is slow, resembling that of intact β_{2a}, whereas channel inactivation with β_{2a}(L93P) + β_1ΔF′ is fast, resembling that of intact β_1. Previous work indicated that the palmitoylated N terminus of $\beta 2_a$ controls inactivation (De Waard et al., 1994; Qin et al., 1998; Restituito et al., 2000), but our work indicates that the β-SH3-GK module transduces this modulation.

All MAGUKs contain at least one PDZ domain in addition to the SH3-GK module (Sheng and Sala, 2001). The VGCC β subunits are the only proteins recognized to contain a split SH3 fold that do not contain a PDZ domain. One model of β_{1b} suggests that V1 may contain a PDZ-like motif (Hanlon et al., 1999), but this variable region is only large enough in the longest isoforms of a few β subunits to accommodate a PDZ domain. Thus, a PDZ domain is not a general feature of the β subunit family. However, the homology between β subunits precedes the initiation of the SH3 fold by more than 40 amino acids, so this region may contain other critical structural elements. Although the structures for the first and third variable regions (V1 and V3, respectively) are unknown, some work suggests that these regions mediate subcellular trafficking (Bogdanov et al., 2000). An acidic motif unique to the V3 region of β_{1b} is responsible

for plasma membrane association of the subunit. Furthermore, the V1 and V3 regions of β_4 appear to mediate synaptic localization of the β subunit in transfected hippocampal neurons (Wittemann et al., 2000).

Experiments in this study and elsewhere (Wittemann et al., 2000) suggest that the C1V2C2 (here named the β-SH3GK) region is the functional core of the β subunits. The sequence homology of β-GK to the GK domain from MAGUKs is limited. In PSD-95, the GK domain lends structural stability to the SH3 fold but makes few direct contacts with any of the β strands (McGee et al., 2001; Tavares et al., 2001). Furthermore, the GK domain of PSD-95 interacts with a specific set of proteins of the neuronal cytoskeleton, including guanylate kinase-associated protein (Kim et al., 1997) and the microtubule-associated protein MAP1A (Brenman et al., 1998). Although β-SH3 and β-GK share binding characteristics with the SH3 and GK regions of MAGUKs, the β-GK may otherwise be structurally and functionally distinct.

By examining the function of VGCC β subunits with a perspective provided by the structural and biochemical characteristics of the SH3-GK module of PSD-95, this study demonstrates that the altered inactivation associated with the VGCC β_{2a} subunit requires the structural elements shared with MAGUKs. As the SH3-GK module of MAGUK proteins regulates several interacting ion channels, this work should promote an improved understanding of MAGUKs. The core SH3 domain of CASK has been reported to bind the tail of the α_{1B} calcium channel subunit (Maximov et al., 1999). On the other hand, the GK domains of SAP-97 and PSD-95 modulate the activity of associated K^+ channels (Hibino et al., 2000) and NMDA receptors (Yamada et al., 1999), respectively. In light of the slowing of inactivation by β_{2a}-SH3GK, it is especially intriguing that the PSD-95 SH3-GK domain binds the glutamate receptor subunit KA2 and reduces channel desensitization (Garcia et al., 1998). Future studies are needed to determine how these distantly related SH3-GK modules bind and influence ion channels.

Experimental Procedures

Construction of cDNA Plasmids

For yeast two-hybrid experiments, DNA sequences corresponding to the amino acids indicated for rat CACB2A were amplified by PCR with primers containing endonuclease restriction sites. Products were digested with the appropriate enzymes and ligated into pGBKT7 to produce DNA binding domain fusions or into pGADT7 to produce activation domain fusions. For in vitro transcription, PCR products were subcloned into pGEM-HE in a similar fashion. The point mutation L93P was generated by sequential PCR and subcloned as above (McGee and Bredt, 1999). The β_1 (L134P) and β_1 ΔF′ oocyte expression constructs were generated by PCR mutagenesis (Stratagene) from β_{1a} (Pitt et al., 2001).

Yeast Two-Hybrid Analysis

Yeast cotransformation was performed as described in the MatchMaker3 Library protocol (Clontech) with the yeast strain AH109. Binding was analyzed using streaks of yeast colonies transformed with the indicated plasmids on –LHWA and –LW plates. Interactions were scored as robust (++) if the resultant streaks yielded densely packed colonies, positive (+) if many (>20–200) individual colonies were observed, weak (+/−) if very few colonies were observed, and negative (−) if no colonies were present on the –LWHA plate. Control plates (–LW) were used to verify efficient cotransformation of all pairs.

Interactions of Bacterially Expressed Proteins

For bacterial expression of β subunit domains or the α_1 2.1 I-II intracellular loop (I-II_A), DNA sequences encoding the appropriate regions (β-SH3 18–137; β-GK 203–411; and I-II_A 382–477) were amplified with PCR primers containing endonuclease restriction sites. Products were digested with the appropriate enzymes and ligated into pET-28a(+) or pGEX4-T1 for the β subunit domains and I-II_A loop, respectively. The resultant plasmids were transformed into BL-21 bacteria that were grown to an $OD_{600} = 0.6$ before induction with IPTG. Efficient expression of β-GK required coexpression with the I-II_A loop. Bacterial cell lysates were prepared by passage through a French pressure cell. For purification of the 6xHis-tagged β subunit domains, the lysates were centrifuged at 100,000 × g for 90 min, and the supernatants were then applied to Talon metal affinity resin (Clontech). Eluted protein was aliquoted and stored at −20°C in 25% glycerol for further use. The I-II_A GST fusion protein was bound to glutathione Sepharose (Amersham) as described (Pitt et al., 2001). Pull-down assays with the β subunit domains were performed in 150 mM NaCl, 50 mM Tris, and 0.1% Triton X-100 (pH 7.4) as described (Pitt et al., 2001). Retained 6xHis-tagged β subunit was detected by immunoblotting with an anti-6xHis antibody (Covance).

Gel filtration was performed over a Superdex 200 HR 10/30 column on an AKTA FPLC (Amersham Biosciences) in 500 mM NaCl, 20 mM Tris (pH 7.5). The following protein standards (Amersham Biosciences) were used to calibrate the elutions: 158 kDa, aldolase; 67 kDa, albumin; 43 kDa, ovalbumin; and 26 kDa, chymotrypsinogen.

Electrophysiology

The plasmid encoding the rabbit α_{1A} subunit (GenBank accession number: X57477) used for expression in *Xenopus* oocytes was a kind gift of J. Yang (Columbia University). In vitro transcription and microinjection into *Xenopus* oocytes of α_{1A}, $\alpha_2\delta$, and the indicated β subunits were performed essentially as described (Zuhlke et al., 2000), and recordings were performed 2–4 days after injection. For injection of β protein, purified protein was mixed with α_1 2.1 mRNA, and recordings were performed 2–5 days after injection. To quantify current amplitude (Figure 5D), injections were performed into oocytes from the same donor at the same time, and recordings were performed for all constructs in the same session. Before recordings, oocytes were injected with 25–50 nl of 100 mM BAPTA solution (pH 7.4) to minimize contaminating Ca^{2+}-activated Cl^- currents. I_{Ba} recordings were performed as described (Zuhlke et al., 2000) with a standard two-electrode voltage clamp configuration using an oocyte clamp OC-725C amplifier (Warner Instrument Corp.) connected through a Digidata 1322A A/D interface (Axon Instruments) to a personal computer. Ionic currents were filtered at 1 kHz by an integral four-pole Bessel filter, sampled at 10 kHz, and analyzed with Clampfit 8.1. Adequate voltage control was ensured by analyzing the voltage records from each experiment. For the largest barium currents recorded (peak of the I-V for most constructs was ∼+10 mV), the difference between the maximum and minimum voltages recorded during the plateau phase of the 2000 ms steps was less than 0.5 mV, and the time required to achieve 99% of the 100 mV difference in potential (from $V_h = -90$ mV to +10 mV) was less than 1.0 ms. For all other steps, the deviation in V_m and time to reach 99% of V_{cmd} were less.

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