Inactivation of R-type Calcium Channel

**Background:** Membrane anchoring underlies inhibition of voltage-dependent inactivation (VDI) of calcium channels by the β_{2a}-subunit.

**Results:** A polybasic segment of β_{2a} slows VDI without membrane association. This effect is abolished by charge neutralization.

**Conclusion:** VDI inhibition by the β_{2a}-subunit can occur without membrane anchoring by a mechanism that relies on positively charged residues.

**Significance:** A novel mechanism underlying inhibition of VDI in calcium channels is revealed.

Besides opening and closing, high voltage-activated calcium channels transit to a nonconducting inactivated state from which they do not re-open unless the plasma membrane is repolarized. Inactivation is critical for temporal regulation of intracellular calcium signaling and prevention of a deleterious rise in calcium concentration. R-type high voltage-activated channels inactivate fully in a few hundred milliseconds when expressed alone. However, when co-expressed with a particular isoform, β_{2a}, inactivation is partial and develops in several seconds. Palmitoylation of a unique di-cysteine motif at the N terminus anchors β_{2a} to the plasma membrane. The current view is that membrane-anchored β_{2a} immobilizes the channel inactivation machinery and confers slow inactivation phenotype. β-Subunits contain one Src homology 3 and one guanylate kinase domain, flanked by variable regions with unknown structures. Here, we identified a short polybasic segment at the boundary of the guanylate kinase domain that slows down channel inactivation without relocating a palmitoylation-deficient β_{2a} to the plasma membrane. Substitution of the positively charged residues within this segment by alanine abolishes its slow inactivation-conferring phenotype. The linker upstream from the polybasic segment, but not the N- and C-terminal variable regions, masks the effect of this determinant. These results reveal a novel mechanism for inhibiting voltage-dependent inactivation of R-type calcium channels by the β_{2a}-subunit that might involve electrostatic interactions with an unknown target on the channel’s inactivation machinery or its modulatory components. They also suggest that intralinker interactions occlude the action of the polybasic segment and that its functional availability is regulated by the palmitoylated state of the β_{2a}-subunit.

The entry of calcium ions into the cell triggers a multitude of cellular responses that rely on a tight spatio-temporal regulation of the calcium transient spreading within the cell for their coordination (1, 2). High voltage-activated (HVA) calcium channels open in response to large membrane depolarization and constitute the major entry pathway for calcium into excitable cells. The largest component of HVA channels, α_{1}-subunit (Ca_{v}α_{1}), contains the ion conduction pore, the voltage sensor, and multiple intracellular domains that regulate calcium influx and provide interaction sites for regulatory proteins. Two subfamilies of HVA Ca_{v}α_{1} are recognized as follows: Ca_{v}1.x or L-type channels and Ca_{v}2.x, also referred as to neuronal channels (Ca_{v}2.1 encoding P/Q-type; Ca_{v}2.2 encoding N-type; and Ca_{v}2.3 encoding R-type) (3). Following strong depolarization and calcium permeation, HVA channels enter into an inactivated nonconducting state that constrains the amount of calcium influx and protects the cells from the cytotoxic effects of an excessive calcium rise. Inactivation of HVA channels is triggered by both calcium increase and prolonged membrane depolarization, referred as to calcium-dependent inactivation and voltage-dependent inactivation (VDI), respectively (4). Although calcium-dependent inactivation depends on the binding of calmodulin to a conserved site among HVA channels (5), VDI is an intrinsic property of Ca_{v}α_{1}, but it is strongly modulated by the regulatory β-subunit (Ca_{v}β) (6–11) that binds to a site highly conserved among HVA channels, termed the α-interaction domain (12).

Crystallographic studies from three of the four known Ca_{v}β isoforms (Ca_{v}β_{1} to Ca_{v}β_{4}) revealed the molecular aspects of this interaction. Ca_{v}β shares a common structural arrangement with members of the membrane-associated guanylate kinase family (MAGUK), a highly conserved Src homology 3 (SH3) and guanylate kinase (GK) domain flanked and joined by variable segments (13–15). The binding motif in the pore-
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EXPERIMENTAL PROCEDURES

Construction of cDNA and Protein Expression—cDNA encoding all Ca_{v}β_{2a} (Swiss-Prot accession number Q8VG3C-2, described elsewhere (27)) and Ca_{v}β_{2axo} (Swiss-Prot accession number Q91630) derivatives were subcloned by PCR methods into pRSET vector (Invitrogen) to include the N-terminal histidine tag and Xpress epitope tag. The proteins were overexpressed in bacteria and purified by metal-affinity chromatography, followed by size-exclusion chromatography onto a Superdex S-200 column (GE Healthcare), as described previously (27). Proteins were concentrated to 0.7–5.0 mg/ml by ultrafiltration, fast frozen, and stored at −80 °C until use. For confocal fluorescence microscopy, the same cDNAs encoding for the different Ca_{v}β_{2a} derivatives used for protein expression were subcloned into pcDNA 3.1 vector and fused to YFP. All constructs were verified by DNA sequencing. The human form of the Ca_{v}2.3 subunit (Swiss-Prot accession number Q15878) used in this study has been previously described (28, 29).

Western Blot Analysis—For Western blot analysis of β_{2a}-derivative protein constructs, five noninjected control oocytes and five oocytes were injected with purified His-β_{2a} protein constructs 5 days before recording. Oocytes were transferred to a 1.5-ml tube and homogenized by pipetting up and down with 50 μl of lysis buffer (100 mM sodium phosphate, pH 7.2), supplemented with 0.1% Triton X-100 and protease inhibitor mixture (Sigma). After incubation on ice for 15 min (15 s vortexing every 5 min), the samples were centrifuged twice at 12,000 rpm for 10 min at 4 °C. The supernatant was diluted with 5× SDS loading buffer and loaded on a 12% acrylamide gel. After SDS-PAGE, the proteins were electrically transferred to ECL Plex membranes (GE Healthcare) for 1 h. Membranes were blocked using 3% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) and incubated with primary antibody diluted according to the manufacturer’s instructions in TBS supplemented with 1% BSA. Three primary antibodies were used depending on the β-construct to be detected as follows: anti-calcium channel β- subunit antibody (Sigma) when the C terminus was present (β_{2a} C3S,C4S, β_{2a} SH3-PBLK-GK + CT, and β_{2a} APBLK), anti-CACNB2 antibody (Abcam) only when the full linker sequence was present (β_{2a} SH3-LK-GK), and anti-Xpress antibody (Invitrogen) for the rest of the constructs (β_{2a} SH3-PBLK-GK, β_{2a} SH3-PBLK-GK + NT^C3S,C4S, β_{2a} SH3-PBLK_{Ala}-GK, and β_{2a} SH3-GK). Immunodetection was carried out using HRP-conjugated secondary antibody (Pierce Antibodies, Thermo Scientific) diluted 1:40,000 to 1:60,000 and a chemiluminescence detection kit (SuperSignal West Femto Chemiluminescent Substrate, Thermo Scientific). Membranes were exposed between 15 and 60 s into a GeneGnome chemiluminescence imaging system (SYNGENE).

Oocytes Injection and Electrophysiological Recordings—The cRNA encoding for Ca_{v}2.3 α_{1}-subunit was synthesized using a capping RNA transcription kit (mMESSAGE mMACHINE, Thermo Scientific).
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Ambion) and injected into *Xenopus laevis* oocytes, as reported previously (30). To minimize cRNA degradation, the protein was injected 1 h after cRNA injection, using the same method as for cRNA injection. Electrophysiological recordings were performed using the cut-open oocyte technique (31) with a CA-1B amplifier (Dagan Corp., Minneapolis, MN), as described previously (27). Data acquisition and analysis were performed using the pCLAMP system and software (Axon Instruments Inc., Foster City, CA). Linear components were eliminated by P/4 pre-pulse protocol. All experiments were carried out in at least two batches of *X. laevis* oocytes from two different frogs. The external solution contained (in mM), 10 BaCl₂, 96 n-methylglucamine, and 10 HEPES, and the internal solution contained 120 n-methylglucamine, 10 EGTA, and 10 HEPES. Both solutions were adjusted to pH 7.0 with methanesulfonic acid.

Confocal Fluorescence Microscopy—Live cell confocal imaging was carried out with a ×40 oil immersion objective on a Leica inverted confocal microscope equipped with an argon ion laser using transiently transfected tsA201 cells. Cells were cultivated on a glass coverslip and imaged 18–48 h after transfection using Lipofectamine™ (Invitrogen) according to the manufacturer’s instructions. For excitation of YFP, the 514-nm laser was used, and the emitted light was monitored between 520 and 560 nm. β2a-YFP has been described elsewhere (32).

RESULTS

**Co-injection of Full-length β-Protein with α₁-cRNA Fully Reconstitutes β-Subunit Modulation of Activation and Inactivation of CaV2.3 R-type Calcium Channel Expressed in Xenopus Oocytes**—Instead of injecting the cRNA encoding for CaV_{2.3}α₁ and CaV_{2.3}β, i.e. the widespread strategy to study β-modulation of calcium channels, we injected β-derived constructs as purified proteins together with the cRNA encoding the α₁-subunit (27, 29, 30). With this strategy we were able to study isolated domains and short constructs of β_{2a} that are not functional when injected as cRNA (29) but that regulate calcium channel function when injected as purified proteins. To confirm the integrity and folding of the recombinant proteins, all constructs used in this study were subjected to SDS-reducing PAGE and size-exclusion chromatography (supplemental Fig. S3).

Fig. 1 illustrates the strategy followed in this study to investigate the effect of different β-subunit derivatives on VDI of R-type CaV_{2.3} channels. Protein constructs were injected into *Xenopus* oocytes 1 h later than the cRNA encoding the α₁-subunit, so as to avoid RNA degradation. Electrophysiological recordings were performed 5–6 days after cRNA/protein injection, corresponding to the time necessary for the α₁-subunit to be assembled in the plasma membrane (Fig. 1A).

As observed from earlier co-injection experiments of cRNA encoding α₁ and β-subunits, β_{2a} inhibits VDI of CaV_{2.3} channels expressed in *Xenopus* oocytes, and β_{3xo} stimulates it (8, 33–35). This effect is reflected in much greater values of τ_{0.5} for CaV_{2.3}/β_{2a} channel complexes than CaV_{2.3}/β_{3xo} and in steady-state inactivation curves, exhibiting opposing voltage shifts with respect to the CaV_{2.3} α₁-subunit alone (Fig. 1, B and C, and Table 1). See Equation 1.

\[
I(V) = I_{\text{max}} \cdot \left( \frac{l_{\text{res}} + \frac{z^F}{1 + e^{\pi V - V_{\text{m}}}} \cdot \left(1 - l_{\text{res}}\right)}{1 + e^{\pi V - V_{\text{m}}}} \right)
\]

(Eq. 1)

where \(I_{\text{max}}\) is the current at peak; \(l_{\text{res}}\) is the noninactivating current; \(F\) is the Faraday’s constant; \(R\) is the universal constant of gases; \(t\) is temperature (298 K), and \(V_{\text{m}}\) is the membrane voltage. \(V_{\text{m}}\), \(z_1\), and \(z_2\) are the parameters defining each Boltzmann component. \(V_{\text{m}}\) is then obtained by solving numerically \(K(V) = I_{\text{max}}/I_{\text{res}}\) corresponds to the fraction of channels that
do not inactivate at extreme positive voltages. Moreover, by shifting the steady-state inactivation curve to more depolarizing potentials, β2a also induces a residual noninactivating current component that is unique to this isoform (Fig. 1C). In contrast to their differences in the channel inactivation curve, β2a and β3,30 shift the activation curve to the same extent and direction (Fig. 1D and supplemental Table S1). This change in the midpoint of the activation curve toward more hyperpolarizing potentials is the signature of β-subunit modulation of HVA calcium channels (33, 36), and we used it as a parameter to confirm the functional state of the protein construct within the oocytes. We conclude that the effect of β-subunit protein on CaV2.3 channels is undistinguishable from one of the cRNA constructs, consisting of only SH3 and GK domains (β2a SH3-GK expressing oocytes. These results identify the distal polybasic linker segment of CaV2.3 channels and reveal divergences in the modulation of kinetic and steady-state parameters.

### Distal Linker Segment of CaVβ2a

**Comprising the Amino Acid Sequence HSKEKMPFKK** Slows Down VDI of CaV2.3 Channels—Previous results from Qin et al. and Richards et al. (7, 22) suggested that the linker joining SH3 and GK domains of the β-subunit plays a role in the regulation of VDI. However the variability in length and sequence of this region makes it difficult to define further the structural determinant of VDI inhibition. Eye inspection of the amino acid sequence of the linker region of β2a reveals a short polybasic segment at the boundary of the GK domain, comprising residues 202–213, hereby referred to as polybasic linker segment (PBLK, Fig. 2A) that is absent in β3,30. To isolate the potential impact of the polybasic linker segment on VDI regulation, we removed all other variable regions of β2a, including the N and C termini and the segment upstream PBLK that encompasses a long serine-rich sequence (PSLK, Fig. 2A), containing several predicted phosphorylation sites. The result is a construct that consists of SH3 and GK domains joined only by PBLK (β2a SH3-PBLK-GK, Fig. 2B). We found that this construct confers the slow inactivation phenotype, despite lacking the palmitoylatable NT segment that is commonly recognized as responsible for this effect.

To test if indeed the polybasic segment is responsible for this conferring phenotype, we generated a linker-less β-subunit construct, consisting of only SH3 and GK domains (β2a SH3-GK, Fig. 2B) that has been proposed earlier to be the functional unit of the β-subunit (13, 37). In contrast to β2a SH3-PBLK-GK, β2a SH3-GK confers a fast inactivating phenotype to CaV2.3 channel complexes, even faster than the channel alone (Fig. 2C and Table 1). As performed for the full-length proteins (see Fig. 1D), the functional state of the β-derivative constructs 5 days following injection into oocytes was tested by measuring the activation curves of the different CaV2.3 channel complexes. Both β2a SH3-PBLK-GK and β2a SH3-GK produced a left shift in the activation curve of around 10 mV accompanied by an increase in the slope, and relative contribution of the first component of the sum of two Boltzmann distributions that fit the activation curves (supplemental Fig. S1 and supplemental Table S1). We also verified the integrity of the β-derivatives by Western blot analysis (Fig. 2B, inset). Although no signal was detected in lysates from noninjected oocytes, a strong band that migrated at the same position as the purified protein was visible in lysates from injected Xenopus oocytes showing that β-derivatives constructs were stable for at least 5 days within the oocyte (Fig. 2B, inset).

**Although only β2a SH3-PBLK-GK slowed inactivation, both constructs shifted the voltage dependence of VDI toward more negative voltages, compared with CaV2.3 expressed alone.** The voltage for 50% inactivation (V1/2) was shifted by about 8 mV to the left by the presence of β2a SH3-PBLK-GK and 20 mV by β2a SH3-GK (Fig. 2D and Table 1). Nevertheless, as with wild-type β2a, a residual current can still be detected at the end of a 10-s pulse in CaV2.3/β2a SH3-PBLK-GK expressing oocytes. These results identify the distal polybasic linker segment of CaV2β2a as a structural determinant for slowing down inactivation of CaV2.3 channels and reveal divergences in the modulation of kinetic and steady-state parameters.

**Polybasic Linker Segment That Slows Down Inactivation Does Not Confer Membrane Anchoring**—The interaction of membrane-associated proteins with lipid membranes can be driven by electrostatic interactions between positively charged residues and negatively charged lipid membranes (38). The basic nature of PBLK might provide attachment of the β-subunit to the plasma membrane, mimicking the mechanism proposed for palmitoyl-membrane-anchored β2a (17). To investigate the cellular location of β2a SH3-PBLK-GK, we fused it to YFP (β2a SH3-PBLK-GK-YFP), expressed it in mammalian cells, and
Short polybasic linker segment at the boundary of the GK domain slows down voltage-dependent inactivation of CaV2.3 channels.

**A**. A schematic representation of the domain structure of the β-subunit. The highly conserved SH3 and GK domains are flanked by three variable regions, N-terminal (NT), C-terminal (CT), and the linker region joining both domains (LK). Numbers denote amino acid position of the rat β2a isoform. The two cysteine residues at positions 3 and 4 (C46) that undergo palmitoylation in β2a are highlighted. We divided LK into two segments, a large polyserine segment (PSLK), followed by a short polybasic segment (PBLK) at the boundary of GK domain (shown in red). Positively charged residues within PBLK are highlighted in red. **B**. Representative current traces from Xenopus oocytes injected with CaV2.3 encoding cRNA and the indicated β-protein construct following the same protocol as in Fig. 1B. β2a SH3-PBLK-GK consists of SH3 and GK domains joined by a PBLK segment, although in β2a SH3-GK no linker segment is present between both domains. The insets show anti-β antibody Western blot analysis of crude lysate from Xenopus oocytes injected with β2a SH3-PBLK-GK (left panel) or β2a SH3-GK (right panel), as described under “Experimental Procedures”; left lane, crude lysate from five noninjected oocytes; middle lane, crude lysate from five oocytes injected with the corresponding β-construct; and right lane, purified β-construct before injection (80 ng loaded). C. Bar plot of average T50 values for oocytes expressing CaV2.3 plus the indicated β-construct. Values are expressed as means ± S.E. The dashed line corresponds to T50 values for CaV2.3 channels expressed alone. **D**, voltage-dependent inactivation curves from oocytes injected with CaV2.3 encoding cRNA and the indicated β-protein measured and fitted as in Fig. 1C. The curve for CaV2.3 alone is shown by a dotted line.

Positively Charged Amino Acids within the Polybasic Linker Segment Are Required for Slowing Down Inactivation of CaV2.3 Channels—Because the most striking property of this segment is a relatively high density of positively charged residues, we rationalized that they might still be functionally relevant although not for surface membrane association. To test this idea, we substituted all positively charged residues within this segment by alanine (β2a SH3-PBLKAla-GK, see Fig. 4A). This β-derivative remains intact after 5 days of injection into Xenopus oocytes, as judged by Western blot analysis (Fig. 4A) and left shifts of the channel activation curve (supplemental Fig. S1 and supplemental Table S1). Channel complexes bearing β2a SH3-PBLKAla-GK inactivate nearly as fast as the construct lacking this segment (Fig. 4, B and C, and Table 1). These results demonstrate that the basic residues within the PBLK segment are required for conferring a slow inactivation phenotype to nonmembrane-anchored β-subunit and suggest the involvement of electrostatic interactions in VDI modulation.

Linker Region Upstream from the Polybasic Linker Segment Masks Its Effect on VDI—It remains to be explained why the palmitoylated β2a C3S,C4S double mutant (β2a-C3S,C4S) containing the polybasic linker segment confers fast inactivation to CaV2.3 channels (Fig. 5, A and E) (17, 29). This observation implies that other parts of the nonmembrane-anchored β-proteins counteract the effect of the polybasic linker segment. We followed a simple strategy to identify variable segments of the protein that may occlude the effect of the polybasic linker segment. This involved adding to β2a SH3-PBLK-GK to the rest of the variable regions, one at a time (Fig. 5 and see also Fig. 2A). Three new constructs were designed as follows: β2a SH3-PBLK-GK plus NT variable region carrying the double cysteine substitution (β2a SH3-PBLK-GK + NT(3S,C4S)), β2a SH3-PBLK-GK plus CT variable region (β2a SH3-PBLK-GK + CT), and β2a SH3-PBLK-GK lacking NT and CT but carrying the full linker (β2a SH3-LK-GK).

Adding NT or CT to β2a SH3-PBLK-GK did not interfere with the ability of the PBLK segment to confer a slow inactivation phenotype, and in the presence of either construct, the channel voltage dependence of inactivation is closer to the parent protein than to β2a C3S,C4S (Fig. 5, A–C and E and F, and...
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FIGURE 3. β2a SH3-PBLK-GK located within the cytoplasm in mammalian cells. Confocal images of fluorescently labeled β2a (β2a-YFP), β2a SH3-PBLK-GK (β2a SH3-PBLK-GK-YFP), and β2a C3S,C4S (β2a C3S,C4S-YFP). All β2a derivatives were fused to YFP and expressed in tsA201 cells. Only β2a-YFP shows strong membrane localization, and the others are distributed within the cytoplasm.

FIGURE 4. Substitution of the basic residues by alanine within the polybasic linker segment eliminates slow-inactivation conferring phenotype of β2a SH3-PBLK-GK. A, schematic representation of β2a SH3-PBLK-GK showing the amino acid sequence of native PBLK and PBLK with all positively charged residues (shown in red) substituted by alanine (β2a SH3-PBLK-Ala-GK). The inset shows Western blot analysis using anti-β antibody of β2a SH3-PBLK-Ala-GK, as described under “Experimental Procedures”: left lane, crude lysate from five noninjected oocytes; middle lane, crude lysate from five oocytes injected with β2a SH3-PBLK-Ala-GK; and right lane, purified β2a SH3-PBLK-Ala-GK after injection (80 ng loaded). B, representative current traces from oocytes injected with β2a SH3-PBLK-Ala-GK and right lane, purified β2a SH3-PBLK-Ala-GK before injection (80 ng loaded). B, representative current traces from oocytes injected with β2a SH3-PBLK-Ala-GK following the same protocol as in Fig. 1B. For comparison, the current recording for CaV2.3/β2a SH3-PBLK-Ala-GK channel complexes from Fig. 2B is shown in light gray. C, average T50 values for oocytes expressing CαV2.3/β2a SH3-PBLK-Ala-GK channel complexes. The dotted line denotes the average T50 for CaV2.3/β2a SH3-GK channels. T50 decreases from 0.98 ± 0.16 s for CaV2.3/β2a SH3-PBLK-Ala-GK to 0.07 ± 0.01 s for CaV2.3/β2a SH3-PBLK-GK channel complexes. Values are expressed as mean ± S.E. D, voltage-dependent inactivation curves from oocytes injected with CaV2.3 and β2a SH3-PBLK-Ala-GK obtained as in Fig. 1C. For comparison, the steady-state inactivation curve from oocytes expressing CaV2.3/β2a SH3-PBLK-GK shown in Fig. 2D is included (dotted line).

Table 1). In contrast, β-subunit carrying a full-length linker while lacking the N and C termini gives rise to relatively fast inactivating CaV2.3 channels, and the inactivation curve is shifted even further to the left than with β2a C3S,C4S, indicating that the sequence upstream from the polybasic segment occludes its effect on VDI (Fig. 5, D–F, and Table 1).

The idea that a post-translational modification may be involved in occluding a slow inactivation phenotype is appealing because it might explain why a nonmembrane-anchored β-subunit can slow inactivation when acutely applied to already expressed CaV2.3 channels (29). Because the linker region upstream from the polybasic segment contains several predicted phosphorylation sites, there is a possibility that the addition of phosphate groups would hinder potential electrostatic interactions that might, in turn, be responsible for VDI inhibition by the polybasic segment. We substituted all serine and threonine residues by alanine within PSLK in the β2a-C3S,C4S background, and no effect on T50 or the inactivation curve was observed (supplemental Fig. S2 and supplemental Table S1). This indicates that phosphorylation events within the region upstream from the PBLK do not participate in VDI modulation.

Although the molecular mechanism, by which the upstream linker region hinders the effect of the distal polybasic segment, remains elusive, the above results provide a clear explanation why nonmembrane-anchored full-length proteins containing the polybasic segment do not manifest their phenotype.

Palmitoylated β2a Lacking the Polybasic Linker Segment Inhibits Voltage-dependent Inactivation of CaV2.3 Channel Complex to a Lesser Extent than Does the Wild-type Protein—It is well accepted that membrane anchoring by itself suffices to confer slow inactivation to the CaV2.3 subunit. This idea is based on results showing that removal of the N-terminal double cysteine motif in β2a abolishes membrane localization and the slow inactivation-conferring phenotype that is rescued by artificially anchoring β2a C3S,C4S to the membrane (see also Figs. 3 and 5) (17, 19). Within this framework, the functional relevance of PBLK on the membrane-anchored β2a-subunit comes into question. To provide an answer to this, we removed the PBLK from wild-type β2a containing the double cysteine motif (β2a ΔPBLK). This protein was also stable within the oocyte, as judged by Western blot analysis and its effect on the channel’s voltage dependence of activation (Fig. 6A and supplemental Table S1). CaV2.3/β2a ΔPBLK channels inactivated slightly faster than the CaV2.3-β2a channel complex, and when fused to YFP, β2a ΔPBLK still localized at the plasma membrane (Fig. 6, A–C). Thus, dissociation of the membrane does not explain this
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**FIGURE 5.** Polyserine linker region but not the N- or C-terminal variable sequences mask the effect of the polybasic segment on voltage-dependent inactivation. A, schematic representation of $\beta_{2a}$ C3S,C4S double mutant and representative current traces from oocytes expressing CaV2.3/$\beta_{2a}$ C3S,C4S channel complexes following the same pulse protocol as in Fig. 1B. The inset shows Western blot analysis using anti-\beta antibody, as described under “Experimental Procedures”; left lane, crude lysate from five noninjected Xenopus oocytes; middle lane, crude lysate from five oocytes injected with $\beta_{2a}$ C3S,C4S, and right lane, purified $\beta_{2a}$ C3S,C4S before injection (80 ng). B, same as A but using $\beta_{2a}$ SH3-PBLK-GK that includes the N-terminal region of $\beta_{2a}$ C3S,C4S ($\beta_{2a}$, SH3-PBLK-GK + NT<sup>C3S,C4S</sup>). C, same as A but using $\beta_{2a}$ SH3-PBLKGK containing the C-terminal region of $\beta_{2a}$ (SH3-PBLK-GK + CT). D, same as A but the $\beta_{2a}$ construct encompassing the complete linker region but missing the N- and C-terminal regions ($\beta_{2a}$ SH3-LK-GK). E, bar plot of average $T_{0.5}$ values from oocytes expressing CaV2.3 plus the indicated \beta-derivative construct: NT, $\beta_{2a}$ SH3-PBLK-GK + NT<sup>C3S,C4S</sup> (black bar); CT, $\beta_{2a}$ SH3-PBLK-GK + CT (blue bar); LK, $\beta_{2a}$ SH3-LK-GK (red bar); and C3S,C4S, $\beta_{2a}$ SH3-LK-GK (green bar). The dashed line denotes average $T_{0.5}$ for oocytes expressing CaV2.3/$\beta_{2a}$ SH3-PBLK-GK channel complexes. Values are expressed as mean ± S.E. F, voltage-dependent inactivation curves from oocytes expressing the different subunit combinations shown in E. For comparison, the steady-state inactivation curve from oocytes expressing CaV2.3/$\beta_{2a}$ SH3-PBLK-GK shown in Fig. 2D is included (dotted line).

... that regulate inactivation of voltage-gated calcium channels. This added complexity barred a full mechanistic account of voltage-dependent inactivation in CaV2.x channels. A simple ball-and-chain model in which the membrane anchoring of the auxiliary subunit hinders the movement of the ball is rather simplistic and cannot explain why binding of a large nonmembrane-anchored subunit may facilitate the process. Although our present study does not provide a definitive answer to this question, it does however contribute to a better understanding of the modulation of inactivation by the regulatory $\beta$-subunit.

Four conclusions can be drawn from this work as follows: (i) there is an additional structural determinant in $\beta_{2a}$ isoform, the distal polybasic linker segment, besides the N-terminal palmitoylatable region, that contributes to slowing down inactivation; (ii) the polybasic segment inhibits inactivation in a membrane-anchoring independent manner and the basic residues within are required for this effect; (iii) the linker sequence upstream from the polybasic segment masks the contribution of the latter, and (iv) the effect of the palmitoylated N terminus of the...
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\[ \text{FIGURE 7. Model for modulation of voltage-dependent inactivation by} \ \beta_{2a}\text{-subunit. Two molecular determinants of} \ \beta\text{-subunit control voltage-dependent inactivation as follows: palmitoylated N terminus and the polybasic linker segment (shown in "red"). The} \ \beta\text{-subunit binds through the GK domain to the conserved} \alpha\text{-interaction domain in the} \ C_{a}\text{-} \alpha_{1} \text{subunit. According to current models, anchoring of} \ \beta_{2a} \text{to the membrane restricts the movement of the inactivation particle, and based on our present data, it exposes the polybasic segment that further inhibits VDI (left panel). In the absence of a palmitoylated N terminus, the region upstream from the polybasic linker segment occludes its functional association with the inactivation machinery (right panel).} \]

\( \beta\text{-subunit predominates over the PBLK-mediated inhibition, but both act synergistically to inhibit VDI.} \)

Work by others has also shown that altering the length and sequence of the linker impacts VDI (7, 22, 23). Qin et al. (7) were the first to identify the linker region as a secondary determinant of VDI. Richards et al. (22) reported that in the palmitoylation-deficient mutant, removal of the linker speeds inactivation, which is expected for a linker encompassing a slow inactivation conferring sequence, such as PBLK. Stotz and co-workers (23) transferred the linker and GK domain of \( \beta_{3} \) into \( \beta_{4} \) and found that VDI of \( C_{a}\cdot2.2 \) N-type calcium channel is accelerated. They also found that the N terminus of the \( \beta_{3}\text{-subunit confers fast inactivation to} \ C_{a}\cdot2.2 \text{channels and that transferring this region to} \ \beta_{3} \text{is sufficient to accelerate VDI. Interestingly, this 14-amino acid region contains five negatively charged residues but no positively charged ones. Here, we propose that an electrostatic interaction between the polybasic linker and a putative acidic region within either the channel’s inactivation machinery or some of its regulatory determinants slows VDI. An interesting concept is that acceleration of inactivation might involve also electrostatic interactions but of opposite signs, involving a positively charged receptor site in the pore-forming subunit.} \)

We envisage that membrane anchoring not only contributes to immobilize the inactivation particle, as commonly accepted, but also exposes the polybasic distal linker segment that adds an additional component to VDI inhibition through an electrostatic repulsion of a putative inactivation particle (Fig. 7). In the absence of palmitoylation, as the protein is no longer anchored to the plasma membrane, the linker may fold over, occluding the positive charges of the PBLK and masking their functional association with a potential target within \( C_{a}\cdot\alpha_{1} \). Membrane anchoring would give rise to an extended conformation of the linker that exposes the positively charged residues of the PBLK (Fig. 7).

Another explanation is that the polybasic distal linker segment has no direct action on the inactivation machinery but confers a permissive orientation to SH3/GK functional unit. This idea appears unlikely in light of our data, showing that several \( \beta\)-constructs bearing no linker or linker segments that differ in length and sequence composition (\( \beta_{3xo}, \ \beta_{2a}\cdot3H3-GK, \ \beta_{2a}\cdot3H3-LK-GK, \ \beta_{2a}\cdot3H3-PBLK_{\alpha_{1}}-GK \)) confer fast inactivation phenotype. Moreover, crystallographic studies did not reveal any structural differences between the \( \beta\)-subunit alone and complexes to the peptide corresponding to the binding site in the \( \alpha_{1}\)-subunit. Unless the relative orientation of SH3/GK domain is different in vivo, one should conclude that there are no major conformational changes associated with the binding of the \( \beta\)-subunit to the channel and that relative orientation of SH3 and GK is irrelevant to inactivation.

There is, to date, no direct structural data on the variable regions of \( \beta\)-subunit. Here, we show that N- and C-terminal variable segments do not interfere with the effect of PBLK on inactivation, and we conclude that such regions do not physically interact with the linker.

An unexpected observation from this study is that constructs capable of inducing a large increase in \( T_{0.5} \), such as \( \beta_{2a}\cdot3H3-PBLK-GK \), produced a modest change in \( V_{1/2} \). Thus, slowing the kinetics of inactivation is not always accompanied by a shift in the voltage dependence of inactivation toward positive voltages, as one would expect if VDI inhibition occurs by disfavoring inactivated states. Moreover, \( \beta_{2a}\cdot3H3-PBLK-GK \) that confers a rather slow inactivation phenotype shifts the steady-state inactivation curve to the left when compared with \( C_{a}\cdot2.3 \) alone, as if the equilibrium for the occupation of inactivated states would be favored by the presence of this construct. This suggests that the \( \beta_{2a}\)-subunit and its derivatives influence not only the stability of the inactivated state but also the energy barriers that the channel must overcome in its way to inactivation.

All the available data show that shifting the inactivation curve toward more depolarizing potentials and provoking noninactivating components at positive voltages require a membrane-anchored \( \beta\)-subunit. Consistent with this view, our studies also show that only palmitoylatable \( \beta_{2a} \) displays this behavior. In contrast, the ability to module \( T_{0.5} \) does not require palmitoylation, leading us to conclude that although membrane anchoring destabilizes the channel inactive state, the linker is geared at regulating the movement of the inactivation gate. This suggests that while the channel transits to the inactive conformation, PBLK comes transiently into close contact with the inactivation machinery.

In a previous work, we showed that acute exposure of \( C_{a}\cdot2.3 \) channels to the GK domain of \( \beta_{2a} \) alone was sufficient to slow down inactivation when applied to the \( \alpha_{1}\)-subunit already assembled into the plasma membrane (29). Unfortunately, the GK domain turned out to be unstable, and we could not test whether a long term exposure of this domain, i.e. the cRNA/protein co-injection strategy, would be sufficient to confer fast inactivation. We reported that \( \beta\)-subunits accelerating VDI acquired this phenotype only when co-injecting the protein with \( \alpha_{1}\cdot\text{cRNA}. \) In light of our present results that short constructs also accelerate VDI when co-injected, we rationalized that this could be the case for GK. In favor of this possibility is that the SH3 domain does not have an impact on channel function but on surface expression (25, 29). In this scenario, GK would become competent to accelerate
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inactivation following a post-translational modification, yet to be established.

In summary, we were able to identify a new determinant for the inhibition of inactivation within the linker joining the GK and SH3 domains present in all β2- subunits and conserved across different species. This linker has already been pointed out as a potential regulator for inactivation (7, 22, 23). Here, we defined a short segment located in this region that includes positively charged residues. These basic amino acids are required for modulation of VDI through a mechanism that remains to be elucidated and that may involve electrostatic interactions. The functional capability of this segment in non-membrane-anchored β2- subunits remains elusive. One possibility is that the accessibility of PBLK positively charged residues may indeed come about through post-translational modifications of the protein.

REFERENCES

1. Clapham, D. E. (2007) Calcium signaling. Cell 131, 1047–1058
2. Carafoli, E. (2002) Calcium signaling. A tale for all seasons.
3. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu. Rev. Cell Dev. Biol. 16, 521–555
4. Cens, T., Rousset, M., Leyris, J. P., Fesquet, P., and Charnet, P. (2006) Voltage- and calcium-dependent inactivation in high voltage-gated Ca2+ channels. Prog. Biophys. Mol. Biol. 90, 104–117
5. Liang, H., DeMaria, C. D., Erickson, M. G., Mori, M. X., Alseikhan, B. A., and Yue, D. T. (2003) Unified mechanisms of Ca2+ regulation across the Ca2+ channel family. Neuron 39, 951–960
6. Olcese, R., Qin, N., Schneider, T., Neely, A., Wei, X., Stefani, E., and Birnbaumer, L. (1994) The amino termini of calcium channel β-subunits set rates of inactivation independently of their effect on activation. Neuron 13, 1433–1438
7. Qin, N., Olcese, R., Zhou, J., Cabello, O. A., Birnbaumer, L., and Stefani, E. (1996) Identification of a second region of the β-subunit involved in regulation of calcium channel inactivation. Am. J. Physiol. 271, C1539–C1545
8. Jones, L. P., Wei, S. K., and Yue, D. T. (1998) Mechanism of auxiliary subunit modulation of neuronal α1E calcium channels. J. Gen. Physiol. 112, 125–143
9. Cens, T., Restituito, S., Galas, S., and Charnet, P. (1999) Voltage and calcium use the same molecular determinants to inactivate calcium channels. J. Biol. Chem. 274, 5483–5490
10. Sokolov, S., Weiss, R. G., Timin, E. N., and Hering, S. (2000) Modulation of slow inactivation in class A Ca2+ channels by β-subunits. J. Physiol. 527, 445–454
11. Hering, S., Berjukov, S., Sokolov, S., Marksteiner, R., Weiss, R. G., Kraus, R., and Timin, E. N. (2000) Molecular determinants of inactivation in voltage-gated Ca2+ channels. J. Physiol. 528, 237–249
12. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Sutich, T. P., and Campbell, K. P. (1994) Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α2-subunit. Nature 368, 67–70
13. Opatowsky, Y., Chen, C. C., Campbell, K. P., and Hirsch, J. A. (2004) Structural analysis of the voltage-dependent calcium channel β-subunit functional core and its complex with the α1 interaction domain. Neuron 42, 387–399
14. Chen, Y. H., Li, M. H., Zhang, Y., He, L. L., Yamada, Y., Fitzmaurice, A., Shen, Y., Zhang, H., Tong, L., and Yang, I. (2004) Structural basis of the α1-β-subunit interaction of voltage-gated Ca2+ channels. Nature 429, 675–680
15. Van Petegem, F., Clark, K. A., Chatelain, F. C., and Minor, D. L., Jr. (2004) Structure of a complex between a voltage-gated calcium channel β-subunit and an α-subunit domain. Nature 429, 671–675
16. Williams, M. E., Marubio, L. M., Deal, C. R., Hans, M., Brust, P. F., Phillips, L. H., Miller, R. J., Johnson, E. C., Harpold, M. M., and Ellis, S. B. (1994) Structure and functional characterization of neuronal α1E calcium channel subtypes. J. Biol. Chem. 269, 22347–22357
17. Qin, N., Platano, D., Olcese, R., Costantin, J. L., Stefani, E., and Birnbaumer, L. (1998) Unique regulatory properties of the type 2a Ca2+ channel β-subunit caused by palmitoylation. Proc. Natl. Acad. Sci. U.S.A. 95, 4690–4695
18. Chien, A. J., Carr, K. M., Shirokov, R. E., Rios, E., and Hosey, M. M. (1996) Identification of palmitoylation sites within the L-type calcium channel β2a-subunit and effects on channel function. J. Biol. Chem. 271, 26465–26468
19. Restituito, S., Cens, T., Barrere, C., Geib, S., Galas, S., De Waard, M., and Charnet, P. (2000) The β2a-subunit is a molecular grom for the Ca2+ channel inactivation gate. J. Neurosci. 20, 9046–9052
20. Hurley, J. H., Cahill, A. L., Currie, K. P., and Fox, A. P. (2000) The role of dynamic palmitoylation in Ca2+ channel inactivation. Proc. Natl. Acad. Sci. U.S.A. 97, 9293–9298
21. Stotz, S. C., Jarvis, S. E., and Zamponi, G. W. (2004) Functional roles of cytoplasmic loops and pore lining transmembrane helices in the voltage-dependent inactivation of HVA calcium channels. J. Physiol. 554, 263–273
22. Richards, M. W., Leroy, J., Pratt, W. S., and Dolphin, A. C. (2007) The HOOK domain between the SH3 and the GK domains of Ca2+β subunits contains key determinants controlling calcium channel inactivation. Channels 1, 92–101
23. Stotz, S. C., Barr, W., McRory, J. E., Chen, L., Jarvis, S. E., and Zamponi, G. W. (2004) Several structural domains contribute to the regulation of N-type calcium channel inactivation by the β3-subunit. J. Biol. Chem. 279, 3793–3800
24. Berrou, L., Klein, H., Bernatchez, G., and Parent, L. (2002) A specific tryptophan in the I-II linker is a key determinant of β-subunit binding and modulation in Ca(V)2.3 calcium channels. Biophys. J. 83, 1429–1442
25. Gonzalez-Gutierrez, G., Miranda-Laferte, E., Neely, A., and Hidalgo, P. (2007) The Src homology 3 domain of the β-subunit of voltage-gated calcium channels promotes endocytosis via dynamin interaction. J. Biol. Chem. 282, 2156–2162
26. Gonzalez-Gutierrez, G., Miranda-Laferte, E., Naranjo, D., Hidalgo, P., and Neely, A. (2008) Mutations of nonconserved residues within the calcium channel α1-interaction domain inhibit β-subunit potentiation. J. Gen. Physiol. 132, 383–395
27. Hidalgo, P., Gonzalez-Gutierrez, G., Garcia-Olivares, J., and Neely, A. (2006) The α-β subunit interaction that modulates calcium channel activity is reversible and requires a competent α-association domain. J. Biol. Chem. 281, 24104–24110
28. Schneider, T., Wei, X., Olcese, R., Costantin, J. L., Neely, A., Palade, P., Perez-Reyes, E., Qin, N., Zhou, J., Crawford, G. D., et al. (1994) Molecular analysis and functional expression of the human type E neuronal Ca2+ channel α1-subunit. Receptors Channels 2, 255–270
29. Gonzalez-Gutierrez, G., Miranda-Laferte, E., Nothmann, D., Schmidt, S., Neely, A., and Hidalgo, P. (2008) The guanylate kinase domain of the β-subunit of voltage-gated calcium channels suffices to modulate gating. Proc. Natl. Acad. Sci. U.S.A. 105, 14198–14203
30. Neely, A., Garcia-Olivares, J., Voswinke, S., Horstkott, H., and Hidalgo, P. (2004) Fixing of active calcium channel β1(b)-subunit by size-exclusion chromatography and its role on channel function. J. Biol. Chem. 279, 21689–21694
31. Tagilatelle, M., Toro, L., and Stefani, E. (1992) Novel voltage clamp to record small, fast currents from ion channels expressed in Xenopus oocytes. Biophys. J. 61, 78–82
32. Miranda-Laferte, E., Gonzalez-Gutierrez, G., Schmidt, S., Zeug, A., Ponomaskin, E. G., Neely, A., and Hidalgo, P. (2011) Homodimerization of the Src homology 3 domain of the calcium channel β-subunit drives dynamin-dependent endocytosis. J. Biol. Chem. 286, 22203–22210
33. Olcese, R., Neely, A., Qin, N., Wei, X., Birnbaumer, L., and Stefani, E. (1996) Coupling between charge movement and pore opening in vertebrate neuronal α1E calcium channels. J. Physiol. 497, 675–686
34. Parent, L., Schneider, T., Moore, C. P., and Talwar, D. (1997) Subunit regulation of the human brain α1E calcium channel. J. Membr. Biol. 160, 127–140
35. Cens, T., Restituito, S., Rousset, M., and Charnet, P. (2005) in Voltage-
Determinant for Modulation of Inactivation in $\beta_{2\alpha}$-Subunit

36. Neely, A., Wei, X., Olcese, R., Birnbaumer, L., and Stefani, E. (1993) Potentiation by the $\beta$-subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* **262**, 575–578

37. Opatowsky, Y., Chomsky-Hecht, O., Kang, M. G., Campbell, K. P., and Hirsch, J. A. (2003) The voltage-dependent calcium channel $\beta$-subunit contains two stable interacting domains. *J. Biol. Chem.* **278**, 52323–52332

38. Ben-Tal, N., Honig, B., Peitzsch, R. M., Denisov, G., and McLaughlin, S. (1996) Binding of small basic peptides to membranes containing acidic lipids. Theoretical models and experimental results. *Biophys. J.* **71**, 561–575