FEBS Letters 583 (2009) 1969–1975

FEBS Letters

journal homepage: www.FEBSLetters.org

Functional dissection of the intramolecular Src homology 3-guanylate kinase domain coupling in voltage-gated Ca^{2+} channel β-subunits

Yu-hang Chen [1], Lin-ling He [1], Douglas R. Buchanan, Yun Zhang, Aileen Fitzmaurice, Jian Yang *

Department of Biological Sciences, 917 Fairchild Center, MC2462, Columbia University, New York, NY 10027, USA

ARTICLE INFO

Article history:
Received 10 February 2009
Revised 25 March 2009
Accepted 4 May 2009
Available online 8 May 2009

Edited by Maurice Montal

Keywords:
Calcium channel
Auxiliary subunit
Membrane-associated guanylate kinase
Regulation
X-ray structure
Electrophysiology

ABSTRACT

The β-subunit of voltage-gated Ca^{2+} channels is essential for trafficking the channels to the plasma membrane and regulating their gating. It contains a Src homology 3 (SH3) domain and a guanylate kinase (GK) domain, which interact intramolecularly. We investigated the structural underpinnings of this intramolecular coupling and found that in addition to a previously described SH3 domain β strand, two structural elements are crucial for maintaining a strong and yet potentially modifiable SH3–GK intramolecular coupling: an intrinsically weak SH3–GK interface and a direct connection of the SH3 and GK domains. Alterations of these elements uncouple the two functions of the β-subunit, degrading its ability to regulate gating while leaving its chaperone effect intact.

1. Introduction

High voltage-activated (HVA) Ca^{2+} channels are composed of the pore-forming α_1 subunit and ancillary $\alpha_2\delta$, β and, in skeletal muscles, γ subunits [1]. The calcium channel β-subunit ($Ca_v\beta$) is essential for transporting the channel complexes to the plasma membrane and modulating their gating properties. There are four subfamilies of $Ca_v\beta$s, each with multiple splicing isoforms and unique modulatory functions [2]. Previous studies show that $Ca_v\beta$ has a modular structure consisting of five distinct domains or regions: the N-terminus, an Src homology 3 (SH3) domain, a HOOK region, a guanylate kinase (GK) domain and the C-terminus [3–6]. The SH3–HOOK–GK module is also present in membrane-associated guanylate kinases (MAGUKs), a large family of PDZ domain-containing scaffold proteins important for the synaptic localization and clustering of various membrane receptors and ion channels [7,8].

Previous studies indicate that $Ca_v\beta$ also functions modularly. The GK domain binds tightly to a region called the α interaction domain (AID) in the cytoplasmic loop connecting the first two repeats of the α_1-subunit [9–11]. This AID–GK domain interaction is necessary for trafficking HVA Ca^{2+} channels to the cell surface [9,10,12–18]. The SH3–HOOK–GK core module is responsible for modulating the activation properties [16] and the HOOK and N-terminus play a predominant role in modulating inactivation, either directly by interacting with the α_1-subunit and/or indirectly through palmitoylation [16,19–29].

A feature revealed by the $Ca_v\beta$ crystal structures is that the SH3 and GK domains are coupled intramolecularly [3,5,6]. This structural feature is shared by the MAGUK SH3–HOOK–GK module [7,8]. Disruption of the SH3–GK interaction in MAGUKs has severe functional consequences [30,31]. The SH3–GK intramolecular coupling is also important for $Ca_v\beta$ functions, including gating modulation [32–35] and surface trafficking [35]. The β5 strand of the SH3 domain has been found to be critical for the SH3–GK intramolecular coupling [6,32–36].

In this work we investigated additional structural underpinnings of the SH3–GK intramolecular coupling in $Ca_v\beta$. Taking full advantage of the $Ca_v\beta$ crystal structures, we made specific point mutations, deletions and insertions to create various mutant forms or fragments of $Ca_v\beta$ and tested their functions. Our results shed new light on understanding the structural design and function of the SH3–GK intramolecular coupling in $Ca_v\beta$.

2. Materials and methods

P/Q-type Ca^{2+} channels containing wild-type (WT) or mutant β_{2a} were expressed in *Xenopus* oocytes. The boundaries of the five

Abbreviations: $Ca_v\beta$, calcium channel β-subunit; SH3, Src homology 3; GK, guanylate kinase; MAGUK, membrane-associated guanylate kinase; HVA, high voltage-activated; AID, α interaction domain; TEVC, two-electrode voltage-clamp; WT, wild-type

* Corresponding author. Fax: +212 531 0425.
E-mail address: jy160@columbia.edu (J. Yang).
[1] These authors contributed equally to this work.

0014-5793/$36.00
doi:10.1016/j.febslet.2009.05.001

domains/regions of β_{2a} are: N-terminus: M1-P59; SH3 domain: V60-S120 and P219-P224; HOOK region: P121-P218; GK domain: S225-T410; C-terminus: H411-Q604. Currents were recorded with cell-attached patch-clamp or two-electrode voltage-clamp (TEVC) recording. Full details are available in Supplementary materials and methods.

3. Results

The functional properties we examined included the voltage-dependence (the midpoint $V_{1/2}$ and slope factor K) of activation and steady-state inactivation (protocols illustrated in Fig. 1A–D) and the kinetics of inactivation. All experiments were performed on P/Q-type Ca^{2+} channels produced in *Xenopus* oocytes expressing $Ca_v2.1$, $\alpha_2\delta$, and a WT or mutant β_{2a}, using the cell-attached patch-clamp recording with Ba^{2+} as the charge carrier. β_{2a} was used because the difference in the inactivation properties is the largest between the WT and the GK–C module [16]. This allowed us to more easily detect changes caused by the point mutations, deletions and insertions.

3.1. The SH3–GK interface interactions are important for inactivation regulation

The $Ca_v\beta$ structures show that SH3 and GK domains associate with each other through limited interactions [3,5,6] [Supplementary Fig. S1]. To examine the role of the SH3–GK interface interactions in $Ca_v\beta$ functions, we simultaneously mutated seven amino acids in β_{2a} that are directly involved in these interactions to alanine (this mutant is referred to as β_{2a}_Mut7) (Fig. 1E). These residues are highly conserved among all four $Ca_v\beta$ subfamilies (Supplementary Fig. S2) and the hepta-mutation is very likely to severely disrupt the SH3–GK domain association.

β_{2a}_Mut7 clearly stimulated channel surface expression (Supplementary Fig. S3). However, compared with channels containing WT β_{2a}, those containing β_{2a}_Mut7 exhibited much faster inactivation (Fig. 1F and G, and Table 1). The voltage-dependence of steady-state inactivation was shifted toward more hyperpolarized potentials (Fig. 1H and Table 1). However, there was no change in the voltage-dependence of activation (Table 1). The fact that the hepta-mutation specifically affected β_{2a}'s modulation of inactivation without compromising its ability to regulate channel expression and activation suggests that the mutation did not significantly perturb protein folding. Instead, the mutation probably altered the quaternary arrangement of the N–SH3–HOOK module with respect to the GK–C module. This change may render the N-terminus and the HOOK region less efficient in regulating inactivation [16,19–29].

3.2. The β_5 strand of the split SH3 domain is required for the SH3–GK intramolecular coupling

Are the SH3–GK interface interactions alone sufficient for maintaining a strong SH3–GK intramolecular coupling? To address this question, we divided β_{2a} into two fragments, one containing the N–SH3–HOOK module (β_{2a}_N–SH3–HOOK) and the other containing the GK–C module (β_{2a}_GK–C) (Fig. 2A). We then tested whether these two parts could coassemble into a complex that could fully recapitulate the functions of WT β_{2a}.

We first examined the functionality of each individual component. β_{2a}_N–SH3–HOOK was completely ineffective in stimulating channel expression on the plasma membrane, as determined by TEVC (data not shown). This is expected since the AID–GK interaction is required for this effect [9,10,12–17]. In contrast, β_{2a}_GK–C fully retained the chaperone function, as we previously observed

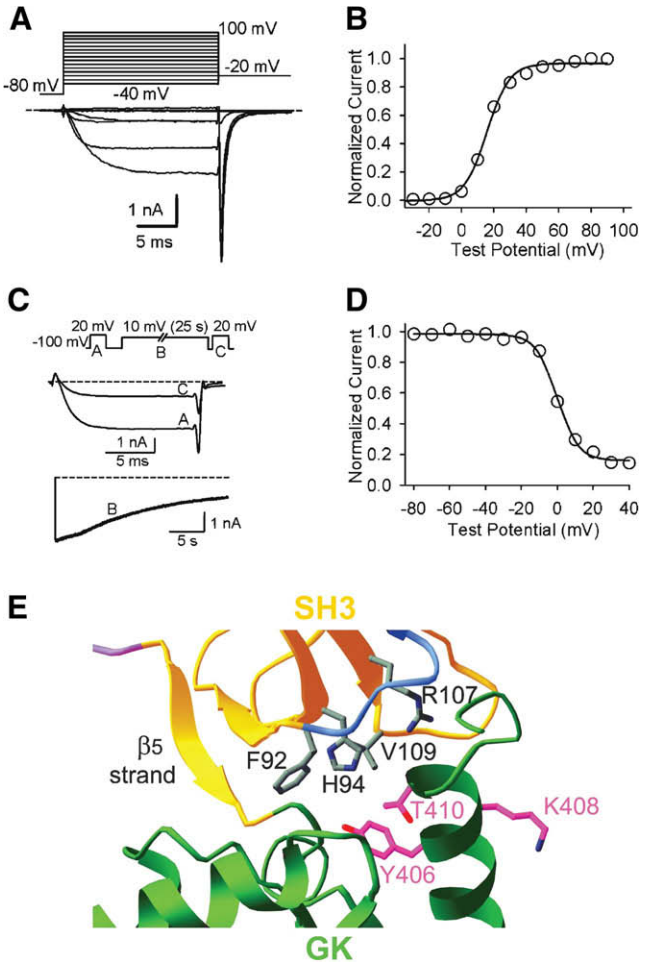

Fig. 1. Effect of disrupting β_{2a} SH3–GK interface interactions on P/Q-type Ca^{2+} channel inactivation. (A and B) Voltage protocol and representative current traces (A) of channels containing WT β_{2a} for constructing the voltage-dependence of activation (B). Currents were evoked by 20-ms depolarizations ranging from –40 mV to +100 mV in 10-mV increments. Smooth line in (B) represents a single Boltzmann fitting curve. (C and D) Voltage protocol and representative current traces (C) of channels containing WT β_{2a} for constructing the voltage-dependence of steady-state inactivation (D). Smooth line in (D) represents a single Boltzmann fitting curve. See Supplementary methods and materials for details. (E) Close-up of the β_{2a} SH3–GK interface. The 7 mutated residues are indicated. This and subsequent ribbon diagrams were generated with the RIBBONS program based on coordinates in the PDB file 1T0J. (F) Representative current trace of channels containing WT β_{2a} or β_{2a}_Mut7. In this and the following similar figures, current was evoked by a 25-s depolarization to +20 mV from a holding potential of −100 mV, and the dashed line indicates the 0 current level. To highlight differences in the inactivation kinetics among different constructs, only the first 5 s is shown. (G) Comparison of the inactivation kinetics of channels containing WT β_{2a} or β_{2a}_Mut7. The current traces in (F) were normalized by the peak amplitude. (H) Voltage-dependence of steady-state inactivation of channels containing WT β_{2a} or β_{2a}_Mut7. N = 7–8. In this and all subsequent similar plots, smooth lines represent single Boltzmann fitting curves.

[16]. This contradicts the lack of trafficking effect of the GKC construct of Takahashi et al. [34,35]. The reasons underlying this discrepancy are unclear. One possibility is the difference in the expression system (*Xenopus* oocytes vs. HEK 293 cells). Channels containing β_{2a}_GK–C inactivated rapidly (Fig. 2B and Table 1) and exhibited a ~27-mV negative shift in the half-inactivation voltage compared to WT (Fig. 2C and Table 1). These results confirm that the GK–C module cannot bestow WT inactivation and reinforce the critical role of the N-terminus and the HOOK in regulating inactivation [16,19–27,29].

We next expressed β_{2a}_N–SH3–HOOK and β_{2a}_GK–C together. Surprisingly, the resultant channels behaved exactly like those

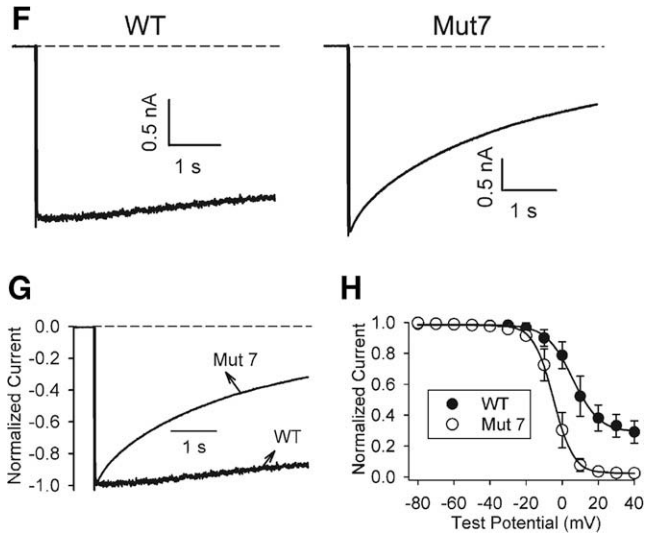

Fig. 1 (*continued*)

obtained when β_{2a}_GK–C was expressed alone (Fig. 2B and C, and Table 1). This result could come about if the β_{2a}_N–SH3–HOOK protein was not synthesized efficiently or folded properly. This possibility seemed highly unlikely since a construct similar to β_{2a}_N–SH3–HOOK but missing the last 9 amino acids including the β5 strand of the SH3 domain (and hence was more likely to be defunct) was synthesized efficiently and folded properly (see below). Our hypothesis was that the SH3–GK interface interactions alone were intrinsically too weak to hold β_{2a}_N–SH3–HOOK and β_{2a}_GK–C together, so that the surface channels contained only β_{2a}_GK–C; meanwhile, β_{2a}_N–SH3–HOOK itself could not bind α_1 tightly enough to exert its influence on inactivation.

To test this hypothesis, we examined the SH3–GK interface interactions biochemically. Indeed, purified β_{2a}_N–SH3–HOOK protein (tagged with glutathione S-transferase (GST)) failed to bind β_{2a}_GK–C (tagged with maltose binding protein (MBP)) in an in vitro pull down assay (Fig. 2F, lane 5).

So what other structural elements are also involved in maintaining the strong SH3–GK intramolecular coupling? It was noted in the $Ca_v\beta$ structure work that unlike canonical SH3 domains, which consist of five contiguous β strands, the $Ca_v\beta$ SH3 domain has a split configuration in which the fifth β strand (β5) is separated from the rest of the SH3 domain by a HOOK region [3,5,6]. The β5 strand interacts weakly with the GK domain but strongly with other parts of the SH3 domain (Supplementary Fig. S4). Since the β5 strand is directly connected to the GK domain in the primary amino acid sequence, these interactions bring the SH3 and GK domains in close proximity and thereby greatly increase the effective affinity between the two domains (Fig. 1E).

Previous studies suggest that the β5 strand plays a crucial role in the SH3–GK intramolecular coupling [6,32–36]. To further investigate its role, we divided β_{2a} into two other fragments, referred to as β_{2a}_N–SH3–HOOK (−β5) and β_{2a}_GK–C (+β5) (Fig. 2A). As expected, β_{2a}_GK–C (+β5) increased Ca^{2+} channel current (Supplementary Fig. S3) and β_{2a}_N–SH3–HOOK (−β5) did not (data not shown). The channels containing β_{2a}_GK–C (+β5) behaved the same as those containing β_{2a}_GK–C did, exhibiting rapid inactivation and negatively shifted half-inactivation voltage (Fig. 2D and E, and Table 1), indicating that the β5 strand itself does not change the inactivation properties. However, when β_{2a}_N–SH3–HOOK (−β5) was expressed together with β_{2a}_GK–C (+β5), the resulting channels behaved similarly as channels containing WT β_{2a} did (Fig. 2D and E, and Table 1), indicating the association of β_{2a}_N–SH3–HOOK (−β5) and β_{2a}_GK–C (+β5) and functional reconstitution. Consistent with these results, purified β_{2a}_N–SH3–HOOK (−β5) protein was able to pull down β_{2a}_GK–C (+β5) (Fig. 2F, lane 3).

Taken together, these results indicate that in addition to the direct contact between the SH3 and GK domains, the β5 strand of the SH3 domain is indispensable for forging a strong SH3–GK intramolecular coupling. Biochemical association and functional reconstitution have already been demonstrated for several other singly cleaved $Ca_v\beta$ fragments [6,32–36]. It is notable that in all the cases of successful complementation, the β5 strand was always included together with the GK–C module.

3.3. The lack of a flexible linker between the SH3 and GK domains strengthens the SH3–GK intramolecular coupling

It is evident from the $Ca_v\beta$ crystal structures that the SH3 and GK domains are juxtaposed without a flexible linker (Fig. 1E). We suspected that this structural feature might also be an important element in maintaining a strong SH3–GK intramolecular coupling.

Table 1
Gating properties of Ca^{2+} channels produced by the coexpression of $Ca_v2.1$, $\alpha_2\delta$ and the indicated WT or mutant β_{2a}.

Construct	Activation		Inactivation		
	$V_{1/2}$ (mV)	K (mV)	$V_{1/2}$ (mV)	K (mV)	$T_{1/2}$ (s) (20 mV)
β_{2a}_WT	15.6 ± 1.1 (10)	7.5 ± 0.7 (10)	3.8 ± 1.2 (8)	−5.2 ± 0.5 (8)	25.43 ± 9.40 (8)
β_{2a}_Mu7	14.8 ± 2.0 (8)	7.6 ± 0.6 (8)	−4.7 ± 3.2 (7)**	−5.1 ± 0.3 (7)	1.71 ± 0.61 (7)**
β_{2a}_GK-C	14.7 ± 1.8 (10)	7.0 ± 0.6 (10)	−23.0 ± 5.9 (10)**	−5.2 ± 0.8 (10)	0.21 ± 0.06 (10)**
β_{2a}_N-SH3-HOOK + β_{2a}_GK-C	14.8 ± 2.3 (5)	7.4 ± 0.5 (5)	−21.1 ± 3.1 (9)**	−4.6 ± 0.6 (9)	0.22 ± 0.04 (9)**
β_{2a}_GK-C (+β_5)	14.7 ± 1.1 (6)	7.7 ± 0.5 (6)	−20.7 ± 4.0 (8)**	−4.9 ± 0.7 (8)	0.21 ± 0.05 (8)**
β_{2a}_N-SH3-HOOK (−β_5) + β_{2a}_GK-C (+β_5)	14.9 ± 1.7 (11)	7.0 ± 0.3 (11)	4.3 ± 2.1 (8)	−5.5 ± 0.4 (8)	15.4 ± 2.6 (8)*
β_{2a}_WT_linker3	15.6 ± 1.1 (12)	7.1 ± 0.7 (12)	−4.8 ± 1.8 (10)**	−5.4 ± 0.5 (10)	1.98 ± 0.47 (10)**
β_{2a}_WT_linker6	15.9 ± 1.1 (9)	7.2 ± 0.5 (9)	−10.5 ± 3.1 (8)$^{\#\#}$	−5.1 ± 0.2 (8)	0.93 ± 0.19 (8)$^{\#\#}$
β_{2a}_WT_linker12	15.1 ± 1.2 (10)	7.2 ± 0.8 (10)	−15.6 ± 2.4 (8)$^{\#\#}$	−5.0 ± 0.5 (8)	0.61 ± 0.08 (8)$^{\#\#}$
β_{2a}_WT_linker21	15.0 ± 1.2 (8)	7.2 ± 0.6 (8)	−17.1 ± 1.9 (7)	−4.3 ± 0.2 (7)	0.43 ± 0.05 (7)$^{\#\#}$
β_{2a}_WT_linker30	15.1 ± 1.6 (9)	7.2 ± 0.6 (9)	-17.5 ± 3.7 (4)	−4.5 ± 0.2 (4)	0.42 ± 0.05 (5)
β_{2a}_Mu7_linker3	15.2 ± 1.9 (11)	7.5 ± 0.7 (11)	−11.3 ± 2.5 (12)**	−4.9 ± 0.4 (12)	0.74 ± 0.12 (12)**
β_{2a}_Mu7_linker6	14.6 ± 1.1 (6)	7.0 ± 0.8 (6)	−13.2 ± 1.9 (15)	−4.8 ± 0.5 (15)	0.66 ± 0.14 (15)
β_{2a}_Mu7_linker12	14.6 ± 2.0 (12)	7.2 ± 0.7 (12)	−15.6 ± 3.1 (10)	−4.7 ± 0.5 (10)	0.46 ± 0.10 (10)$^{\#\#}$
β_{2a}_Mu7_linker21	12.8 ± 1.6 (4)	7.2 ± 0.5 (6)	−18.9 ± 1.8 (15)$^{\#\#}$	−4.8 ± 0.4 (15)	0.44 ± 0.09 (15)
β_{2a}_Mu7_linker30	15.3 ± 1.2 (10)	7.3 ± 0.9 (10)	−22.1 ± 3.8 (8)$^{\#}$	−4.6 ± 0.6 (8)	0.34 ± 0.09 (8)$^{\#}$

The parameters include the midpoint ($V_{1/2}$) and slope factor (K) of current activation and inactivation and the time for the current to decay to 50% of the peak value at +20 mV ($T_{1/2}$ of inactivation at +20 mV). Data represent mean ± S.D. (number of patches). Significance tests were performed between the WT and a given mutant or a pair of mutants ($^{*}P < 0.05$ or $^{**}P < 0.01$), or between a longer linker construct and its immediate shorter neighbor ($^{\#}P < 0.05$ or $^{\#\#}P < 0.01$).

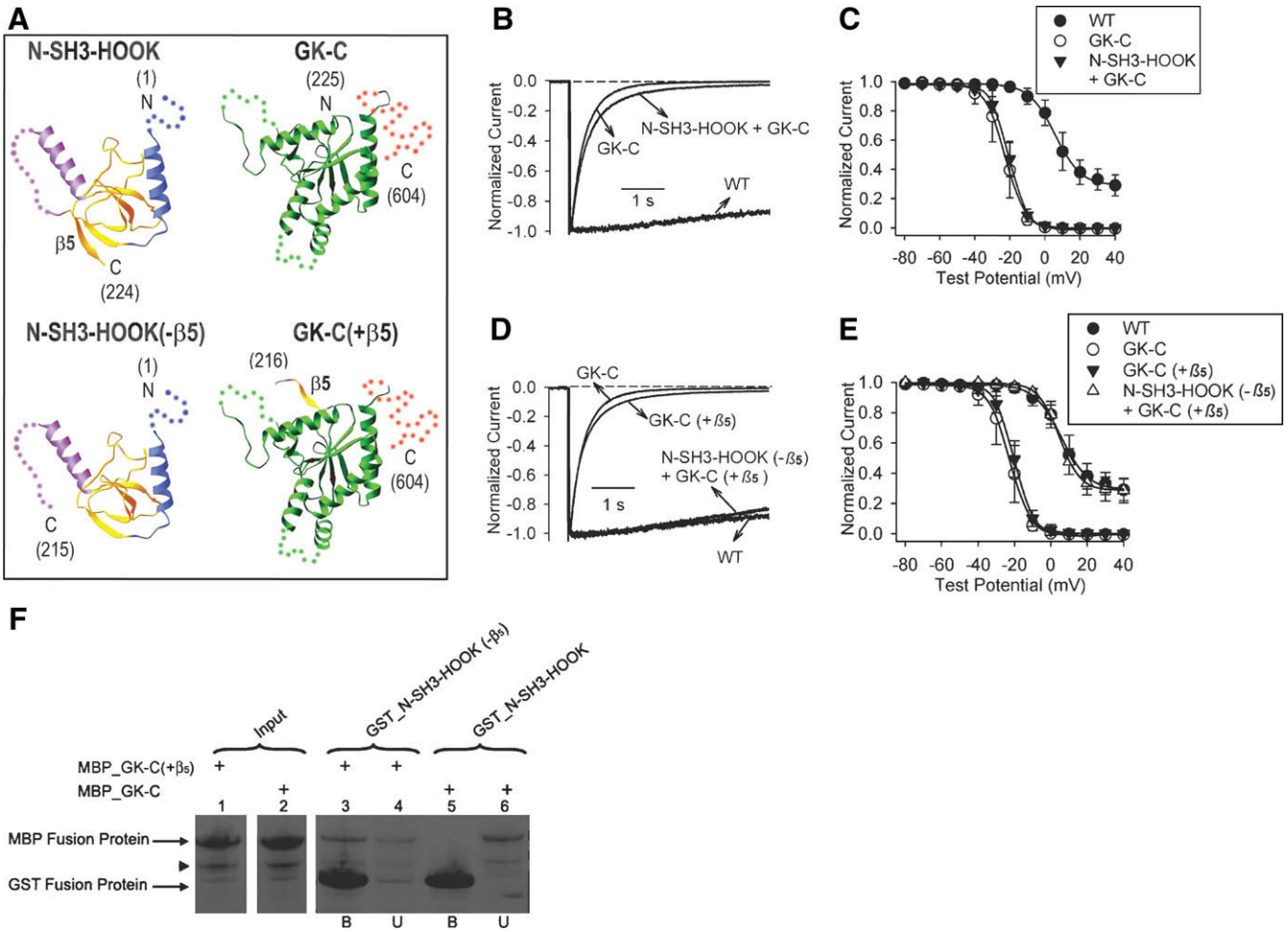

Fig. 2. Importance of the β_5 strand of the SH3 domain in regulating P/Q-type Ca^{2+} channel inactivation. (A) Design and structure of the β_{2a} fragments tested. Dashed lines represent unresolved regions. (B and C) Comparison of the inactivation kinetics (B) and voltage-dependence of steady-state inactivation (C) of channels produced by the coexpression of the indicated β_{2a} fragments and $Ca_v2.1$ and $\alpha_2\delta$. $N = 8–10$. (D and E) Comparison of the inactivation kinetics (D) and the voltage-dependence of steady-state inactivation (E) of channels produced by the coexpression of the indicated β_{2a} fragments and $Ca_v2.1$ and $\alpha_2\delta$. $N = 8–9$. Representative original current trace for each channel type is presented in Supplementary Fig. S5. (F) Coomassie blue staining showing binding between GST_N-SH3-HOOK (−β5) and MBP_GK-C (+β5) (lane 3) and the lack of binding between GST_N-SH3-HOOK and MBP_GK-C (lane 5). Glutathione sepharose beads were used to pull down the GST-fusion proteins. Lanes 4 and 6 show unbound proteins in the flow-through solution. Lanes 1 and 2 mark the size of MBP_GK-C (+β5) and MBP_GK-C, respectively; the two smaller and fainter bands are presumably degraded products. B, bound to the beads; U, unbound. There was a vast excess of GST-fusion proteins.

To test this hypothesis, we inserted a flexible linker of variable length (containing 3, 6, 12, 21 and 30-amino acids) at the SH3–GK junction, between P224 and S225 (Fig. 3A). These constructs were called β_{2a}_linker3, β_{2a}_linker6, etc. Our preceding results show that the SH3–GK interface interactions are too weak to glue the two domains together. Thus, when a flexible linker is inserted in between the two domains, the N–SH3–HOOK module is likely to separate from the GK–C module, which is anchored to the α_1 subunit via its high-affinity binding to the AID. With increasingly longer linkers, the effective local concentration of the N–SH3–HOOK module near the α_1-subunit would decrease gradually and the properties affected by this module would change incrementally.

The results came out as expected. With a linker as short as 3 amino acids, inactivation of the resultant channels became significantly faster than that of WT channels (Fig. 3B and Table 1) and the half-inactivation voltage was shifted to more hyperpolarized potential (Fig. 3C and Table 1) (compare β_{2a}_WT and β_{2a}_linker3). With longer linkers, inactivation became progressively faster (Fig. 3D and Table 1) and the half-inactivation voltage was shifted progressively to more hyperpolarized potentials (Fig. 3E and Table 1). This graded change and the fact that the voltage-dependence of activation was unaffected (Table 1) suggest that the effects on inactivation are unlikely non-specific effects produced by the linkers themselves.

When a linker was inserted into β_{2a}_Mut7 (the resulting construct was referred to as β_{2a}_Mut7_linker3, β_{2a}_Mut7_linker6, etc.), the changes in inactivation were even more dramatic. This was the case for both short (Fig. 3F and G, and Table 1) and long linkers (Fig. 3H and I, and Table 1). The graded change in the kinetics and voltage-dependence of inactivation with increasingly longer linkers was also more robust in the β_{2a}_Mut7 background (Fig. 3J and K, and Table 1). Indeed, channels containing β_{2a}_Mut7_linker30 behaved almost the same as channels containing β_{2a}_GK–C did (Fig. 3H–K, and Table 1). Thus, it appears that with a 30-amino acid flexible linker, the effective concentration of the N–SH3–HOOK module near the surface channels is too low to exert its modulatory function, even though it is still tethered to the GK–C module. These results not only demonstrate the importance of a direct physical connection of the SH3 and GK domains, but also further support the notion of an intrinsically weak SH3–GK interface.

We next performed surface biotinylation and Western blot to confirm that β_{2a}_Mut7_linker30 was associated with surface Ca^{2+} channels and was intact. Indeed, β_{2a}_Mut7_linker30 was pulled

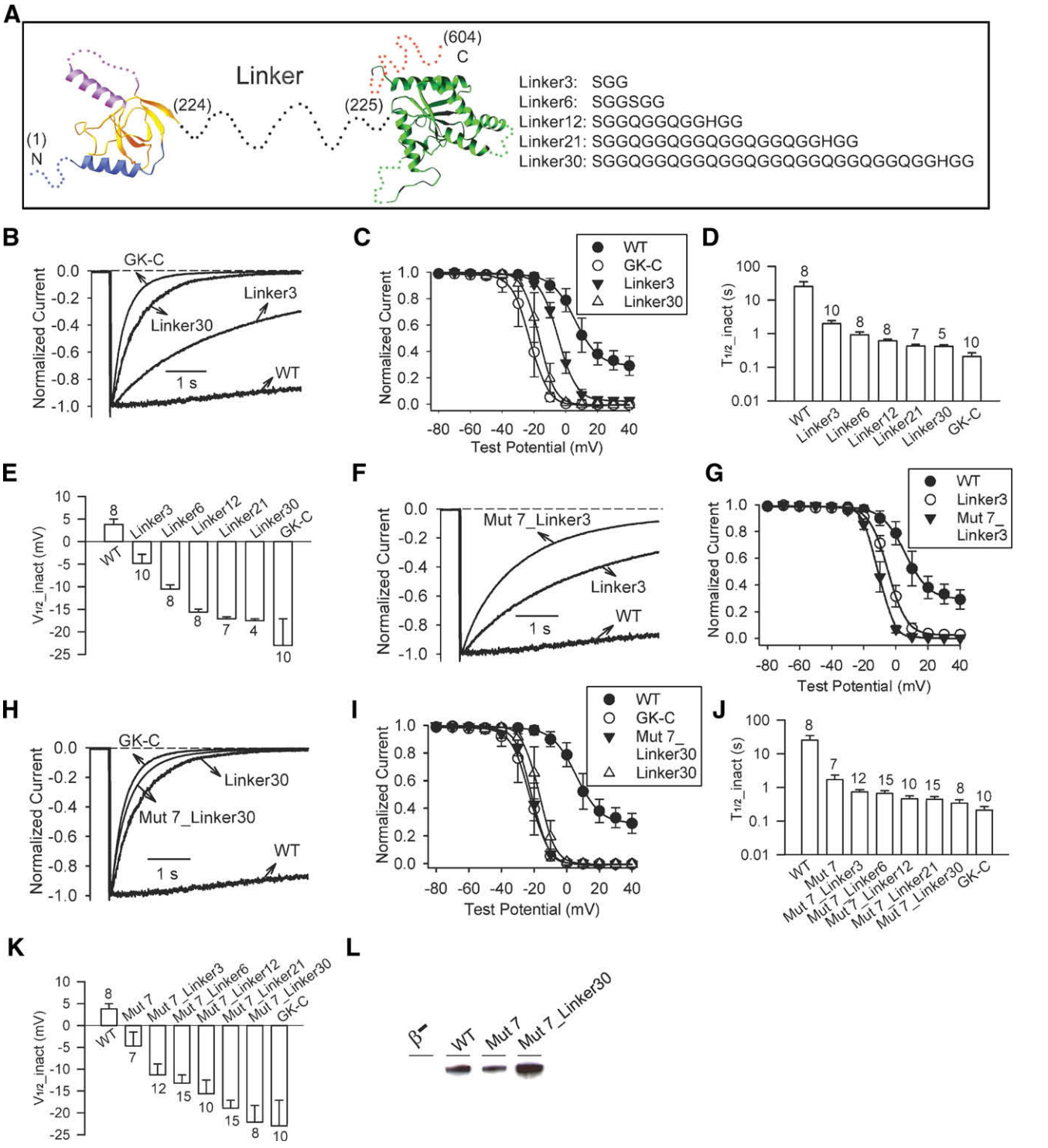

Fig. 3. Effect of inserting a linker between the SH3 and GK domain of β_{2a} on P/Q-type Ca^{2+} channel inactivation. (A) Design and nomenclature of the β_{2a} insertion constructs tested. We mainly used an alternating sequence of serine and glycine (SGG) or glutamine and glycine (QGG) as the linker because they confer both flexibility and solubility. (B and C) Comparison of the inactivation kinetics (B) and the voltage-dependence of steady-state inactivation (C) of channels produced by the coexpression of the indicated β_{2a} constructs and $Ca_v2.1$ and $\alpha_2\delta$. $N = 5–10$. (D and E) Comparison of the $T_{1/2}$ of inactivation at +20 mV (D) and the $V_{1/2}$ of steady-state inactivation (E) of channels produced by the coexpression of the indicated β_{2a} constructs and $Ca_v2.1$ and $\alpha_2\delta$. (F–I) Comparison of the inactivation kinetics (F, H) and the voltage-dependence of steady-state inactivation (G, I) of channels produced by the coexpression of the indicated β_{2a} constructs and $Ca_v2.1$ and $\alpha_2\delta$. $N = 5–15$. (J and K) Comparison of the $T_{1/2}$ of inactivation at +20 mV (J) and the $V_{1/2}$ of steady-state inactivation (K) of channels produced by the coexpression of the indicated β_{2a} constructs and $Ca_v2.1$ and $\alpha_2\delta$. Representative original current trace for some channel types is presented in Supplementary Fig. S5. (L) Western blot showing staining by an anti-β_{2a} antibody of surface proteins isolated from oocytes expressing $Ca_v2.1$ and $\alpha_2\delta$ alone (β^-) or together with the indicated β_{2a} constructs. Surface Ca^{2+} channels were biotinylated and then isolated with streptavidin beads. The indicated β_{2a} constructs were pulled down due to their tight association with surface $Ca_v2.1$.

down together with biotinylated surface α_1- and/or $\alpha_2\delta$-subunits by streptavidin, indicating β_{2a}_Mut7_linker30 was present in surface Ca^{2+} channel complexes (Fig. 3L). Furthermore, the molecular weight of the pull-down β_{2a}_Mut7_linker30 remained as expected (Fig. 3L), indicating that the N–SH3–HOOK module was not cleaved off by proteolysis as a result of linker insertion.

4. Discussion

Our results indicate that the strong SH3–GK intramolecular coupling in $Ca_v\beta$ is orchestrated by three structural elements: a direct but weak SH3–GK interface, a split SH3 domain with a β strand (β_5) that engages in both intradomain (SH3) as well as interdomain (SH3–GK) interactions, and a direct linkage of SH3 and GK domains in the primary amino acid sequence. This intramolecular coupling is not necessary for the chaperone function of $Ca_v\beta$ – every mutant construct with disrupted SH3–GK coupling was still able to traffic Ca^{2+} channels to the surface membrane as long as it contained the GK domain. This notion is consistent with recent findings that the GK domain alone is sufficient to carry out the chaperone function [16,18]. On the contrary, a strong SH3–GK intramolecular coupling is essential for the modulation of gating (especially inactivation) by $Ca_v\beta$. Through this coupling, the N–SH3–HOOK module is tightly bound to the GK–C module and as such is anchored to the α_1-subunit. This enables the N-terminus and the HOOK region to interact with the α_1-subunit, interactions that are of intrinsic low affinity but are essential for the modulation of inactivation.

A unique property of β_{2a} (from rat and human) is that its N-terminus can be palmitoylated, which anchors β_{2a} to the plasma membrane and contributes partly to its depolarizing shift of the steady-state inactivation voltage and slowing of the inactivation kinetics [23–25,29]. Our results show that the N–SH3–HOOK module was functionally mute when it was expressed alone, or coexpressed with β_{2a}_GK–C (Fig. 2B and C, and Table 1), or tethered to the GK–C module via a 30-amino acid linker (Fig. 3B–E and Table 1). These observations suggest that palmitoylation alone is insufficient to promote the SH3–GK intramolecular coupling and confer functionality to the N–SH3–HOOK module. On the other hand, it has been reported that in tsA-201 cells palmitoylation of β_{2a} is able to endow it the ability to modulate gating of a mutated $Ca_v2.2$ with a severely weakened AID–GK interaction [29]. The discrepancy between our work and this work is probably because in tsA-201 cells, due to its relatively small size, overexpression and palmitoylation of β_{2a} greatly increases its effective concentration near the channels on the plasma membrane, and thus, enabling it to modulate gating.

Three lines of biochemical and, more important, functional evidence indicate that the SH3–GK interface interactions are intrinsically weak: β_{2a}_N–SH3–HOOK and β_{2a}_GK–C did not bind each other in vitro (Fig. 2F) and did not complement each other functionally when expressed together (Fig. 2B and C) or when linked together via a long linker (Fig. 3B–E). An intrinsically weak SH3–GK interface may confer additional versatility to $Ca_v\beta$ modulation of HVA Ca^{2+} channel gating. Under normal conditions, the SH3 and GK domains are likely to act as a rigid body owing to the strong SH3–GK intramolecular coupling forged synergistically by the three structural elements mentioned above. This rigid body structure is essential for the gating modulation. However, if any region of the N–SH3–HOOK module were engaged in a high-affinity interaction with another protein, it might generate enough force to disrupt the intrinsically weak SH3–GK interface, which would result in dramatic changes in the inactivation of the channels. Similar functional consequences could also be produced by naturally occurring mutations that severely disrupt the SH3–GK intramolecular coupling. It would be interesting to identify such proteins or mutations in future studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.05.001.

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