Molecular basis for differential modulation of BK channel voltage-dependent gating by auxiliary γ subunits

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Large conductance Ca^{2+}- and voltage-activated potassium (BK) channels are comprised of pore-forming α subunits and various regulatory auxiliary subunits. The BK channel auxiliary γ (BKγ) subunits are a newly identified class of proteins containing an extracellular leucine-rich repeat domain (LRRD), a single transmembrane (TM) segment, and a short cytoplasmic C-terminal tail (C-tail). Although each of the four BKγ proteins shifts the voltage dependence of BK channel activation in a hyperpolarizing direction, they show markedly different efficacies, mediating shifts over a range of 15–145 mV. Analyses of chimeric BKγ subunits created by swapping individual structural elements, and of BKγ deletion and substitution mutants, revealed that differential modulation of BK gating by the four BKγ subunits depends on a small region consisting of the TM segment and the adjacent intracellular cluster of positively charged amino acids. The γ1 and γ2 TM segments contributed approximately −100 mV, and the γ1 and γ3 C-tails contributed approximately −40 mV, to shifting the voltage dependence of BK channel activation, whereas the γ3 and γ4 TM segments and the γ2 and γ4 C-tails contributed much less. The large extracellular LRRDs were mainly functionally interchangeable, although the γ1 LRRD was slightly less effective at enhancing (or slightly more effective at attenuating) the shift in BK channel voltage-dependent gating toward hyperpolarizing potentials than those of the other BKγ subunits. Analysis of mutated BKγ subunits revealed that juxta-membrane clusters of positively charged amino acids determine the functions of the γ1 and γ3 C-tails. Therefore, the modulatory functions of BKγ subunits are coarse- and fine-tuned, respectively, through variations in their TM segments and in the adjacent intracellular positively charged regions. Our results suggest that BK channel modulation by auxiliary γ subunits depends on intra- and/or juxta-membrane mechanisms.

INTRODUCTION

The large conductance Ca^{2+}- and voltage-activated potassium (BK) channel is widely expressed and plays important roles in many physiological processes, including neuronal firing and neurotransmitter release (Gribkoff et al., 2001), and frequency tuning of auditory hair cells (Ramanathan et al., 1999). The BK channel features exceptionally large single-channel conductance and dual activation by membrane depolarization and elevation in intracellular free calcium ($[Ca^{2+}]_i$). Auxiliary membrane proteins are potent and critical regulators of ion channels (Gurnett and Campbell, 1996; Sun et al., 2012; Yan and Tomita, 2012). BK channels consist of the homotetrameric pore-forming voltage- and calcium-sensing α subunits (BKα) alone or in association with regulatory tissue-specific auxiliary β or γ subunits. The newly identified BK channel auxiliary γ (BKγ) subunits are a group of leucine-rich repeat (LRR)-containing membrane proteins, called γ1 (LRRC26), γ2 (LRRC52), γ3 (LRRC55), and γ4 (LRRC38) (Yan and Aldrich, 2010, 2012). The four BKγ subunits have different tissue-specific mRNA expression and may broadly modulate BK channels in different tissues, including brain, which expresses the γ1 and γ3 mRNAs (Yan and Aldrich, 2012). The as yet limited studies on the mechanisms and physiological functions of BKγ subunits have mainly focused on γ1, which has been reported to regulate BK channels in prostate cancer (Gessner et al., 2006; Yan and Aldrich, 2010), salivary gland cells (Almassy and Begenisich, 2012), and also likely in airway epithelial cells (Manzanares et al., 2014) and arterial smooth muscle cells (Evanson et al., 2014). The mouse γ2 subunit was found to function as an accessory subunit of the sperm-specific mouse Slo3 channels (Yang et al., 2011).

The BKγ subunits are structurally unrelated to the double-membrane–spanning BK channel β subunits. The four BKγ subunits are similar to each other in terms of their overall protein sequences (Fig. 1), and they all contain an N-terminal signal peptide, a relatively large

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Abbreviations used in this paper: BK, large conductance Ca^{2+}- and voltage-activated potassium; $[Ca^{2+}]_i$, intracellular free calcium; C-tail, C-terminal tail; HA, Horrigan and Aldrich; HEK, human embryonic kidney; LRR, leucine-rich repeat; LRRD, LRR domain; TM, transmembrane.

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extracellular LRR domain (LRRD), a single transmembrane (TM) segment, and a short intracellular C-terminal tail (C-tail) (Figs. 1 and 2). Unlike the complex effects and mechanisms of different BK channel β (BKβ) subunits on many aspects of BK channel gating (Wallner et al., 1999; Brenner et al., 2000; Meera et al., 2000; Xia et al., 2000; Zeng et al., 2003; Savalli et al., 2007; Contreras et al., 2012; Sun et al., 2012), the action of the γ1 subunit is remarkable in its mechanistic simplicity and modulatory magnitude (Yan and Aldrich, 2010; Zhang and Yan, 2014). An analysis of the γ1 subunit's effects on different BK channel gating parameters within the framework of an allosteric Horrigan and Aldrich (HA) model (Horrigan and Aldrich, 2002) suggested that this subunit modulates the BK channel mainly by an ∼20-fold enhancement in the allosteric coupling factor between voltage sensors and the channel pore (Yan and Aldrich, 2010). The γ1 subunit also exhibited an "all-or-none" regulatory effect on BK channels independent of the molar ratio of injected BKα/γ1 RNA in *Xenopus laevis* oocytes (Gonzalez-Perez et al., 2014). This action is fundamentally different from that of BKβ subunits, which regulate the voltage dependence of BK channel activation in a titration-dependent mode (Wang et al., 2002).

In spite of the above interesting structural and functional features of the BKγ subunits, the molecular basis and mechanisms underlying BK channel regulation by BKγ subunits are largely unknown. The four BKγ subunits have distinct capabilities in shifting the voltage dependence of BK channel activation toward hyperpolarizing voltages by ∼140 mV (γ1), 100 mV (γ2), 50 mV (γ3), and 20 mV (γ4) in the absence of calcium (Yan and Aldrich, 2010, 2012). Identification of key structural elements underlying BKγ subunits' different modulatory effects on BK channels could provide important insight into the molecular basis and mechanisms underlying BK channel regulation by BKγ subunits. In this study, we generated chimeric BKγ proteins by swapping individual structural elements among the four BKγ subunits. Together with deletion and point mutations, we systematically analyzed the functional contributions of different structural elements to the BKγ subunits' modulatory effects on BK channel voltage-dependent gating. We found that the differential modulatory functions of BKγ subunits are mainly determined by a small region consisting of the TM segment and its adjacent intracellular cluster of positively charged residues. Our results suggest involvement of major intra- and/or juxtamembrane mechanisms in the auxiliary γ subunits' activating effects on BK channels.

MATERIALS AND METHODS

Expression of BKα and BKγ proteins in human embryonic kidney (HEK)-293 cells

Recombinant cDNA constructs of human BKα (hSlo), γ1 (LRRC26), γ2 (LRRC52), γ3 (LRRC55), and γ4 (LRRC38) subunits were used for heterologous expression studies in HEK-293 cells. To divide

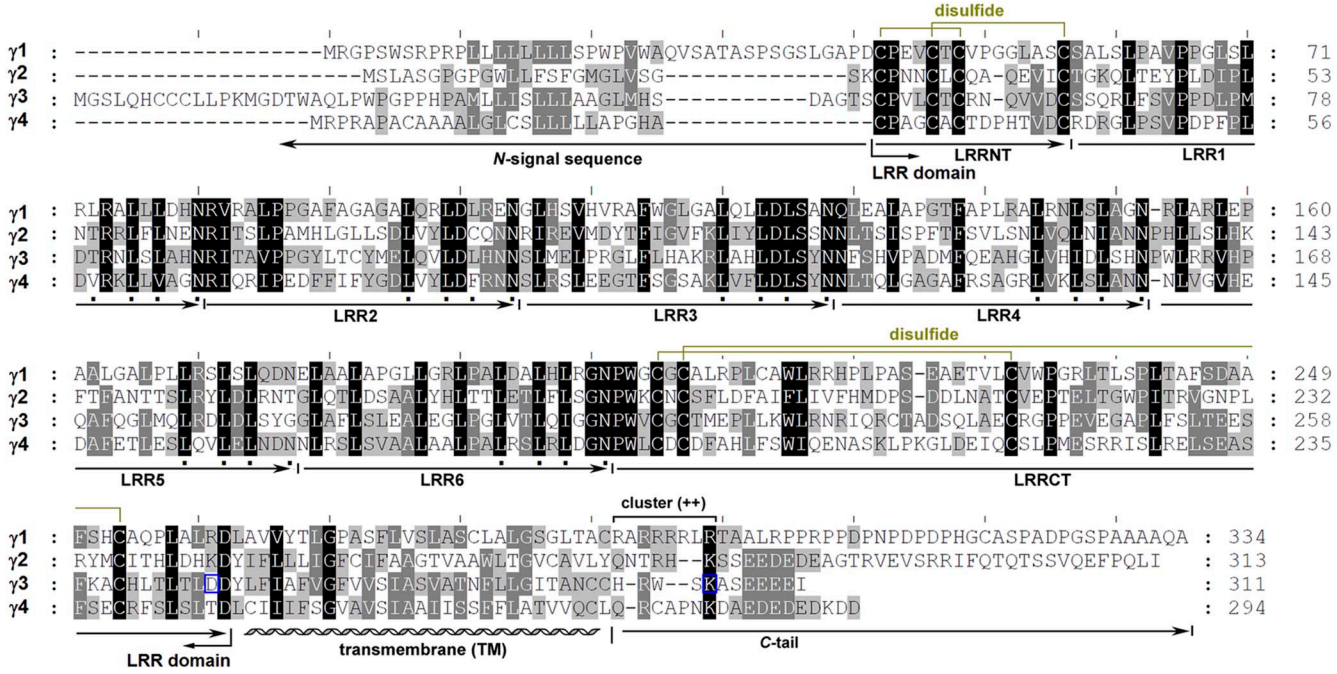

Figure 1. Protein sequence alignment of BKγ subunits in humans. The corresponding amino acid sequences for different domains or segments are assigned and used for the generation of chimeric proteins. Cluster (++) refers to the cluster of positively charged amino acids. The key residues of the consensus sequence (LxxLxLxxN) in each LRR unit are marked by black squares below. Four cysteine pairs for potential disulfide formation are indicated. The two residues in γ3 used for point mutation analyses, D269 and K304, are outlined in blue.

each BKγ subunit into four structural elements for generation of chimeric BKγ proteins, we defined the N-signal peptide sequence as the N-terminal sequence before the first conserved disulfide-forming Cys residue of the LRRNT unit, the TM segment as a relatively hydrophobic region of 29–amino acid residues flanked by charged amino acid residues, LRRD as the sequence located between N-signal peptide and the TM segment, and C-tail as the sequence on the C-terminal side of the TM segment (Fig. 1). Synthetic cDNA sequences of chimeric BKγ subunits were subcloned into the mammalian expression vector of pCDNA6, with V5 tags attached at their C termini. As described previously (Yan and Aldrich, 2010, 2012), fusion cDNA constructs, which encode precursor fusion proteins of human BKα and C-terminal–tagged BKγ proteins, were generated with the pCDNA6 vector and used to facilitate the co-translational assembly of BKα–BKγ protein complexes after endogenous cleavage by peptidases at the linker (signal peptide) region in the mature proteins. This previously established co-translational assembly strategy produced comparably reproducible results as the other strategies that generated over- or super-expression of BKγ relative to BKα in a single HEK-293 cell using either an IRES (internal ribosome entry site)-based single bicistronic expression method or BKα-stable cell line method (Yan and Aldrich, 2010, 2012). HEK-293 cells were obtained from ATCC, transfected with plasmids using Lipofectamine 2000 (Invitrogen), and subjected to electrophysiological assays 16–72 h after transfection.

Electrophysiology

To record the BK channel currents, we used patch-clamp recording in excised inside-out patches of HEK-293 cells with symmetric internal and external solutions of 136 mM $KMeSO_3$, 4 mM KCl, and 20 mM HEPES, pH 7.20. The external solution was supplemented with 2 mM $MgCl_2$, and the internal solution was supplemented with 5 mM HEDTA without Ca^{2+} to create a virtually Ca^{2+}-free solution. Steady-state activation was expressed as the normalized conductance (G/Gmax) calculated from the relative amplitude of the tail currents (deactivation at −120 mV). The voltage of half-maximal activation ($V_{1/2}$) and the equivalent gating charge (z) were obtained by fitting the relations of G/Gmax versus voltage with the single-Boltzmann function $G/Gmax = 1/(1 + e^{-zF(V-V1/2)/RT})$

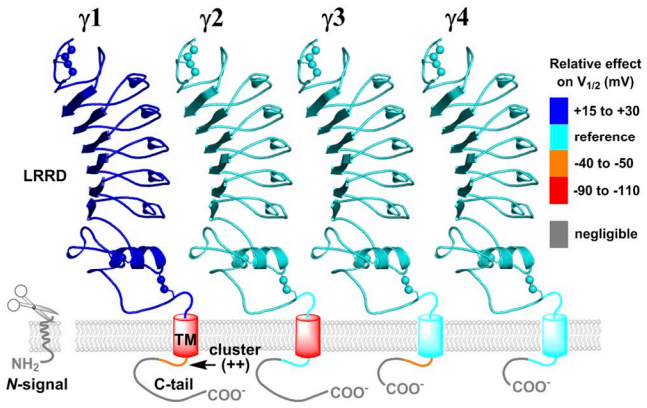

Figure 2. Schematic of the structure, membrane topology, and generalized relative functional contributions of individual structural elements in BKγ subunits. The structural models of the LRRDs were built as described previously (Yan and Aldrich, 2012). The different colors indicate the structural elements' relative contributions (in millivolts) to the BKγ subunit–induced shifts of voltage dependence of BK channel activation. "Reference" means "being used as a reference for comparison," and "negligible" means "can be deleted without influence on modulatory function."

TABLE 1

Boltzmann-fit parameters of the voltage-dependent BK channel activation in the presence of auxiliary BKγ wild types, chimeras, and mutants

Expression	Boltzmann fit parameters		
		z	*n*
	mV		
BKα alone	167 ± 2	1.26 ± 0.07	8
+ γ1	22 ± 4	1.75 ± 0.09	7
+ γ2	61 ± 3	1.17 ± 0.07	8
+ γ3	115 ± 2	1.36 ± 0.05	6
+ γ4	154 ± 3	1.27 ± 0.07	7
+ γ1/γ4-signal	13 ± 3	1.49 ± 0.06	4
+ γ3/γ1-signal	121 ± 5	1.37 ± 0.06	3
+ γ4/γ1-signal	155 ± 1	1.32 ± 0.03	4
+ γ1/γ2-LRRD	−3 ± 3	1.38 ± 0.04	6
+ γ1/γ3-LRRD	6 ± 3	1.52 ± 0.09	8
+ γ1/γ4-LRRD	−12 ± 3	1.66 ± 0.05	11
+ γ1/γ2-TM&tail	90 ± 2	1.28 ± 0.15	4
+ γ1/γ3-TM&tail	151 ± 3	1.48 ± 0.07	6
+ γ1/γ2-tail	143 ± 3	1.26 ± 0.05	4
+ γ1/γ3-tail	25 (75%)[a]	1.71	3
	126 (25%)	1.06	
	28 (16%)	1.33	8
	150 (84%)	0.95	
+ γ1/γ4-tail	147 ± 3	1.23 ± 0.08	5
+ γ2/γ1-tail	17 ± 2	1.86 ± 0.10	3
+ γ2/γ3-tail	21 ± 2	1.67 ± 0.12	4
+ γ2/γ4-tail	68 ± 2	1.12 ± 0.08	5
+ γ3/γ1-tail	120 ± 2	1.81 ± 0.20	3
+ γ3/γ2-tail	141 ± 5	1.66 ± 0.20	3
+ γ3/γ4-tail	156 ± 4	1.40 ± 0.07	3
+ γ1/γ2-TM	32 ± 3	1.40 ± 0.07	5
+ γ1/γ3-TM	135 ± 2	1.24 ± 0.02	3
+ γ1/γ4-TM	155 ± 4	1.30 ± 0.03	5
+ γ2/γ1-TM	155 ± 4	1.33 ± 0.09	6
+ γ2/γ3-TM	155 ± 5	1.54 ± 0.07	4
+ γ2/γ4-TM	158 ± 7	1.46 ± 0.10	4
+ γ3/γ1-TM	165 ± 4	1.14 ± 0.05	5
	119	1.11	1
+ $\gamma3^{D269R}$/γ1-TM	138 ± 6 (36%)	1.12 ± 0.10	7
	62 ± 3 (64%)	1.11 ± 0.05	
+ $\gamma3^{D269R}$	117 ± 3	1.36 ± 0.11	5
+ γ3/γ2-TM	26 ± 2	1.44 ± 0.06	5
+ γ3/γ4-TM	160 ± 4	1.38 ± 0.06	4
	113 ± 4	1.15 ± 0.11	4
+ γ4/γ2-TM	72 ± 3	1.27 ± 0.06	5
+ γ1-$\Delta tailC^{302–334}$	29 ± 4	1.40 ± 0.09	4
+ γ1-$\Delta tailN^{291–298}$	168 ± 3	1.22 ± 0.11	5
+ γ2-$\Delta tailN^{277–279}$	73 ± 3	1.12 ± 0.04	7
+ γ2-$\Delta tailC^{280–313}$	63 ± 2	1.28 ± 0.09	4
+ γ2-$\Delta tail^{277–313}$	166 ± 6	1.16 ± 0.01	3
+ γ3-$\Delta tailC^{305–311}$	109 ± 3	1.56 ± 0.12	4
+ γ3-$\Delta tailN^{298–304}$	180 ± 3	1.26 ± 0.12	5
+ $\gamma3^{K304N}$	144 ± 2	1.34 ± 0.12	4

n values are the number of recorded excised inside-out patches from different HEK-293 cells.

[a]The indicated percentage in parentheses refers to the portion of the channels' subpopulation that was obtained from a double-Boltzmann function fit.

or with the double-Boltzmann function G/Gmax = a/(1 + $e^{-za_1F(V-Va1/2)/RT}$) + (1 − a)/(1 + $e^{-zb_1F(V-Vb1/2)/RT}$). Experimental values are reported as the mean ± the SEM.

RESULTS

Differential modulation of BK channel voltage-dependent gating by the BKγ subunits

We observed that the four BKγ subunits, when heterologously expressed in HEK-293 cells, produced distinct shifts toward hyperpolarizing potentials in the voltage dependence of BK channel activation (Fig. 3, A and B), which is similar to the findings of a previous report (Yan and Aldrich, 2012). The half-activation voltages ($V_{1/2}$) of BK channels were shifted by 145 ± 5 mV by γ1 ($V_{1/2}$ = 22 ± 4), 107 ± 4 mV by γ2 ($V_{1/2}$ = 61 ± 3 mV), 52 ± 3 mV by γ3 ($V_{1/2}$ = 115 ± 2 mV), and 13 ± 4 mV by γ4 ($V_{1/2}$ = 154 ± 3 mV), compared with the BKα channel alone ($V_{1/2}$ = 167 ± 2 mV) in the virtual absence of $[Ca^{2+}]_i$. The equivalent gating charge (*z*) of BK channel voltage gating was largely unaffected by the γ2 (*z* = 1.17 ± 0.07 e), γ3 (*z* = 1.36 ± 0.05 e) and γ4 (*z* = 1.27 ± 0.07 e) subunits but markedly increased by the γ1 subunit (*z* = 1.75 ± 0.09e), compared with the BKα channel alone (*z* = 1.26 ± 0.07 e) in the virtual absence of $[Ca^{2+}]_i$. For comparison, the Boltzmann fit parameters of the voltage-dependent BK channel activation in the presence of auxiliary BKγ wild types, as well as all chimeras and mutants discussed below, are given in Table 1.

We first determined whether the BKγ subunits' distinct capabilities in modulating BK channel voltage-dependent gating are intrinsic properties of their mature proteins or are caused by differences in their signal peptide functions. We generated chimeric BKγ proteins of the γ3/γ1-signal and γ4/γ1-signal by replacing the N-terminal signal peptide sequences in γ3 and γ4 with that of γ1. Similarly, we generated chimeric BKγ proteins of the γ1/γ4-signal by using the γ4 N-terminal signal peptide sequence to guide the expression of γ1 protein. We observed that swapping the N-terminal signal peptide regions, which elicited changes of <10 mV in the BK channel

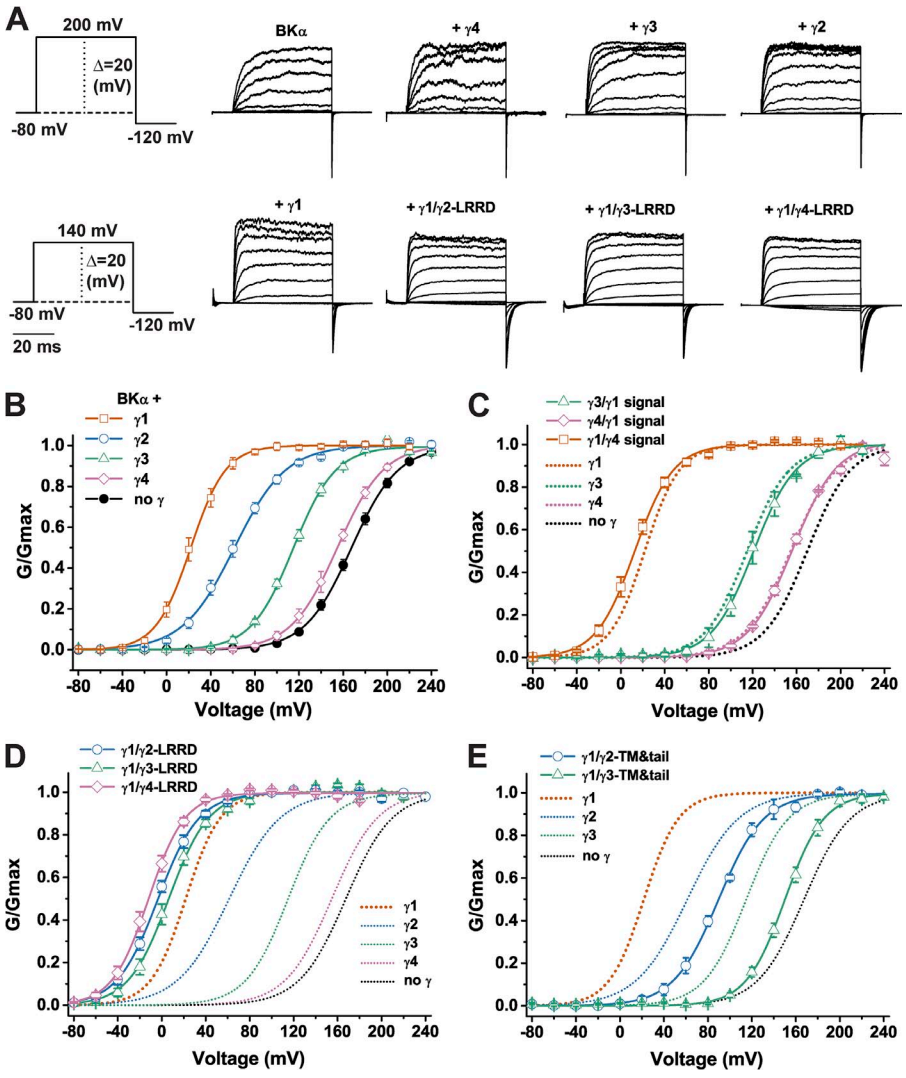

Figure 3. Effects of swapping N-signal peptides and LRRDs on the modulatory functions of BKγ subunits. (A) Representative traces of recorded BK channel currents in response to the depolarization of the membrane potential from −80 mV in 20-mV steps for selected expression constructs. (B–E) Voltage dependence of BK channel activation (plotted from tail currents at −120 mV) in the absence and presence of wild-type BKγ subunits (B); in the presence of γ1 N-signal peptide–swapping chimera (C); in the presence of LRRD-swapping chimera (D); and in the presence of TM and C-tail region–swapping chimera, whose main bodies were from γ1 and whose TM and C-tail regions together were from other BKγ subunits (E). For all indicated given names of chimeric BKγ subunits, the acceptor subunit used to generate the main protein body appears before the slash, and the donor subunit used for a swapped domain or region together with the name of swapped domain or region appears after the slash. All BK channel currents were recorded in the virtual absence of $[Ca^{2+}]_i$ and plotted as the normalized conductance (G/Gmax) against different membrane voltages. Thick dot lines are used for the major wild-type BKγ protein(s) intended for comparison. Error bars represent ± SEM.

$V_{1/2}$ values, had no significant effect on the BKγ subunits' modulatory functions (Fig. 3 C). The BK channel $V_{1/2}$ values in the virtual absence of $[Ca^{2+}]_i$ were 121 ± 5 mV with the γ3/γ1-signal, $V_{1/2}$ = 155 ± 1 mV with the γ4/γ1-signal, and $V_{1/2}$ = 13 ± 3 mV with the γ1/γ4-signal (Table 1). These results indicate that the much weaker modulatory functions of the γ3 and γ4 subunits, as compared with that of the γ1 subunit, are intrinsic properties of the subunits' mature proteins and independent of their N-terminal signal peptide sequences.

LRRD does not determine the modulatory functions of different BKγ subunits

The relatively large LRRD comprises six tandem LRR structural units (LRR1–6) and two cysteine-rich modules, LRRNT and LRRCT, which are capped on the N- and C-terminal sides, respectively (Fig. 1). We found that substituting the LRRD of γ1 with those of the other three BKγ subunits resulted in no reduction but instead slight to moderate gain-of-function in the γ1 subunit's modulatory effect on BK channel voltage-dependent gating (Fig. 3, A and D). Compared with the unaltered γ1 protein ($V_{1/2}$ = 22 ± 4 mV), the resultant LRRD-swapping chimeras of γ1/γ2-LRRD ($V_{1/2}$ = −3 ± 3 mV), γ1/γ3-LRRD ($V_{1/2}$ = 6 ± 3 mV), and γ1/γ4-LRRD ($V_{1/2}$ = −12 ± 3 mV) caused further shifts of 25, 16, and 34 mV, respectively, in the voltage dependence of BK channel activation toward hyperpolarizing potentials (Fig. 3 D). Because the other BKγ subunits are weaker modulators than γ1 in terms of shifting the voltage dependence of BK channel activation, this result contradicts what one would expect if the LRRD were a determinant of the subunits' different modulatory functions. In contrast, replacing the TM and C-tail regions of γ1 with those of γ2 or γ3 caused a 47% reduction ($V_{1/2}$ = 90 ± 2 mV with γ1/γ2-TM&tail) or a 89% loss ($V_{1/2}$ = 151 ± 3 mV with γ1/γ3-TM&tail) of the γ1 subunit's modulatory function (Fig. 3 E), which suggests that the TM and C-tail regions determine the BKγ subunits' modulatory functions. The modulatory effects of the γ1/γ2-TM&tail and γ1/γ3-TM&tail chimeras were even smaller than those of the unaltered γ2 and γ3 subunits, respectively. These results suggest that compared with the LRRDs of other BKγ subunits, the γ1 LRRD plays a slightly larger inhibitory or less stimulatory role in shifting the BK channel voltage-dependent gating toward hyperpolarizing potentials (Fig. 2).

C-tails partially contribute to the difference in modulatory functions among BKγ subunits

The short C-tails of the four BKγ subunits have different lengths and very low sequence similarities. According to our assignments of the C-terminal borders of the neighboring TM segments, the γ1, γ2, γ3, and γ4 C-tails have 44, 40, 12, and 18 amino acid residues, respectively (Fig. 1). Substituting the C-tail of γ2 with those of the other three BKγ subunits produced fully functional chimeric proteins (Fig. 4 A). The modulatory effect of the γ2/γ4-tail chimera ($V_{1/2}$ = 68 ± 2 mV) was nearly the same as that of unaltered γ2, which suggests that the γ4 C-tail functions similarly as the γ2 C-tail. In contrast, both the γ1 and γ3 C-tails potentiated the γ2 acceptor subunit's modulatory function by approximately −40-mV shifts in the BK channel $V_{1/2}$ (17 ± 2 mV with γ2/γ1-tail and 21 ± 2 mV with γ2/γ3-tail), which became more like that produced by γ1. These modifications in $V_{1/2}$ were accompanied by an increase in the steepness of the G-V

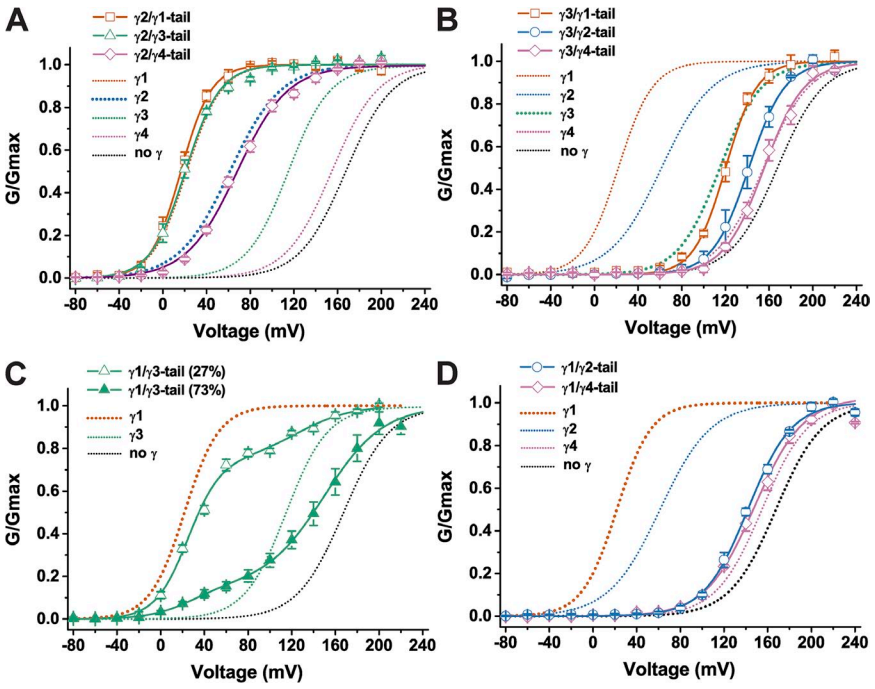

Figure 4. Effects of swapping C-tail regions on the modulatory functions of BKγ subunits. Voltage dependence of BK channel activation in the presence of BKγ subunit chimeras, whose C-tail regions were from different BKγ subunits and whose main bodies were from γ2 (A), γ3 (B), and γ1 (C and D). Thick dot lines are used for the major wild-type BKγ protein(s) intended for comparison. Error bars represent ± SEM.

relationship curve (z = 1.86 ± 0.1 e with γ2/γ1-tail and z = 1.67 ± 0.12 e with γ2/γ3-tail, as compared with z = 1.17 ± 0.07 e with γ2), which approached that of γ1 (z = 1.75 ± 0.09 e). These results suggest that the γ1 and γ3 C-tails are similarly effective, whereas the γ2 and γ4 C-tails are similarly less effective or ineffective, in their contributions to the BKγ subunits' modulatory functions.

Consistent with the above inference, the γ1 C-tail did not cause a significant (i.e., ≥15 mV in $V_{1/2}$) change in the γ3 acceptor subunit's function ($V_{1/2}$ = 120 ± 2 mV with γ3/γ1-tail). In contrast, the γ2 and γ4 C-tails both markedly reduced the γ3 acceptor subunit's modulatory function ($V_{1/2}$ = 141 ± 5 mV with γ3/γ2-tail and $V_{1/2}$ = 156 ± 4 mV with γ3/γ4-tail) to levels similar to that of the weaker γ4 ($V_{1/2}$ = 154 ± 3 mV) (Fig. 4 B). The ∼40-mV difference between the pair of the γ1 and γ3 C-tails and the pair of the γ2 and γ4 C-tails in their influences on the γ2 or γ3 acceptor's modulatory functions agrees well with the differences between γ1 and γ2 and between γ3 and γ4 in their abilities to shift the voltage dependence of BK channel activation. These results indicate that although the C-tail region is not a major determinant of the overall large difference in modulatory functions across the four BKγ subunits, it is very likely a key determinant of the functional differences between γ1 and γ2, and between γ3 and γ4.

γ1 TM segment and other BKγ subunits' C-tails are incompatible

The above results with C-tail–swapped chimeric BKγ proteins showed that the C-tails of γ1 and γ3 function similarly in the presence of the main bodies (i.e., the LRRDs and TM segments) of γ2 and γ3. However, upon replacement of the γ1 C-tail with the γ3 C-tail, the resultant γ1/γ3-tail chimera caused the G-V relationships of BK channels to be fitted to at least two populations of BK channels in all recorded 11 excised patches (Fig. 4 C). In three patches, the G-V relationships were best fitted to a major population (∼75%) of low voltage–activated channels ($V_{1/2}$ of ≈25 mV), similar to those associated with the unaltered γ1 subunit (BKα/γ1) and to a minor population (∼25%) of high voltage–activated channels more similar to those of BKα alone. In the other eight patches, the G-V relationships were best fitted to a minor population (16%) of low voltage–activated BKα/γ1-type channels and to a major population (84%) of high voltage–activated BKα-type channels. These two distinct G-V relationships for different membrane patches have been observed in the same batch of transfected cells. These results suggest that the γ3 C-tail did function as the γ1 C-tail in the γ1/γ3-tail chimera in about half of the recorded BK channels. However, in the other half of the recorded BK channels, the γ3 C-tail caused a drastic reduction (72–88%) in the γ1's modulatory function. Moreover, both the γ2 and γ4 C-tails caused an ∼85% loss of the γ1 acceptor subunit's modulatory function ($V_{1/2}$ = 143 ± 3 mV with γ1/γ2-tail and $V_{1/2}$ = 147 ± 3 mV with γ1/γ4-tail) (Fig. 4 D). Given that the γ1 C-tail in the presence of γ2 and γ3 TM segments produced only a limited contribution (i.e., approximately −40 mV) to the shift of the BK channel $V_{1/2}$, the γ1 C-tail is unlikely a sole determinant of γ1's modulatory function. The drastic reduction in the γ1 subunit's modulatory function upon replacement of its C-tail with those of the other γ subunits was likely caused by some structural or functional incompatibility (i.e., antagonistic effect) between the other γ subunits' C-tails and other regions of the γ1 subunit.

Although both γ1 and γ2 modulate the BK channel very effectively, the γ2/γ1-TM chimera, which resulted from γ1's TM segment being in the presence of γ2's LRRD and C-tail, was ineffective (i.e., the resultant $V_{1/2}$ is within 15 mV of that produced by BKα alone) in modulating BK channel voltage gating ($V_{1/2}$ = 155 ± 4 mV) (Fig. 5 A), suggesting that incompatibility exists between the γ1 TM segment and either the γ2 LRRD or the γ2 C-tail. Given that the γ2 LRRD was fully compatible (i.e., no obvious antagonistic effect) with the rest of γ1 in the γ1/γ2-LRRD chimera (Fig. 3 D) and that the γ1/γ2-tail chimera was only slightly effective (i.e., the resultant $V_{1/2}$ is within 15–30 mV of that produced by BKα alone) in modulating the BK channel (Fig. 4 D), this incompatibility must exist between the γ1 TM segment and the γ2 C-tail.

Unexpectedly, replacing the TM segment of γ3 with that of γ1 created a γ3/γ1-TM chimera that completely failed to potentiate γ3's modulatory function and instead caused a loss of γ3's modulatory function in most recorded excised patches ($V_{1/2}$ = 165 ± 5 mV, n = 5; $V_{1/2}$ = 119 mV, n = 1) (Fig. 5 A and Table 1). This outcome suggests that in addition to the partial incompatibility that exists between the γ1 TM segment and the γ3 C-tail, functional incompatibility exists between the γ1 TM segment and the γ3 LRRD. γ3 has a negatively charged residue, D269, which in γ1 is a positively charged residue, R260, located immediately on the N-terminal side of the TM segment (Fig. 1). We arbitrarily included these residues' positions in the LRRDs to construct chimeric BKγ proteins (Fig. 1). The D269R mutation itself had no effect on γ3's modulatory function (Fig. 5 A), but the γ1 TM segment became partially effective in potentiating the $\gamma3^{D269R}$ mutant's modulatory function. The resultant G-V relationship of the BK channels in the presence of the $\gamma3^{D269R}$/γ1-TM chimera could be fitted with a double-Boltzmann function to a major population (64%) of low voltage–activated channels ($V_{1/2}$ = 62 mV, z = 1.1 e) and to a minor population (36%) of high voltage–activated channels ($V_{1/2}$ = 138 mV, z = 1.1 e) (Fig. 5 A and Table 1). Therefore, in the presence of the γ3 C-tail, the γ1 TM segment is also incompatible with the extracellular γ3 juxta-membrane charged residue D269. Overall, the γ1 subunit has a special TM segment that is

fully compatible with the γ1 C-tail, partially compatible with the γ3 C-tail, and fully incompatible with the γ2 and γ4 C-tails.

The TM segment is a key determinant of BKγ subunits' different modulatory functions

The TM segment of γ2, unlike that of γ1, was fully compatible with other BKγ subunits' C-tails. Upon being swapped with other BKγ subunits, the γ2 TM segment fully potentiated the γ3 and γ4 subunits' modulatory functions ($V_{1/2}$ = 26 ± 2 mV with γ3/γ2-TM and $V_{1/2}$ = 72 ± 3 mV with γ4/γ2-TM) to levels nearly as high as those of γ1 and γ2, respectively, but caused little change in γ1's function ($V_{1/2}$ = 32 ± 3 mV with γ1/γ2-TM) (Fig. 5 B). This observation indicates that in the presence of the γ1 or γ3 C-tail, the γ2 TM segment is as effective as the γ1 TM segment in modulating BK channel gating. However, replacing the TM segments of γ1 and γ2 with those of γ3 and γ4 resulted in a 78–92% reduction of γ1 and γ2's modulatory functions, which became more similar to those of γ3 and γ4 (Fig. 5, C and D). The resultant BK channel $V_{1/2}$ values were 135 ± 2 mV with γ1/γ3-TM, 155 ± 4 mV with γ1/γ4-TM, 155 ± 5 mV with γ2/γ3-TM, and 158 ± 7 mV with γ2/γ4-TM (Table 1). The smaller modulatory effect of the γ1/γ3-TM chimera compared with that of γ3 was likely caused by the functional difference between the γ1 and γ3 LRRDs. Therefore, the γ3 and γ4 TM segments are similarly ineffective in modifying BK channel voltage gating in the presence of the γ2 or γ4 C-tails, as indicated by similarly high $V_{1/2}$ values with γ4 and chimeras of γ2/γ3-TM, γ2/γ4-TM, and γ3/γ4-tail. Nevertheless, in the presence of the γ1 or γ3 C-tail, the γ3-TM segment appeared to be slightly more effective than the γ4-TM segment. There was approximately −20-mV difference in the resultant BK channel $V_{1/2}$ between γ1/γ3-TM and γ1/γ4-TM. Likewise, replacing the TM segment of γ3 with that of γ4 caused the functional conversion of γ3 to be more like that of γ4 (160 ± 4 mV with γ3/γ4-TM) in four of the eight examined excised patches (Fig. 5 D). Collectively, the above results suggest that the TM segment is a key determinant of the difference in the modulatory functions between the potent modulators γ1 and γ2 and the much weaker modulators γ3 and γ4.

Effects of the TM segment, C-tail, and LRRD on BK channel voltage-dependent gating

To thoroughly compare the contributions of the LRRD, C-tail, and TM segment to the BKγ proteins' modulatory functions on BK channels, we grouped and plotted the shifts in $V_{1/2}$ ($\Delta V_{1/2}$) of the BK channels caused by all wild-type and chimeric BKγ proteins, except those containing the incompatible γ1-TM segment and other BKγ subunits' C-tails (Fig. 6). The BK channel $V_{1/2}$ values are first determined by the TM segment. The differences between the γ1- and γ2-TM segments in their effects on BK channel $V_{1/2}$ were indistinguishable (i.e., <10 mV) or small (i.e., 10–20 mV), i.e., −9 mV between γ1 and γ1/γ2-TM and −20 mV between γ1/γ2-LRRD and γ2/γ1-tail (Fig. 6). As noted above, the differences between the γ3- and γ4-TM segments in their effects on the BK channel $V_{1/2}$ in the presence of the γ1 or γ3 C-tail were limited.

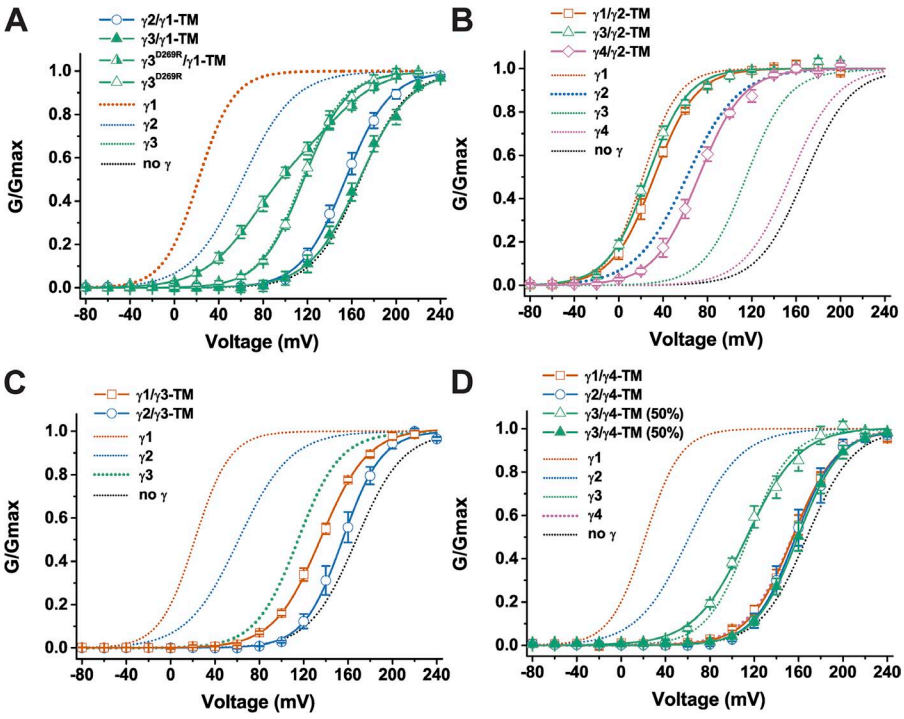

Figure 5. Effects of swapping TM segments on the modulatory functions of BKγ subunits. Voltage dependence of BK channel activation in the presence of BKγ subunit chimeras, whose main bodies were from different γ subunits and whose TM segments were from γ1 (A), γ2 (B), γ3 (C), and γ4 (D). Thick dot lines are used for the major wild-type BKγ protein(s) intended for comparison. Error bars represent ± SEM.

However, the difference between these two pairs of TM segments (γ1 and γ2 vs. γ3 and γ4) in their effects on the BK channel $V_{1/2}$ was very large (~100 mV). The differences between the γ1- and γ3-TM segments in their effects on the BK channel $V_{1/2}$ were more than −110 mV, for example, a −112-mV difference between γ1 and γ1/γ3-TM and a −114-mV difference between γ1/γ3-LRRD and γ3/γ1-tail. Similarly, the average difference between the γ2- and γ3-TM segments in their effects on BK channel $V_{1/2}$ was about −95 mV, with differences of −103 mV between γ1/γ2-TM and γ1/γ3-TM, −89 mV between γ3/γ2-TM and γ3, and −94 mV between γ2 and γ2/γ3 TM. The differences between the γ2- and γ4-TM segments in their effects on the BK channel $V_{1/2}$ were −97 mV between γ2 and γ2/γ4-TM and −82 mV between γ4/γ2-TM and γ4.

The shifts in BK channel $V_{1/2}$ values were second determined by the C-tail regions (Fig. 6). The differences between the γ1 and γ3 C-tails and between the γ2 and γ4 C-tails in their effects on the BK channel $V_{1/2}$ were nearly indistinguishable (i.e., <10 mV) in the presence of the γ2-TM segment; for example, the differences between γ2/γ1-tail and γ2/γ3-tail and between γ2 and γ2/γ4-tail were −4 and −7 mV, respectively. Nevertheless, the differences between these two pairs of C-tails in their effects on BK channel $V_{1/2}$ were at least as high as 45 mV, including differences of −58 mV between γ1/γ2-TM and γ1/γ2-TM and C-tail (γ1 C-tail vs. γ2 C-tail), −45 mV between γ2/γ1 C-tail and γ2 (γ1 C-tail vs. γ2 C-tail), and −48 mV between γ2/γ3-tail and γ2/γ4-tail (γ3 C-tail vs. γ4 C-tail). In the presence of the γ3 TM segment, the differences between the C-tails of individual subunits within each subunit pair (i.e., γ1 and γ3; γ2 and γ4) in their effects on the BK channel $V_{1/2}$ were either small or indistinguishable (e.g., 5 mV between γ3/γ1-tail and γ3 and −16 mV between γ1/γ3-TM and γ1/γ3-TM and C-tail for the difference between the γ1 and γ3 C-tails, and −15 mV between γ3/γ2-tail and γ3/γ4-tail for the difference between the γ2 and γ4 C-tails). The differences between these two pairs of C-tails in their effects on the BK channel $V_{1/2}$ were still significant (~20–40 mV) in the presence of the γ3- or γ4-TM segments (e.g., −41 mV between γ3 and γ3/γ4-tail for the difference between γ3 and γ4 C-tails, −21 mV between γ3/γ1-tail and γ3/γ2-tail for the difference between the γ1 and γ2 C-tails, and −26 mV between γ3 and γ3/γ2 C-tail for the difference between the γ3 and γ2 C-tails), although overall they were smaller than those in the presence of the γ2-TM segment. Therefore, in the presence of different BKγ (i.e., γ2, γ3, and γ4) TM segments, we observed a similar trend: the C-tails of individual subunits within each subunit pair (i.e., γ1 and γ3; γ2 and γ4) had more similar functionality, whereas the two pairs themselves differed significantly in functionality.

Collectively, the shifts in BK channel $V_{1/2}$ values were least affected by differences in LRRDs (Fig. 6). An analysis of six pairs of comparable chimeras formed with a TM segment and C-tail from the same BKγ subunit indicated that the LRRDs of γ2, γ3, and γ4 had little difference in their influences on the BK channel $V_{1/2}$ values, eliciting an average change of only 10 mV. A slightly larger difference of 18 mV was observed only between the γ3 and γ4 LRRDs in the presence of the γ1-TM segment and C-tail (i.e., γ1/γ3-LRRD vs. γ1/γ4-LRRD). However, compared with the LRRDs of the other three BKγ subunits, the γ1 LRRD caused a larger shift in the BK channel $V_{1/2}$ of 14–36 mV (mean, 23 mV) toward depolarizing voltages among seven pairs of comparable chimeras. Therefore, LRRDs are not major determinants of the BKγ subunits' different modulatory functions, and they instead may slightly alleviate the functional difference between γ1 and other BKγ subunits.

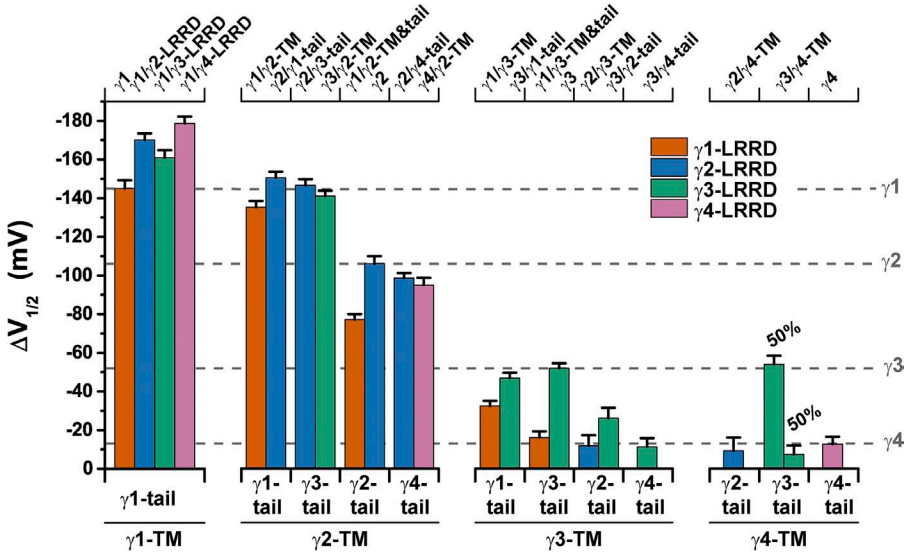

Figure 6. Shifts in BK channel $V_{1/2}$ ($\Delta V_{1/2}$) values caused by different wild-type, chimeric, or mutant BKγ proteins. The names of the BKγ proteins appear above the graph, and the corresponding compositions of the TM and C-tail regions from different BKγ subunits appear below the graph. Each LRRD is indicated by a different color. The $\Delta V_{1/2}$ values with BKγ wild types are indicated by dotted gray lines. Error bars represent ± SEM.

Juxta-membrane positively charged residues determine the C-tail functions of γ1 and γ3

Each of the four BKγ subunits' C-tails contains a cluster of positively charged residues that is adjacent to the C terminus of the TM segment (Fig. 1). This residue cluster might be involved in the proper insertion and orientation of the TM segment, which follows the general "positive-inside rule" (White and von Heijne, 2004). The juxta-membrane cluster of positively charged amino acids in γ1 is heavily charged with six arginine residues within a peptide sequence of eight residues (^{291}RARRRRLR^{298}), whereas the clusters in γ2 and γ3 contain only one arginine, one lysine, and one histidine in short sequences of ^{277}RHK^{279} (γ2) and ^{298}HRWSK^{304} (γ3). To determine whether these clusters are major contributors to the modulatory functions of C-tails, we performed deletion and substitution mutations in the C-tail regions and assessed the mutated C-tails' effects on BK channel voltage-dependent gating. Deleting most or all residues on the C-terminal side of the clusters in γ1 and γ3 did not significantly change the subunits' modulatory functions ($V_{1/2}$ = 29 ± 4 mV with γ1-ΔtailC$^{302–334}$ and $V_{1/2}$ = 109 ± 3 mV with γ3-ΔtailC$^{305–311}$) (Fig. 7 A). However, similar to a previous report (Yan and Aldrich, 2012), deletion of the polyarginine region in γ1 caused a 100% loss of γ1's modulatory function ($V_{1/2}$ = 168 ± 3 mV with γ1-ΔtailN$^{291–298}$). These results suggest that the juxta-membrane positively charged region is more than an important contributor to the γ1-induced shift in BK channel voltage-dependent gating; rather, the region is essential for the whole γ1 protein function, presumably by determining the protein's proper folding, surface expression, or assembly with BKα. Similarly, deletion of the positively charged region ^{298}HRWSK^{304} in γ3 resulted in a loss of γ3's modulatory function, yielding a $V_{1/2}$ (180 ± 3 mV with γ3-ΔtailN$^{298–304}$) close to that of BKα alone (Fig. 7 A). Neutralization of the last lysine residue (K304) in this cluster also reduced γ3's modulatory effect on the BK channel $V_{1/2}$ by nearly 30 mV ($V_{1/2}$ = 144 ± 2 mV with γ3^{K304N} mutant vs. 115 ± 2 mV with wild type) (Fig. 7 A).

For the γ2 C-tail, the deletion of either the three-residue cluster ^{277}RHK^{279} or the rest of the C-tail region to the C-terminal end alone did not significantly alter γ2's modulatory function ($V_{1/2}$ = 73 ± 3 mV with γ2-ΔtailN$^{277–279}$ and $V_{1/2}$ = 63 ± 2 mV with γ2-ΔtailC$^{280–313}$). However, the deletion of both regions resulted in a 100% loss of γ2's modulatory function ($V_{1/2}$ = 166 ± 6 mV with γ2-Δtail$^{277–313}$) (Fig. 7 B). These results suggest that some amino acid residues on the C-tail—not necessarily the positively charged residues immediately following the TM segment—are still needed for γ2 to be functional. There are multiple positively charged and negatively charged residues on the C-terminal side (residues 280–313) of the three-residue cluster ^{277}RHK^{279} in γ2's C-tail region.

DISCUSSION

In this study, by swapping structural elements among BKγ subunits and by performing deletion and substitution mutations, we were able to systematically analyze the functional contributions of the BKγ subunits' individual structural elements to the subunits' different modulatory functions. We identified the TM segment and the adjacent intracellular clusters of positively charged amino acids as key determinants of the distinct effects that different BKγ subunits have on BK channel voltage gating. Our results suggest that BK channel modulation by BKγ subunits involves major intra- and/or juxta-membrane mechanisms. Our findings also reveal a strategy in which the modulatory functions of regulatory membrane proteins of voltage-gated ion channels can be coarse- and fine-tuned, respectively, through variations in their TM segments and adjacent intracellular positively charged region.

A previous study showed that deletion of a signal peptide, TM segment, or C-tail, or even a single LRR unit of the LRRD, resulted in a nearly 100% loss of the γ1's modulatory function (Yan and Aldrich, 2010), which could be complicated by disruptions in the subunit's 3-D structure. To maintain structural integrity, in this study, we systematically swapped individual structural elements among the four BKγ subunits to identify the molecular determinants of their different modulatory

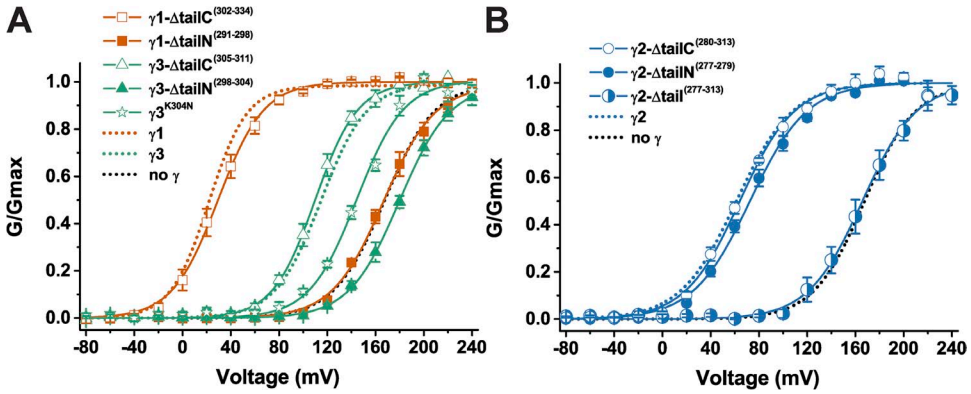

Figure 7. Effects of deletion and substitution mutations within the C-tail regions on the modulatory functions of BKγ subunits. The voltage dependence of BK channel activation in the presence of γ1 and γ3 (A) or in the presence of γ2 (B) mutants is shown. Error bars represent ± SEM.

functions. Of the 37 BKγ chimera or mutants we examined, 20 effectively modulated BK channels and produced G-V curves that could be fitted by a single Boltzmann function with a normal slope ($z > 1$) and more than 30-mV shifts in $V_{1/2}$ compared with that formed by BKα alone. According to the previously described "all-or-none" gating shift for the γ1 subunit (Gonzalez-Perez et al., 2014), the property of effective shift in $V_{1/2}$ and the resultant normal G-V slope is a good indication that these 20 BKγ chimera or mutants formed functional complexes with BKα that reflect full stoichiometric assembly. Indeed, four of the other chimera and mutants resulted in two populations of channels, one with a significant shift in voltage-dependent gating and the other with a voltage dependence more similar to the channel formed by BKα alone. Similar variable ratio of two populations of BK channels, whose G-V curves were either shifted to a full extent or unchanged ("all-or-none"), was reported when the molar ratio of the injected BKα/γ1 RNA to *Xenopus* oocytes was varied (Gonzalez-Perez et al., 2014). Therefore, these proteins were likely partially defective in folding, surface expression, or proper assembly with BKα. For three of these four chimera and mutants, we unexpectedly observed some patch-to-patch variation that resulted in two distinct G-V relationships; e.g., the recorded BK channels were dominated by a low $V_{1/2}$ population in three excised membrane patches but by a high $V_{1/2}$ population in another eight patches in the presence of γ1/γ3-tail (Table 1). This variation might arise from patch-to-patch variation caused by differences in cell membrane composition (such as the presence or absence of lipid rafts) and/or cell-to-cell variation in protein trafficking machinery. The remaining 13 BKγ chimera or mutants resulted in $V_{1/2}$ values within 30 mV of that produced by the BKα alone; these proteins may be only slightly effective in modifying the BK channel voltage gating or may have failed to fold, express on the cell surface, or assemble with BKα properly. Because we designed the present study to identify key determinants of the apparent differential modulatory effects of the four BKγ subunits on BK channels, we consider these non- or less-modulatory BKγ chimera or mutants, regardless of the causes, still useful when comparing with other chimeras or mutants for the apparent differential modulatory effects.

The BKγ subunits are type I single-pass TM proteins. Similar to the reported essential role of the N-terminal signal peptide in γ1 function (Yan and Aldrich, 2012), we expect that those of other BKγ subunits also play a key role in the subunits' maturation and proper surface expression. An ineffective signal peptide can cause a defect in cellular localization or protein folding, leading to a dysfunctional protein. The sequences of the four BKγ subunits' N-terminal signal peptide regions have very little similarity (Fig. 1). By swapping distinct signal peptide regions among BKγ proteins, we first demonstrated that the BKγ proteins' different modulatory functions are intrinsic properties of the proteins and are independent of the proteins' signal peptide sequences. The four BKγ subunits are closely related to each other mainly because of their LRRDs, which share a considerable level of sequence identity (31–37%) or similarity (43–48%) and have been predicted to have overall similar 3-D structures (Yan and Aldrich, 2012) (Figs. 1 and 2). We initially expected all four BKγ subunits' LRRDs to play a critical role in determining their subunits' differential modulatory functions, but to our surprise, only the γ1 LRRD was slightly different from the other LRRDs in function in BKγ chimeric proteins. The γ1 LRRD may play a slightly inhibitory role, or the other BKγ subunits' LRRDs may play a slightly stimulatory role, in BK channel voltage-gated activation.

On the basis of the results of our functional analyses of the LRRDs, TM segments, and C-tails in the BKγ subunit chimeras, we concluded that the TM and the C-tail regions are the main determinants of the four BKγ subunits' differential modulatory functions. As seen in Fig. 6, the differences in the different γ subunit–induced shifts of the BK channel $V_{1/2}$ are first determined by the TM segments, in which the γ1 and γ2 TMs produced low $V_{1/2}$ BK channels, whereas the γ3 and γ4 TM segments all resulted in high $V_{1/2}$ channels. The differences between the TM segments of γ1 and γ2 and between those of γ3 and γ4 in their effects on BK channel $V_{1/2}$ were small, but the difference between the TM segments of the two BKγ subunit pairs themselves was large (∼100 mV). The C-tails further adjust the modulatory functions of the four BKγ subunits. Although the differences between the C-tails of γ1 and γ3 and between those of γ2 and γ4 in their effects on BKγ modulatory functions were small, the difference between the two pairs' C-tails in their effects on BK channel $V_{1/2}$ could be big (∼40–50 mV), which could be largely accounted for by the different modulatory functions between γ1 and γ2 and between γ3 and γ4. We also found that the functions of the γ1 and γ3 C-tails are determined by the juxta-membrane clusters of positively charged amino acids. On the basis of these findings, we generalized the relative contributions of the four BKγ subunits' individual structural elements to the subunits' modulatory functions in BK channel voltage-dependent gating (Fig. 2).

Our findings demonstrate that the TM segments of γ1 and γ2 play a major role in setting these subunits' high modulatory efficacies, and that the γ1 and γ3 subunits' juxta-membrane clusters of positively charged amino acids also significantly contribute to these subunits' modulatory functions. It was reported previously that only the TM segment is critical for the physical association of γ1 with BKα (Yan and Aldrich, 2010). Therefore, the physical association or interaction between the BKγ TM segments and BKα may be a main mechanism by which the γ1 and γ2 subunits modulate BK channel

voltage-dependent gating. In principle, this possibility is consistent with the previous notion that γ1 mainly affects the allosteric coupling (D-factor) between the voltage-sensor domain and the pore (Yan and Aldrich, 2010), which likely occurs in the membrane. The other BKγ subunits, particularly γ2, whose TM segment is similar to that of γ1 in function, may have a similar mechanism underlying their BK channel modulation. According to the HA allosteric model (Horrigan and Aldrich, 2002), an increase in D-factor may slightly steepen the slope of the G-V relationship curve, as reflected by an increased z value obtained from a Boltzmann fit. We found that, compared with other BKγ subunits, γ1 increased the z value of the BK channels to a greater degree. When the C-tail of γ2 was replaced with that of γ1 or γ3, γ2 elicited $V_{1/2}$ and z values approaching those of γ1, suggesting that the C-tail has a large impact on the slope of the G-V curve. However, because a slight modification in the voltage-sensing parameters Z_J or J_0 can have a marked effect on the slope of the G-V curve, de-convolving the G-V curve to identify contributions from different HA model gating parameters to its slope is difficult.

The way in which the BKγ subunits' C-tails contribute to the subunits' modulatory functions remains unclear. The C-tails may contribute to the modulatory functions of BKγ subunits through two distinct mechanisms. First, the C-tail or its juxta-membrane cluster of positively charged amino acids may be required for BKγ to be functional, presumably by ensuring the proper insertion or orientation of the TM segment in accordance with the positive-inside rule (White and von Heijne, 2004). This possibility would explain why the deletion of C-tails or even the juxta-membrane clusters of positively charged amino acids led to a nearly 100% loss of the modulatory function of γ1 and γ3 in the present study. Second, through one or more unknown mechanisms, the juxta-membrane positively charged residues may directly affect BK channel gating independently of their influence on the TM segments. This would account for the additional shift in the BK channel $V_{1/2}$ (approximately −40 mV) and the increase in the steepness of the G-V curve caused by the γ1 and γ3 C-tails as compared with the γ2 and γ4 C-tails in the presence of γ2's LRRD and TM segments. Alternatively, the juxta-membrane clusters of positively charged amino acids in γ1 and γ3 may indirectly affect BK channel gating by interacting with the TM segments. This indirect mechanism might be similar to the positive-inside rule mechanism in that it alters the structure of the TM segment to enhance the function of the BKγ subunit, which would be consistent with our observation that the effects of individual C-tails on BK channel voltage gating somehow varied in a TM segment–dependent manner. The functional incompatibility of γ1's TM segment with other BKγ subunits' C-tails suggests their strong interactions. However, a precise delineation of the juxta-membrane border between the TM segment and intracellular C-tail will be needed in future studies to clearly define their separate modulatory functions and determine their interactions.

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