KCNE4 domains required for inhibition of KCNQ1

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Voltage-gated potassium (K_v) channels are modulated in distinct ways by members of the KCNE family of single transmembrane domain accessory subunits. KCNE4 has a dramatic inhibitory effect on KCNQ1 that differs substantially from the activating effects of KCNE1 and KCNE3. The structural features of KCNE4 that enable this behaviour are unknown. We exploited chimeras of KCNE1, KCNE3 and KCNE4 to identify specific domains responsible for the inhibitory effects on heterologously expressed KCNQ1. Previous structure–function analysis of KCNE1 and KCNE3 identified a critical tripeptide motif within the transmembrane domain that accounts for the differences in KCNQ1 modulation evoked by these two KCNE proteins. Swapping the transmembrane tripeptide motif of KCNE4 with the corresponding amino acid sequence of KCNE1 did not influence the behaviour of either protein. Similarly, exchanging the tripeptide regions of KCNE3 and KCNE4 further demonstrated that this transmembrane motif does not explain the activity of KCNE4. Using a more systematic approach, we demonstrated that the KCNE4 C-terminus was critical for KCNQ1 modulation. Replacement of the KCNE1 or KCNE3 C-termini with that of KCNE4 created chimeric proteins that strongly inhibited KCNQ1. Additional evidence supported a cooperative role of the KCNE4 transmembrane domain. Although the C-terminus was necessary for KCNE4 activity, we demonstrated that a surrogate transmembrane domain derived from the cytokine receptor CD8 did not enable inhibition of KCNQ1, indicating that the KCNE4 C-terminus alone was not sufficient for KCNQ1 modulation. We further demonstrated that the KCNE4 C-terminus interacts with KCNQ1. Our data reveal important structure–function relationships for KCNE4 that help advance our understanding of potassium channel modulation by KCNE proteins.

(Received 11 August 2008; accepted after revision 21 November 2008; first published online 24 November 2008)
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KCNE proteins exert diverse functional effects on K_V channels ranging from activation to inhibition of channel activity (Sanguinetti *et al.* 1996; Barhanin *et al.* 1996; Tinel *et al.* 2000; Schroeder *et al.* 2000; Grunnet *et al.* 2002; Angelo *et al.* 2002). The first identified member of this family, KCNE1 (originally named minK) (Takumi *et al.* 1988), partners with KCNQ1 (K_V7.1) in heart to generate the slow component of the cardiac delayed rectifier current (I_{Ks}), important for cardiomyocyte repolarization (Sanguinetti *et al.* 1996; Barhanin *et al.* 1996). Four paralogues initially called minK-related peptides (MiRPs) and encoded by human genes *KCNE2*, *KCNE3*, *KCNE4* and *KCNE5* were later identified (Abbott *et al.* 1999; Piccini *et al.* 1999). All members of the KCNE gene family have been linked to inherited disorders of cardiac rhythm (Splawski *et al.* 1997; Bianchi *et al.* 1999; Isbrandt *et al.* 2002; Yang *et al.* 2004; Ma *et al.* 2007; Lundby *et al.* 2008; Ravn *et al.* 2008) illustrating their importance in normal physiological processes and emphasizing the need to learn more about the fundamental basis for their functions.

Previous studies have examined the determinants of K_V channel modulation compartmentalized within the three major structural domains of KCNE proteins, the extracellular N-terminus, the transmembrane (TM) domain and the intracellular C-terminus. Little functional importance has been attributed to the N-terminus, other than potential effects of N-linked glycosylation in KCNE1 and KCNE3 (Freeman *et al.* 2000; Gage & Kobertz, 2004), while the TM domain and C-terminus have been demonstrated to contain critical elements necessary for function of KCNE1 and KCNE3.

One notable structure–function relationship is the correlation of a tripeptide motif in the TM domain with functional differences between KCNE1 and KCNE3. Using KCNE1–KCNE3 molecular chimeras, Melman and

This paper has online supplemental material.

DOI: 10.1113/jphysiol.2008.161281

Table 1. Primers for chimera construction

Chimera	Template	Primer	Sequence
E4E1E1	KCNE4	Forward	CGTCAGATCCGCTAGCGCGGCCGCCATGCTGAAAAT
		Reverse	TGGAGCGGATGTAGCTCAGCATGATGCCCAGGGTGAAGAAGCCGAAGAATCCCAGTACC ATGAGGACGTAGAGGGCCTCGTTGCCATTGCCGCT
E1E4E4	KCNE1	Forward	CGTCAGATCCGCTAGCGCGGCCGCCATGATCCTGTCTAA
		Reverse	ACTCCAGCTTGCCGTCACCGC
E1E4E1	KCNE1	Forward*	CGTCAGATCCGCTAGCGCGGCCGCCATGATCCTGTCTAA
		Reverse	AACCAGAATGTAGAAGTACTCCAGCTTGCCGTCACCGCT
	KCNE4	Forward	GGTGACGGCAAGCTGGAGTACTTCTACATTCTGGTT
		Reverse*	GGCATCGGACTCGATGTAGACGTTGAATGGGTCGTTCGAGTGCTCCAGCTTCTTGGAGCG CATGTAGCCCAGCATGAT
E4E1E4	KCNE1	Forward*	AGCGGCAATGGCAACGAGTACTGCCCTCTACGTCCTCATGGTA
		Reverse	CTTCTCCCGCCTCTTGGATTTGATGTAGCTCAGCATGAT
	KCNE4	Forward	GGCATCATGCTGAGCTACATCAAATCCAAGCGGGAGAA
		Reverse*	AACAGAGGGTGCAGGACA
E1E1E4	KCNE4	Forward	TTCACCCTGGGCATCATGCTGAGCTACATCAAATCCAAGAGGCGGGAGAA
		Reverse	GAGAGGGGCGGATCCCTAGGAATT
E4E4E1	KCNE1	Forward*	GGAATCATGCTGGGCTACATGCGCTCCAAGAAGCTGGAGCACTCGAA
		Reverse	GAGAGGGGCGGATCCTCACTTGTCAT
	KCNE4	Forward	GCCTCCAGCAGCCCCCTGGAGT
		Reverse*	GTGCTCCAGCTTCTTGGAGCGCATGTAGCCCAGCATGATTCCGAT
E3E3E4	KCNE3	Forward*	AACCGTCAGATCCGCTAGCGCGGCCGCCATGGAGACTACCAATGGAACGGAGA
		Reverse	CTTCTTCTCCCGCCTCTTGGATTTGGTGTATCCCAGGATGAGGCT
	KCNE4	Forward	AGCCTCATCCTGGGATACACCAAATCCAAGAGGCGGGAGAAGAA
		Reverse*	GAGAGGGGCGGATCCCTAGGAATT
E4E4E3	KCNE4	Forward*	GCCTCCAGCAGCCCCCTGGAGT
	KCNE4	Reverse	TTGTCCACTTTGCGGGAGCGCATGTAGCCCAGCATGATT
	KCNE3	Forward	AATCATGCTGGGCTACATGCGCTCCCGCAAAGTGGACAA
	KCNE3	Reverse*	GGTCACGGAATCTGGATCCTTAGATCATAGACACACGGTT
CD8_E4CT	CD8	Forward*	AACACCGGCGCCCACCAT
		Reverse	TTCTCCCGCCTCTTGGATTTGTTGCAGTAAAGGGTGATAA2
	KCNE4	Forward	TTATCACCCTTTACTGCAACAAATCCAAGAGGCGGGAGAA
		Reverse*	GAGAGGGGCGGATCCCTAGGAATT

*Asterisk denotes primers utilized in overlap reaction.

colleagues identified three non-conserved residues within the KCNE1 and KCNE3 TM regions that were sufficient to exchange the distinct gating properties conferred upon KCNQ1 by these two accessory subunits (Melman *et al.* 2001, 2002). One residue within this tripeptide motif (threonine-58 in KCNE1) accounts for the majority of this effect (Melman *et al.* 2002). Additional experiments are needed to clarify whether the observations made for KCNE1 and KCNE3 hold true for all other KCNE subunits, particularly KCNE4 which has a dramatic inhibitory effect on KCNQ1 and other K_V channels.

In this study, we determined the structural domains of KCNE4 that are essential for modulation of KCNQ1 current. Our findings indicated that the KCNE4 C-terminus is necessary but not sufficient for KCNQ1 inhibition and that the TM domain is required for the full effect. However, the transmembrane tripeptide motif that functionally differentiates KCNE1 from KCNE3 is not important for KCNE4. Finally, we demonstrated that the KCNE4 C-terminus biochemically interacts with KCNQ1.

Methods

Construction of chimeras and site-directed mutagenesis

Chimeric KCNE1–KCNE4, KCNE3–KCNE4 and CD8–KCNE4 constructs were generated using recombinant polymerase chain reaction (PCR) and point mutations were engineered using QuikChange Mutagenesis (Stratagene, La Jolla, CA, USA). Primer sequences used for constructing chimeras or site-directed mutagenesis are provided in Tables 1 and 2. In some experiments,

Table 2. Mutagenesis primers

Mutation	Mutagenic primer sequence
KCNE1-FLI	CTGGGATTCTTCGGCTTCTTCCTCATAGGCATCATGCTGAGCTAC
KCNE1-L	GGGATTCTTCGGCTTCTTCCTCCTAGGCATCATGCTGAGCTAC
KCNE4-FTL	GTCCTTCTACGGCATTTTCACCCTAGGAATCATGCTGGGCTAC
KCNE4-T	GTCCTTCTACGGCATTTTCACGATCGGAATCATGCTGGGCTAC
KCNE3-FLI	GTCATGTTTCTATTTGCTGTCTTCCTGATAAGCCTCATCCTGGG
KCNE4-TVG	GTCCTTCTATGGAATAACCGTGGGCGGAATCATGCTAGGCTACATG

a C-terminal epitope-tagged KCNE1 (triple FLAG, 3xFLAG; Manderfield & George, 2008) was used to enable biochemical analyses. Chimeric KCNE proteins that contain the C-terminus of KCNE1 have the 3xFLAG epitope. All constructs were subcloned into the pIRES2-EGFP vector (Invitrogen, San Diego, CA, USA) and the complete open reading frames of all KCNE cDNAs were sequenced to verify correct assembly and exclude polymerase errors.

Cell culture and transfection

Chinese hamster ovary cells (CHO-K1, American Type Culture Collection, Rockville, MD, USA) used in electrophysiology experiments were grown at 37°C with 5% CO_2 in F-12 nutrient mixture medium supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA), penicillin (50 units ml^{-1})–streptomycin (50 $\mu g\ ml^{-1}$) and L-glutamine (2 mM). Unless otherwise stated, all tissue culture media were obtained from Invitrogen. CHO cells were transiently transfected using FuGENE-6 (Roche Applied Science, Indianapolis, IN, USA). Full length KCNQ1 was expressed from the pIRES2-dsRed vector (Invitrogen), while all KCNE cDNAs were expressed from pIRES2-EGFP. Forty-eight hours after transfection, successfully co-transfected cells were identified by epi-fluorescence microscopy.

COS-M6 cells used in biochemical experiments were maintained as previously described (Manderfield & George, 2008), transiently transfected using FuGene-6 (Roche Applied Science) and cells were harvested 48 h post-transfection.

Electrophysiology

Whole-cell currents were measured in the whole-cell configuration of the patch clamp technique (Hamill *et al.* 1981). All experimental conditions, pulse generation, data collection and analyses were completed as detailed previously (Lundquist *et al.* 2005). Briefly, currents were elicited from a holding potential of −80 mV to test potentials from −80 to +60 mV in 10 mV increments and recorded for 2000 ms followed by a 1000 ms step to −30 mV to elicit tail currents. The apparent voltage dependence of activation was determined from normalized peak tail currents that were fitted with a Boltzmann function having parameters $V_{1/2}$ (half-maximal activation voltage) and k (slope factor): $I = 1/(1 + \exp(V - V_{1/2})/k)$. Time to half maximum peak current was calculated by measuring current achieved at the end of the +60 mV pulse and then determining the time required to reach half that value during the voltage trace. Activation time constants were determined by fitting a monoexponential function ($A \times \exp(-t/\tau) + C$) where A stands for amplitude and C is a constant to the initial 500 ms of current recorded at +60 mV following the capacitive transient. Deactivation time constants were calculated by fitting a monoexponential function to tail currents recorded during the period between 100 and 850 ms after the voltage step for all chimeras and wild-type KCNE1. Deactivation for KCNQ1 co-expressed with vector alone was fitted with the same function but for the 750 ms period following the peak of the tail current hook. Curve fitting was performed using ClampFit 9.2 (Molecular Devices, Sunnyvale, CA, USA). Statistical comparisons among three or more groups were performed using one-way ANOVA followed by a Tukey post test. Two group comparisons were performed using Student's t test. Significance was assumed for $P < 0.05$.

Biochemistry

All biochemical experiments including the preparation of cellular lysates, immunoprecipitation, cell surface biotinylation and Western blotting were completed as previously described (Manderfield & George, 2008). Briefly, 48 h post-transfection COS-M6 cells were lysed with ice-cold NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 8.0) supplemented with a Complete mini protease inhibitor (Roche Applied Science). For immunoprecipitation, anti-KCNQ1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was chemically cross-linked to Protein G Sepharose 4 Fast Flow (GE Healthcare Life Sciences, Piscataway, NJ, USA) with dimethyl pimelimidate dihydrochloride (Sigma Aldrich, St Louis, MO, USA).

In cell surface biotinylation experiments, 48 h post-transfection COS-M6 cells were incubated with cell-impermeant sulfo-NHS-biotin (Pierce Chemical

Co., Rockford, IL, USA). The reaction was quenched with Dulbecco's phosphate-buffered saline (DPBS) (Gibco/Invitrogen, Grand Island, NY, USA) containing 100 mM glycine. Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-base, pH 7.5, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS). To isolate biotinylated proteins, lysates were incubated overnight with ImmunoPure Immobilized Streptavidin beads (Pierce Chemical Co.) and then eluted with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA). Biotinylated fractions were then subjected to Western blot analysis.

Western blot analysis was performed as previously described (Manderfield & George, 2008). Blots probed for FLAG (KCNE1), KCNE4, CD8 expression constructs, GAPDH or transferrin were incubated overnight at 4°C with the appropriate primary antibody (1 : 2000 M2 FLAG mouse monoclonal antibody (Sigma-Aldrich), 1 : 200 KCNE4 rabbit polyclonal antibody (Manderfield & George, 2008), 1 : 200 CD8-α rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), 1 : 2000 GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology Inc.), 1 : 500 transferrin mouse monoclonal antibody (Zymed Laboratories/Invitrogen Corporation, Carlsbad, CA, USA)). Membranes probed for KCNQ1 were incubated for 1 h at room temperature with a goat anti-KCNQ1 polyclonal antibody (1 : 200, Santa Cruz Biotechnology Inc.). All secondary antibodies were HRP-conjugated, diluted 1 : 5000 and incubated at room temperature for 40 min (KCNE4, donkey anti-rabbit (GE Healthcare Life Sciences), CD8-α, goat anti-rabbit (Santa Cruz Biotechnology Inc.), FLAG, GAPDH and transferrin, goat anti-mouse (Santa Cruz Biotechnology Inc.) and KCNQ1, rabbit anti-goat (Santa Cruz Biotechnology Inc.)).

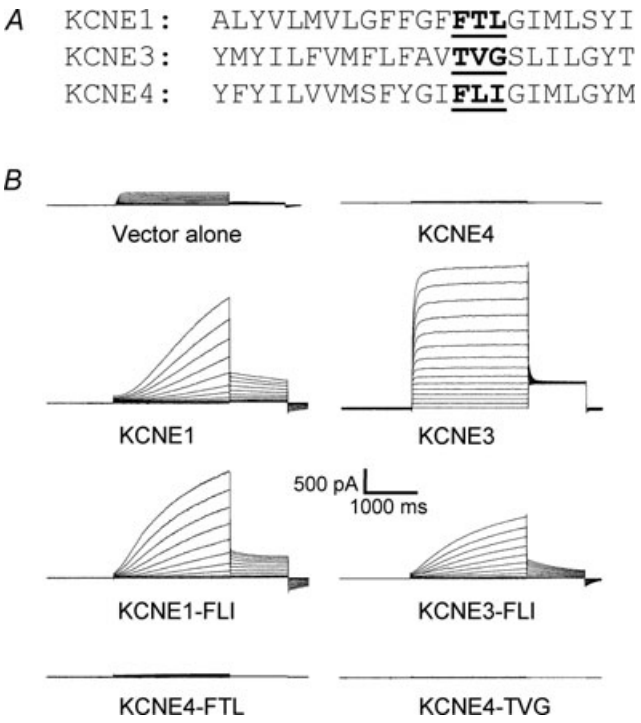

Figure 1. Role of KCNE4 transmembrane tripeptide motif
A, amino acid alignment of KCNE1, KCNE3 and KCNE4 TM domains. The tripeptide region in each sequence is bold and underlined. *B*, representative whole-cell currents recorded from CHO cells expressing KCNQ1 with either vector alone or the KCNE subunit indicated beneath the traces.

Results

Transmembrane tripeptide does not control KCNE4 suppression of KCNQ1

The differential modulation of KCNQ1 by KCNE1 and KCNE3 has been attributed to a tripeptide sequence in the transmembrane (TM) domain of these two accessory proteins (Melman *et al.* 2001). We tested whether the corresponding tripeptide motif of KCNE4 is similarly responsible for the inhibitory effect of this protein on KCNQ1. In KCNE1, the TM tripeptide sequence is Phe^{57}-Thr^{58}-Leu^{59} (FTL) while the corresponding KCNE4 sequence is Phe^{49}-Leu^{50}-Ile^{51} (FLI) (Fig. 1*A*). When the divergent residues within the tripeptide motifs were exchanged between KCNE1 and KCNE4 proteins, there was no transference of their modulatory effect on KCNQ1 (Fig. 1*B*). When KCNQ1 was co-expressed with KCNE1-FLI we observed I_{Ks}-like activity resembling the currents generated by co-expressing the channel with wild-type (WT) KCNE1. Interestingly, replacement of the KCNE3 tripeptide with that of KCNE4 (KCNE3-FLI) also did not impart an inhibitory phenotype but rather evoked I_{Ks}-like current (Fig. 1*B*) which is inconsistent with the previous assertion that a hydroxyl side chain at the central position in the tripeptide motif is essential for KCNE1-like activity (Melman *et al.* 2002). Conversely, when KCNE4-FTL was co-expressed with KCNQ1, there was no measurable current similar to the effect of WT-KCNE4. We observed the same effect with engineered KCNE1 and KCNE4 mutants having only the central residue of the tripeptide motif exchanged (KCNE1-L and KCNE4-T) (Fig. 2*A*). Suppression of KCNQ1 current was also observed when the channel was co-expressed with a KCNE4 chimera having the KCNE3 tripeptide sequence (KCNE4-TVG, Figs 1*B* and 2*B*). These data further indicated that regions other than the TM tripeptide are necessary to explain the main effect of KCNE4. This result prompted us to employ a more systematic approach to define KCNE4 structural determinants.

KCNE4 C-terminus is required for KCNQ1 inhibition

To identify regions necessary to confer KCNQ1 inhibition, a series of domain-exchange KCNE1–KCNE4 chimeras

were studied. Three pairs of chimeras were created by exchanging the N-terminus, TM domain or C-terminus between KCNE1 and KCNE4. In the figures, specific chimeras are designated using simple abbreviations for the KCNE proteins (E1 for KCNE1; E4 for KCNE4) coupled with a modular nomenclature having the format 'ExExEx' in which the first position represents the N-terminus, the middle position represents the TM domain and the last position represents the C-terminus (divergent domains within each chimera are underlined).

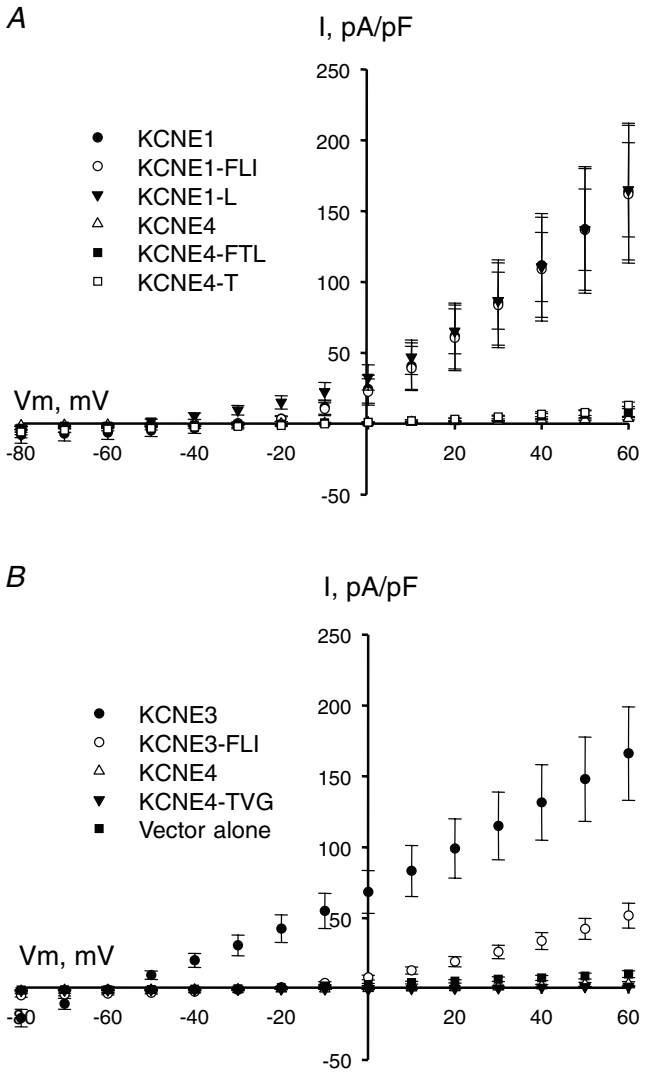

Figure 2. Modulation of KCNQ1 by tripeptide motif chimeras
A, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with KCNE1 (●, $n = 6$), KCNE1-FLI (○, $n = 9$), KCNE1-L (▼, $n = 14$), KCNE4 (△, $n = 15$), KCNE4-FTL (■, $n = 7$), or KCNE4-T (□, $n = 9$). *B*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with KCNE3 (●, $n = 10$), KCNE3-FLI (○, $n = 13$), KCNE4 (△, $n = 8$), KCNE4-TVG (▼, $n = 10$) or vector alone (■, $n = 13$). Whole-cell currents in both panels were measured 1990 ms after the start of the voltage step. Data points are mean values with error bars representing S.E.M.

Figure 3*A* illustrates representative whole-cell current recordings from cells co-expressing KCNQ1 with chimeras having mostly KCNE1 sequence (E4E1E1, E1E4E1, E1E1E4). All chimeras were examined in parallel with WT-KCNE1 (designated as E1E1E1), EGFP plasmid (vector alone) and WT-KCNE4 (E4E4E4). Co-expression of KCNQ1 with E4E1E1 exhibited currents that were not different from cells co-expressing WT-KCNE1, while cells co-transfected with E1E4E1 had current amplitudes similar to KCNQ1 co-expressed with vector alone (Fig. 3*A*). By contrast, co-expression with E1E1E4 completely suppressed KCNQ1 current indicating that the KCNE4 C-terminus conferred an inhibitory phenotype to KCNE1 (Fig. 3*A*).

There were no significant differences in current-voltage *(I–V)* relationships observed in cells expressing KCNQ1 with either WT-KCNE1 or E4E1E1 (Fig. 3*B*). By contrast, cells expressing KCNQ1 with either E1E4E1 or E1E1E4 exhibited significantly lower outward current density compared to co-expression with WT-KCNE1. While the E1E4E1 chimera did not affect KCNQ1 current amplitude (Fig. 3*B*), activation and deactivation kinetics were significantly different from cells co-expressing vector alone and WT-KCNE1 (Table 3) implying functional interactions. Further comparisons of the *I–V* relationships revealed that E1E1E4 suppressed outward current significantly greater than E1E4E1. This result indicated that the KCNE4 C-terminus is the more potent structural domain for suppression of KCNQ1 current, but a contribution of the KCNE4 TM domain also appears important for this effect. Importantly, neither E1E4E1 nor E1E1E4 caused decreased KCNQ1 cell surface expression (Fig. 3*C*). Further, each of these chimeras retained the ability to co-immunoprecipitate with KCNQ1 (Supplemental Fig. S1) demonstrating intact inter-subunit interactions.

Figure 4 illustrates representative whole-cell current recordings and *I–V* relationships made from cells co-expressing KCNQ1 with three chimeras composed of mostly KCNE4 sequence, the converse of the experiments described above. Cells co-expressing KCNQ1 with KCNE4 chimeras having either the N-terminal (E1E4E4) or TM (E4E1E4) domains of KCNE1 generated no current, a phenotype identical to cells co-expressing WT-KCNE4. By contrast, replacing the C-terminus of KCNE4 with that of KCNE1 created a chimeric KCNE protein (E4E4E1) that did not suppress KCNQ1 current density but did exert moderate effects on channel gating kinetics (Table 3). However, current recorded from cells co-expressing KCNQ1 with E4E4E1 did not fully resemble I_{Ks} and there was significantly lower outward current density as compared to cells co-expressing WT-KCNE1 (Fig. 4*B*). None of these chimeric KCNE proteins

impaired KCNQ1 cell surface expression (Fig. 4*C*) and each retained the ability to co-immunoprecipitate with KCNQ1 (Supplemental Fig. S1). These results indicate that replacement of the KCNE4 C-terminus alters the ability of this protein to suppress KCNQ1 current, but that the KCNE1 C-terminal domain is not sufficient in this molecular context to rescue all functional attributes of WT-KCNE1.

We further evaluated the importance of the KCNE4 C-terminus by testing whether transplanting this domain to KCNE3 would also confer an inhibitory phenotype. Figure 5*A* and *B* illustrates representative whole-cell recordings and *I–V* relationships obtained from cells co-expressing KCNQ1 with either WT-KCNE3 (designated E3E3E3) or E3E3E4. Co-expression of WT-KCNE3 with KCNQ1 generates a constitutively active channel exhibiting a linear *I–V* relationship. By contrast, cells expressing KCNQ1 with E3E3E4 exhibited no outward or inward current at any tested membrane potential despite intact KCNQ1 cell-surface expression (Fig. 5*C*). KCNQ1 currents were not suppressed by a KCNE4 chimera in which the native C-terminus was

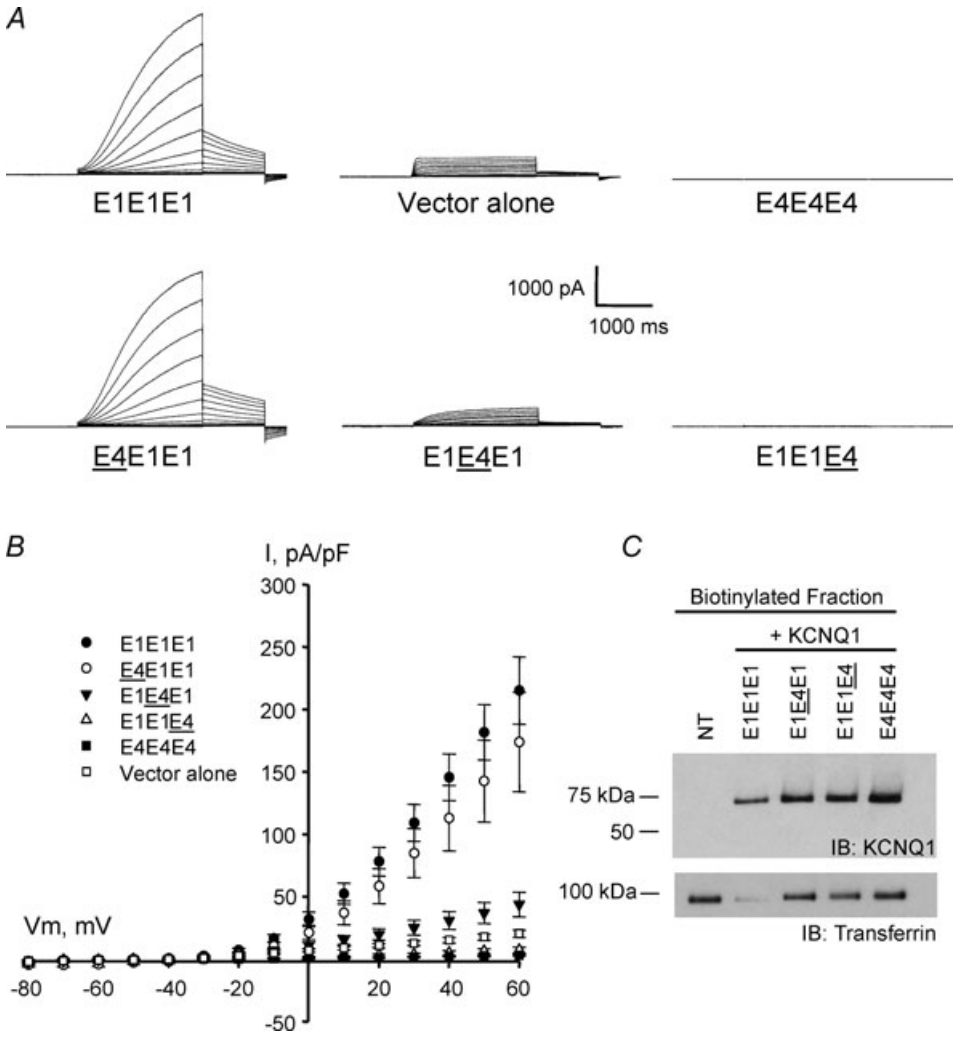

Figure 3. KCNE4 C-terminus converts KCNE1 into a KCNQ1-inhibitory subunit
A, representative whole-cell currents recorded from CHO cells co-expressing KCNQ1 with the indicated KCNE protein, chimera or vector alone. *B*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with E1E1E1 (●, $n = 12$), E4E1E1 (○, $n = 8$), E1E4E1 (▼, $n = 7$), E1E1E4 (△, $n = 6$), E4E4E4 (■, $n = 15$) or vector alone (□, $n = 12$). Whole-cell currents were measured 1990 ms after the start of the voltage protocol. Current density was significantly lower at all test potentials between +10 and +60 mV for E1E4E1 and E1E1E4 ($P \leq 0.001$) when compared to E1E1E1. E1E4E1 exhibits significantly greater current density at all test potentials between −10 and +60 mV (adjusted $P \leq 0.02$) when compared to E1E1E4. *C*, representative immunoblots demonstrating KCNQ1 cell surface expression in the presence of KCNE chimeras (NT, non-transfected). Only biotinylated fractions are shown. The blot was probed with anti-KCNQ1 to demonstrate KCNQ1 expression and with anti-transferrin to demonstrate corresponding levels of all membrane proteins in each sample.

Table 3. Biophysical parameters for KCNE chimeras

	Time to 1/2 max peak (ms)	*n*	Activation time constant	*n*	Deactivation time constant	*n*	$V_{1/2}$ (mV)	*n*	Slope factor (*k*)	*n*
Vector alone	35.0 ± 3.0	24	45.9 ± 3.1	26	1647.3 ± 254.2	17	−11.3 ± 2.1	10	13.9 ± 4.2	10
E1E4E1	232.7 ± 36.9*	7	342.6 ± 52.8*	7	373.8 ± 77.5*	6	−7.9 ± 3.7	7	15.7 ± 1.5	7
E4E4E1	269.7 ± 64.6*	7	403.8 ± 82.4*	6	310.9 ± 40.5*	6	−4.9 ± 3.4	7	12.6 ± 1.8	7
E4E4E3	176.2 ± 31.2*	13	196.8 ± 28.8*	15	439.3 ± 47.0*	13	−5.7 ± 7.2	7	9.9 ± 2.1	7
KCNE1	708.6 ± 83.4	11	1911.9 ± 378.2	10	1377.8 ± 93.8	11	21.5 ± 4.0*	14	16.8 ± 0.5	14
KCNE3	4.0 ± 0.9	14	51.4 ± 2.6	14	181.3 ± 19.9	16	ND		ND	

Data are means ± S.E.M. at +60 mV. Asterisks (*) indicate values significantly different from both vector only and E1E1E1 at $P < 0.001$. No statistical comparisons were made with KCNE3. ND, not determined.

replaced with that of KCNE3 (E4E4E3) (Fig. 5*D* and *E*). Also, KCNQ1 channels modulated by E4E4E3 exhibited kinetic properties that were similar to E1E4E1 and E4E4E1 but significantly different from vector alone or WT-KCNE1 (Table 3). These data indicated that the KCNE4 TM domain can exert a modulatory effect on KCNQ1, but these effects appear to be masked by the inhibitory influence of the C-terminus in wild-type KCNE4. These results further indicate that the KCNE4 C-terminus is critically important for the suppression of KCNQ1.

KCNE4 C-terminus is not sufficient to inhibit KCNQ1

We next sought to determine if the C-terminus alone was sufficient to inhibit KCNQ1 activity. We engineered a CD8–KCNE4 chimera that contained the extracellular N-terminus and TM domain of the single transmembrane lymphocyte CD8 receptor fused to the complete KCNE4 C-terminus (CD8–E4CT). We demonstrated expression of the chimeric protein at the plasma membrane by the successful surface labelling of transfected cells with microbeads coated with CD8 antibody and by immunodetection of the protein in cellular lysates with an antibody directed against the KCNE4 C-terminus (data not shown). However, co-expression of KCNQ1 with CD8–E4CT did not inhibit outward current (Fig. 6*A* and *B*). This finding suggested that the KCNE4 C-terminus fused to a surrogate single TM domain protein is not sufficient for the inhibitory phenotype of this accessory subunit.

KCNE4 C-terminus interacts with KCNQ1

The failure of CD8–E4CT to inhibit KCNQ1 might reflect incompetent biochemical interactions with the pore-forming subunit. However, co-immunoprecipitation experiments demonstrated that the chimeric protein was indeed associated with KCNQ1 (Fig. 6*C*, lane 2) and that this interaction was not the result of non-specific interactions among the KCNQ1 and CD8 proteins (Fig. 6*D*, lane 3). This finding suggests that the KCNE4 C-terminal domain may mediate KCNQ1 inhibition through a physical interaction with the channel.

Discussion

In this study, we investigated the structural determinants required for the dramatic inhibitory effect of KCNE4 on heterologously expressed KCNQ1. Our experiments demonstrated three important points. First, a tripeptide motif within the KCNE4 transmembrane domain corresponding to the amino acid triplet responsible for differential gating modulation of KCNQ1 by KCNE1 and KCNE3 is not important for KCNE4 suppression of KCNQ1 current amplitude. Second, we demonstrated that the KCNE4 C-terminus was necessary but not sufficient for KCNQ1 modulation, and third, that this domain biochemically interacts with KCNQ1. These findings have direct implications for understanding structure–function relationships among KCNE accessory subunits, and provide important new clues regarding potential mechanisms of action for these intriguing proteins.

KCNE4 C-terminus is necessary but not sufficient for KCNQ1 inhibition

The necessity of the KCNE4 C-terminus for KCNQ1 inhibition was clearly demonstrated by the conversions of KCNE1 and KCNE3 to inhibitory subunits when their native C-terminal domains were replaced with that of KCNE4. Previous studies have demonstrated that the C-terminus of KCNE1 is also necessary for KCNQ1 modulation and deletion analysis defined the minimal C-terminal sequence necessary for this effect (Takumi *et al.* 1991; Tapper & George, 2000). A previous study seeking to infer the secondary structure of the KCNE1

cytoplasmic domain using scanning mutagenesis defined a proline residue that divides the C-terminus into two helical subdomains (Rocheleau *et al.* 2006). This proline residue, as well as the proximal ∼20 amino acids adjacent to the TM domain are well conserved among all KCNE proteins except for KCNE4 (Rocheleau *et al.* 2006). The substantial differences between KCNE1 and KCNE4 primary structure particularly within the C-terminal domains (< 20% amino acid sequence identity) suggests that distinct KCNQ1 modulation evoked by these two accessory proteins depends upon the structural divergence of the C-terminus.

Although we were able to establish that the KCNE4 C-terminus is necessary for KCNQ1 inhibition, our results indicated that this domain is not sufficient for activity when presented to the channel tethered to the transmembrane domain of CD8. Similarly, the KCNE1 C-terminus does not modulate KCNQ1 when fused to the transmembrane segment of the sodium channel β1 subunit (Tapper & George, 2000). Our data for KCNE4 and the prior study of KCNE1 suggest that another domain, possibly the TM, is required to mediate KCNQ1 modulation. The functional role of the KCNE4 C-terminus may be in binding to an undetermined structural motif in KCNQ1.

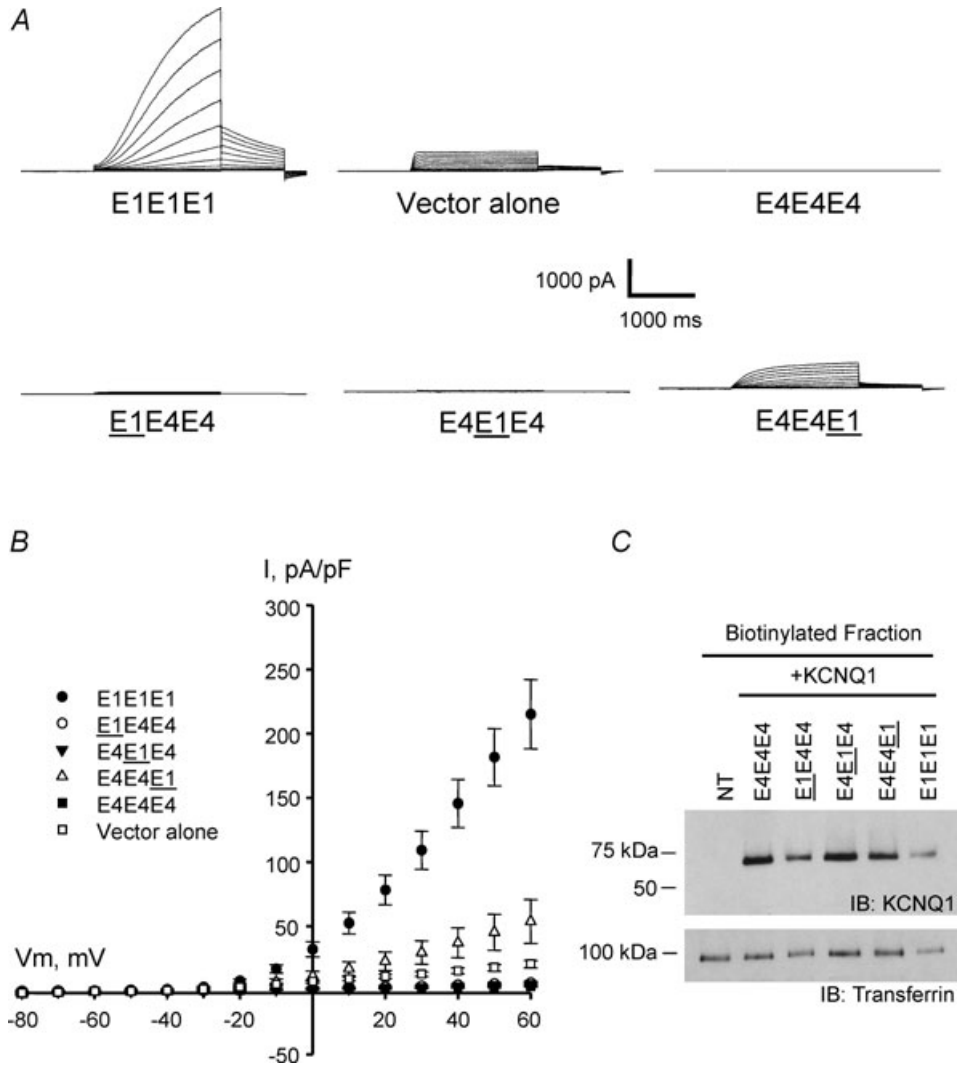

Figure 4. KCNE1 C-terminus does not confer KCNE1-like activity to KCNE4
A, representative whole-cell currents recorded from CHO cells co-expressing KCNQ1 with the indicated KCNE protein, chimera or vector alone. *B*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with E1E1E1 (●, $n = 12$), E1E4E4 (○, $n = 8$), E4E1E4 (▼, $n = 9$), E4E4E1 (△, $n = 7$), E4E4E4 (■, $n = 15$) or vector alone (□, $n = 12$). Current density was significantly lower at all test potentials between 0 and +60 mV for E4E4E1 ($P < 0.001$) when compared to E1E1E1. E4E4E1 had significantly greater current density at all test potentials between 0 and +60 mV (adjusted $P \leq 0.002$) when compared to E4E4E4. *C*, representative immunoblots examining KCNQ1 cell surface expression in the presence of KCNE chimeras.

Multiple structural domains participate in KCNQ1 modulation by KCNE proteins

The apparent co-operativity between the TM domain and C-terminus of KCNE4 to impart full biophysical modulation of KCNQ1 is a consistent theme in structure–function analyses of KCNE proteins. While deletion of the KCNE1 C-terminus abolished the ability to modulate KCNQ1, this structural change did not prevent a cysteine mutation in the transmembrane domain (Gly^{55} to Cys; G55C) from conferring susceptibility to extracellular Cd^{2+} block (Tapper & George, 2000). These data suggested that the KCNE1 C-terminus is necessary for KCNE1 gating modulation but that the TM domain helped mediate association with KCNQ1 (Tapper & George, 2000). In later studies, Gage and Kobertz proposed that KCNE proteins exhibit 'bipartite modulation' of KCNQ1 channels (Gage & Kobertz, 2004). In their model, the TM domain and the C-terminus have different roles in assembly and modulation that are specific to each KCNE protein (Gage & Kobertz, 2004). They further classified TM domains as 'active or passive' and hypothesized that this property determines whether KCNQ1 can be modulated by the C-terminus of the KCNE protein (Gage & Kobertz, 2004).

We have developed a refined model to explain the apparent co-operativity between the C-terminus and TM of KCNE4. In this model, the C-terminus anchors the subunit to a probable intracellular domain of KCNQ1 and enables the TM to functionally interact with the channel. This model is consistent with the requirement by KCNE1 and KCNE4 for their respective C-terminal domains in order to modulate KCNQ1 and the lack of sufficiency of the KCNE1 and KCNE4 C-termini when attached to surrogate TM domains. Our model is also consistent with the idea that a given KCNE TM domain might behave differently depending upon how it is anchored to the rest of the channel as illustrated by the E1E1E4 and E3E3E4 chimeras that act to inhibit rather than activate KCNQ1. In the case of KCNE3, interactions with KCNQ1 may be mediated predominantly by the TM domain as

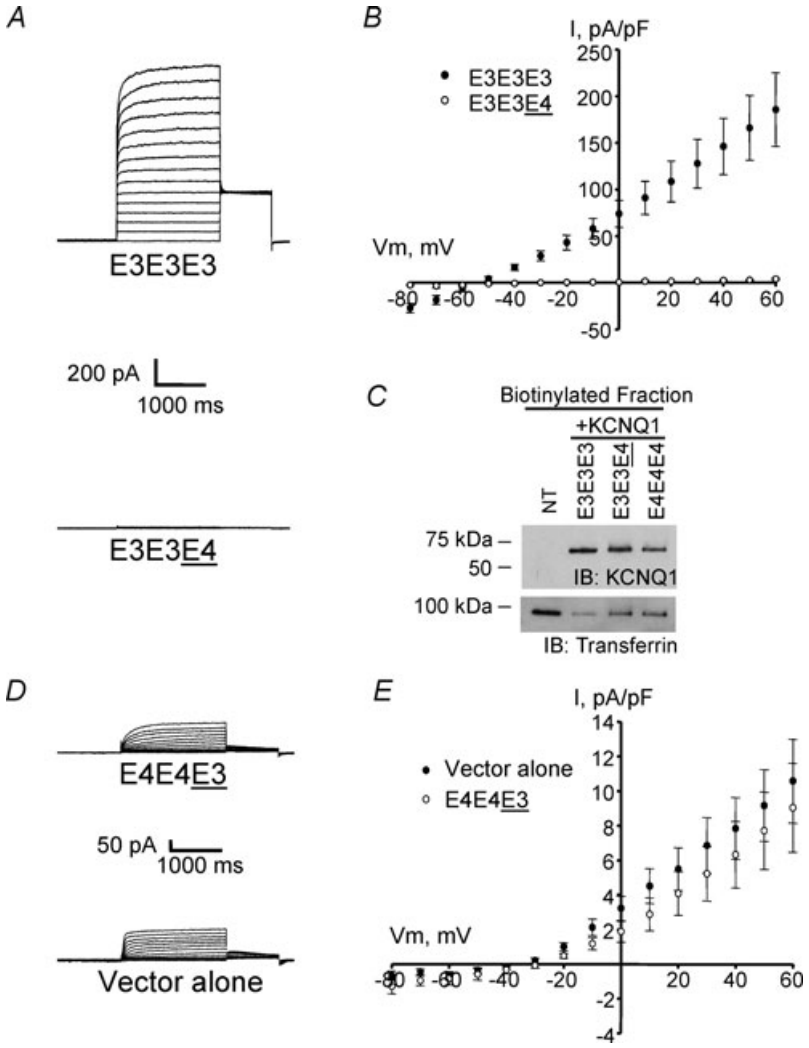

Figure 5. KCNE4 C-terminus converts KCNE3 into a KCNQ1-inhibitory subunit
A, representative whole-cell currents observed in CHO cells expressing KCNQ1 with E3E3E3 or E3E3E4. *B*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with E3E3E3 (●, $n = 9$) or E3E3E4 (○, $n = 10$). Current density in cells expressing KCNQ1 and E3E3E4 was significantly lower at all test potentials except −60 mV ($P \leq 0.001$ for −80 and −40 to +60 mV; $P = 0.01$ for −70 mV; $P = 0.04$ for −50 mV) compared to cells expressing KCNQ1 with E3E3E3. *C*, representative immunoblots examining KCNQ1 cell surface expression. *D*, representative whole-cell currents observed in CHO cells expressing KCNQ1 with E4E4E3 or vector alone. *E*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 co-expressed with vector alone (●, $n = 13$) or E4E4E3 (○, $n = 10$). There were no differences in current density between vector alone and E4E4E3.

supported by the observation that removal of the KCNE3 C-terminus does not greatly perturb the ability of this subunit to modulate KCNQ1 (Gage & Kobertz, 2004). Our E1E1E4 data support the Gage and Kobertz model of the KCNE1 TM domain as a passive element whose ability to modulate KCNQ1 is dependent upon the C-terminus, but our observations regarding E3E3E4 are contrary to the conclusion that the KCNE3 TM domain can modulate KCNQ1 channels independent of the C-terminus (Gage & Kobertz, 2004).

Our model does not preclude multiple contact points between the TM domain of KCNE proteins and various transmembrane segments of KCNQ1 or other K_V channels as recently demonstrated by Xu *et al.* (2008) and suggested by Kang *et al.* (2008). Similarly, evidence suggests that KCNE1 may interact with other specific structures within KCNQ1 such as the pore loop and S6 segment (Melman *et al.* 2004). These other contacts between KCNE proteins and KCNQ1 or other pore-forming subunits probably facilitate and determine the final functional outcome of channel modulation.

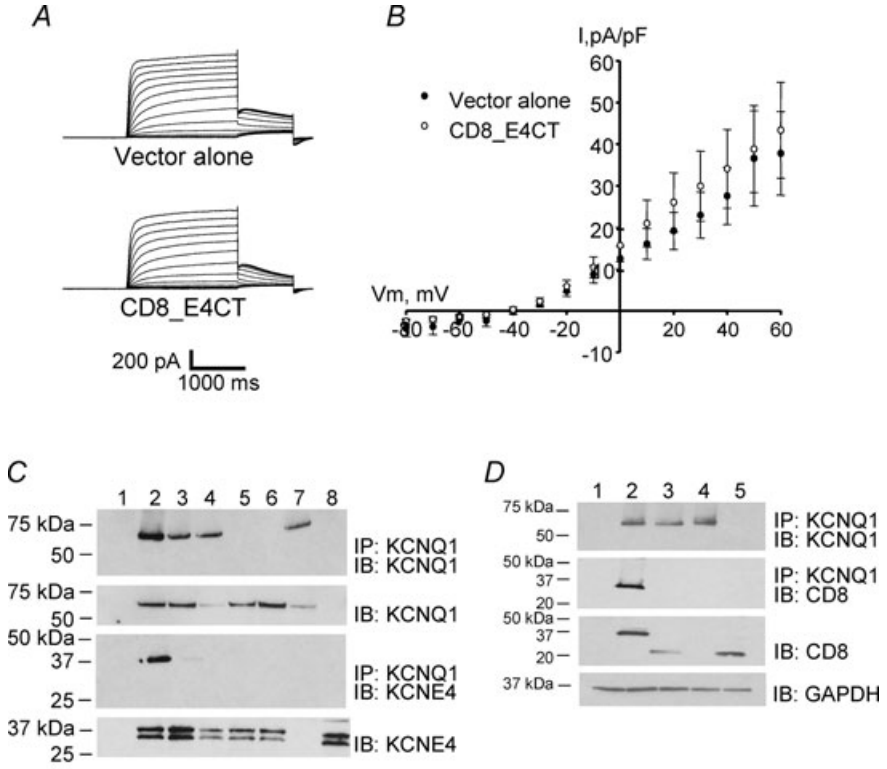

Figure 6. KCNE4 C-terminus is not sufficient for KCNQ1 modulation
A, representative whole-cell currents recorded from CHO cells co-expressing KCNQ1 with an EGFP vector (vector alone) or CD8–KCNE4-C-terminal chimera (CD8_E4CT). *B*, current–voltage relationships (normalized for membrane capacitance) for KCNQ1 alone (●, $n = 13$) or co-expressed with CD8_E4CT (○, $n = 9$). *C*, co-immunoprecipitation of KCNQ1 with CD8_E4CT (IP, immunoprecipitation; IB, immunoblot). Lane 1, non-transfected COS cells. Lane 2, cells transfected with KCNQ1 and CD8_E4CT. Lane 3, cells transfected with KCNQ1 and CD8_E4CT, but the anti-KCNQ1 used for the immunoprecipitation was pre-incubated with antigenic peptide. Lane 4, mixture of lysates from cells expressing either KCNQ1 or CD8_E4CT only and combined prior to immunoprecipitation. Lane 5, KCNQ1 and CD8_E4CT transfected cells immunoprecipitated with Protein-G Sepharose. Lane 6, KCNQ1 and CD8_E4CT transfected cells immunoprecipitated with goat pre-immune serum. Lanes 7 and 8, cells expressing only KCNQ1 or CD8_E4CT, respectively. The top immunoblot depicts samples immunoprecipitated with anti-KCNQ1 and immunoblotted for KCNQ1. The second image is a KCNQ1 immunoblot of the initial lysates demonstrating KCNQ1 expression. The third image is of samples immunoprecipitated with anti-KCNQ1 but probed with anti-KCNE4. The bottom image is a KCNE4 immunoblot of the initial lysates demonstrating CD8_E4CT expression. *D*, KCNQ1 does not interact with CD8 (IP, immunoprecipitation; IB, immunoblot). Lane 1, non-transfected COS cells. Lane 2, cells transfected with KCNQ1 and CD8_E4CT. Lane 3, cells transfected with KCNQ1 and CD8. Lanes 4 and 5, cells expressing only KCNQ1 or CD8, respectively. The top immunoblot depicts samples immunoprecipitated with anti-KCNQ1 and immunoblotted for KCNQ1. The second immunoblot is of samples immunoprecipitated with anti-KCNQ1 and immunoblotted for CD8. The third image is a CD8 immunoblot of the initial lysates demonstrating expression of either the CD8_E4CT subunit (lane 2) or CD8 (lanes 3 and 5). The bottom image is a GAPDH immunoblot of all initial lysates demonstrating total protein expression.

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Acknowledgements

This work was funded by NIH grant HL077188 (A.L.G.) and a pre-doctoral fellowship award to L.J.M. from the American Heart Association Southeast Affiliate.

Conflicts of interest

There are no conflicts of interest.

Supplemental material

Online supplemental material for this paper can be accessed at: http://jp.physoc.org/cgi/content/full/jphysiol.2008.161281/DC1