MinK Subdomains that Mediate Modulation of and Association with KvLQT1

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abstract KvLQT1 is a voltage-gated potassium channel expressed in cardiac cells that is critical for myocardial repolarization. When expressed alone in heterologous expression systems, KvLQT1 channels exhibit a rapidly activating potassium current that slowly deactivates. MinK, a 129 amino acid protein containing one transmembrane-spanning domain modulates KvLQT1, greatly slowing activation, increasing current amplitude, and removing inactivation. Using deletion and chimeric analysis, we have examined the structural determinants of MinK effects on gating modulation and subunit association. Coexpression of KvLQT1 with a MinK COOH-terminus deletion mutant (MinK ΔCterm) in *Xenopus* oocytes resulted in a rapidly activated potassium current closely resembling currents recorded from oocytes expressing KvLQT1 alone, indicating that this region is necessary for modulation. To determine whether MinK ΔCterm was associated with KvLQT1, a functional tag (G55C) that confers susceptibility to partial block by external cadmium was engineered into the transmembrane domain of MinK ΔCterm. Currents derived from coexpression of KvLQT1 with MinK ΔCterm were cadmium sensitive, suggesting that MinK ΔCterm does associate with KvLQT1, but does not modulate gating. To determine which MinK regions are sufficient for KvLQT1 association and modulation, chimeras were generated between MinK and the Na+ channel β1 subunit. Chimeras between MinK and β1 could only modulate KvLQT1 if they contained both the MinK transmembrane domain and COOH terminus, suggesting that the MinK COOH terminus alone is not sufficient for KvLQT1 modulation, and requires an additional, possibly associative interaction between the MinK transmembrane domain and KvLQT1. To identify the MinK subdomains necessary for gating modulation, deletion mutants were designed and coexpressed with KvLQT1. A MinK construct with amino acid residues 94–129 deleted retained the ability to modulate KvLQT1 gating, identifying the COOH-terminal region critical for gating modulation. Finally, MinK/MiRP1 (MinK related protein-1) chimeras were generated to investigate the difference between these two closely related subunits in their ability to modulate KvLQT1. The results from this analysis indicate that MiRP1 cannot modulate KvLQT1 due to differences within the transmembrane domain. Our results allow us to identify the MinK subdomains that mediate KvLQT1 association and modulation.

key words: KCNQ1 • heart • long QT syndrome • potassium channel • KCNE1

INTRODUCTION

The KCNE family is a group of small subunits that modulate voltage-gated potassium channels in heart, cochlea, and small intestine. The first identified member of the KCNE family, MinK, a 129 amino acid protein with a single putative transmembrane domain, was cloned originally from rat kidney and elicits a slowly activating potassium current when expressed in *Xenopus* oocytes (Takumi et al., 1988). Eventually, it was discovered that MinK interacts with and modulates a voltage-gated potassium channel, KvLQT1 (Barhanin et al., 1996; Sanguinetti et al., 1996; Yang et al., 1997). In the heart, MinK (KCNE1) coassembles with KvLQT1, forming functional channels that produce the slowly activating cardiac potassium current, I_{Ks}, that is critical for myocardial repolarization (Sanguinetti and Jurkiewicz, 1990). Mutations in KvLQT1 and MinK are responsible for >50% of congenital long QT syndrome, an inherited cardiac disorder characterized by syncope, arrhythmias, and sudden death (Wang et al., 1996; Shalaby et al., 1997; Splawski et al., 1997). The second member of the KCNE family, MinK related protein-1 (MiRP1),[1] was cloned by homology to MinK (Abbott et al., 1999). MiRP1 appears to coassemble with *human ether-á-go-go–related gene* (HERG) in heart to produce the rapidly activating cardiac potassium current I_{Kr}, that also contributes to myocardial repolarization. MiRP1 does not modulate KvLQT1.

Previous work has been done to explore structure–function relationships in MinK. Before cloning of KvLQT1, mutagenesis experiments suggested that a minimal MinK COOH-terminal sequence is essential for the potassium channel activity observed in *Xenopus* oocytes (Takumi et al., 1991). In another experiment, synthetic peptides from a carboxy terminal region were injected into oocytes, and I_{Ks}-like currents were recorded, indicating that this region may be sufficient for potassium channel activity (Ben-Efraim et al., 1996). However, both studies relied upon the modulation of an endogenous oocyte K+ channel. More recently,

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[1]*Abbreviations used in this paper:* aa, amino acid; HERG, *human ether-á-go-go–related gene*; MiRP1, MinK related protein-1.

Romey et al. (1997) demonstrated (using yeast two-hybrid and immunodetection analysis) that the MinK carboxy terminus may directly interact with the KvLQT1 pore-loop. No interaction with any other region of MinK or KvLQT1 was detected (Romey et al., 1997). Experiments using scanning cysteine mutagenesis demonstrated that cysteine substitution of amino acids within the MinK transmembrane spanning domain rendered I_{Ks} susceptible to partial block by external or internal Cd^{2+}. The Cd^{2+} block appeared to occur through a direct pore blockade mechanism, suggesting that the MinK transmembrane domain may be intimately associated with the KvLQT1 pore (Tai and Goldstein, 1998). More recently, stilbenes and fenamates, small molecule activators of the I_{Ks} channel complex, were found to be dependent on the MinK amino terminus for their pharmacological effect (Abitbol et al., 1999). These efforts have left many unanswered questions regarding how MinK interacts with KvLQT1. Can one MinK subregion be sufficient for the various effects MinK has on KvLQT1? If MinK is intimately associated with the KvLQT1 pore, then which MinK domain is responsible for subunit association? and what is its role in gating modulation? Is there any role for the extracellular amino terminus in KvLQT1 modulation? To modulate gating and current amplitude, MinK presumably must associate with KvLQT1 in a specific manner. Whether MinK-mediated KvLQT1 association and modulation involves one or many MinK subregions is unknown.

We have used chimeric and deletion analysis to systematically determine the structural requirements of KvLQT1 modulation by MinK. We present evidence that the MinK carboxyl terminus is necessary, but not sufficient, for KvLQT1 modulation and demonstrate that gating modulation cannot occur without the MinK transmembrane domain that appears critical for association with KvLQT1. By contrast, we provide data indicating that the MinK amino terminus is not necessary for gating modulation. Furthermore, using chimeric analysis between two members of the KCNE family, we show that the MiRP1 COOH terminus can modulate KvLQT1 in the presence of the MinK transmembrane domain. These results suggest that the major functional properties of MinK can be localized to two structural subdomains, and this knowledge may help elucidate the molecular mechanisms responsible for K^+ channel modulation.

MATERIALS AND METHODS

Construction of MinK Deletion Mutants

MinK deletion mutants were constructed using recombinant polymerase chain reaction. A mutagenesis forward (sense) primer complementary to MinK nucleotides 1–21 with a 5′ "tail" containing a HindIII restriction site (5′-AGATCGATCAAGC-CTATGCCCAGGATGATCCTGTCT-3′) was paired with various

reverse (antisense) primers contained 18–21 MinK nucleotides at the desired region, followed by a stop codon and EcoRI restriction site at the 5′ end (amino acids 67–130 deleted (MinK ΔC-term) 5′-TTATCAGAATTCTCATTAGCGGATGTAGCTCAGCAT-3′, MinK Δ94–129 5′-AGATCGATCGAATTCTCAGGCCTTGTC-CTTCTCTTGCCA-3′, MinK Δ79–129del 5′-AGATCGATCGAAT-TCTCAGAATGGGTCGTTCGAGTGCT-3′). The MinK deletion mutants were amplified via PCR from a human MinK cDNA template, digested with HindIII and EcoRI, gel purified, and cloned into the oocyte expression vector pSP64T. All sequences were verified by automated sequencing.

Construction of β1 Subunit/MinK Chimeras

The β1 subunit/MinK chimeras were constructed using recombinant PCR. For the β1/MinK carboxy terminus chimera that encodes the β1 amino terminus and transmembrane domain [amino acids (aa) 1–182] in frame with the MinK carboxyl terminus (aa 67–129, β1/MinK1), an overlapping β1/MinK forward primer (5′-ATT-GTGGTGTTGACCATATGGCTCGTGGCAGAGATGATTTACT-GCTACCGCTCCAAGAAGCTGGAGCA-3′) and a COOH-terminus MinK reverse primer (5′-TCGATCGAATTCATGTAGGGGTCAT-GGGGAAGGCTT3′) were used to amplify the MinK carboxyl terminus from a human MinK cDNA template. The PCR amplified fragment was digested with NdeI and EcoRI, gel purified, and unidirectionally subcloned into pSP64T hβ1. β/MinK2 encodes the β1 amino terminus and the amino terminal half of the transmembrane domain (aa 1–171) in frame with the carboxyl terminal half of the MinK transmembrane domain and the complete MinK COOH terminus (aa 55–129). An overlapping β1/MinK forward primer (5′-GTGGTGTTGACCTGCTTCTTCACCCTG-GGCATCAT-3′) and the COOH-terminus MinK reverse primer were used to amplify the MinK sequence from a human MinK cDNA template. The amplified region was digested with HincII and EcoRI, gel purified, and unidirectionally subcloned into pSP64T hβ1. β1/MinK-3 encodes the β1 amino terminus (aa 1–160) fused to the MinK transmembrane domain and carboxyl terminus (aa 44–129). To construct chimera 3, the β1 amino terminus was PCR amplified using a β1 forward primer (5′-GATGAATA-CAAGCTTGCTTGTTCT-3′) and an overlapping β/MinK reverse primer (5′-TACCATGAGGACGTAGAGGGCCTCAGACACGATG-GATGCCAT-3′). In a separate PCR reaction, the MinK transmembrane domain and carboxy terminus were amplified using a forward primer complementary to the MinK sequence present in the overlapping β/MinK reverse primer (5′-GCCCTCTACGTC-CTCATGGTA-3′) and the MinK carboxy terminus reverse primer. The PCR products of these two reactions were combined in a subsequent PCR reaction, allowed to anneal, and then amplified using the β1 forward primer and the MinK carboxy terminus reverse primer. The resulting product was digested with HindIII and EcoRI, gel purified, and cloned into pSP64T. All chimera sequences were verified by automated sequencing.

Construction of MiRP1/MinK Chimeras

MiRP1 was cloned from human genomic DNA using PCR. Forward (5′-AGATCGATCAAGCTTATCCCAGGATGTCTACTTTAT-CCAATT-3′) and reverse (5′-GCTAGCTAGGAATTCTTGATGT-TAGCTTGGTGCCTT-3′) primers based on the MiRP1 sequence (GenBank Accession number: AF071002) were used to amplify a 441-bp fragment containing the complete coding sequence. MiRP1 was then digested with HindIII and EcoRI, gel purified, and cloned into the oocyte expression vector, pSP64T. MiRP1 function was verified by whole-cell voltage-clamp recording of oocytes coexpressing HERG and MiRP1. The MiRP1/MinK chimera containing the MiRP1 amino and transmembrane domain (aa 1–72) fused to the MinK carboxy terminus (aa 67–129, MiRP1/

Cterm-MinK) was constructed using recombinant PCR. The MiRP1 amino terminus and transmembrane domain were PCR amplified using the MiRP1 forward primer and an overlapping MiRP1/MinK reverse primer (5′-GTTCGAGTGCTCCAGCTTCT-TGGAGCGCACAGTGCTCACCAGGAT-3′). The resulting product was digested with HindIII and BstXI, gel purified, and unidirectionally subcloned into pSP64TMinK. MinK/Cterm-MiRP1 encoding the MinK amino and transmembrane domain (aa 1–66) fused to the MiRP1 carboxy terminus (aa 73–98) was designed using double overlap PCR. The MiRP1 carboxy terminus was PCR amplified using a MinK/MiRP1 overlapping forward primer (5′-ATCATGCTGAGCTACATCAAATCCAAGAGACGGGAA-5′) and an MiRP1 carboxyl terminus reverse primer (5′-AGATCGATC-GAATTCTCAGCTCTTGTACTTTTCCTGCCA-3′). In another PCR reaction, the MinK amino and transmembrane domains were amplified using the MinK forward primer and a reverse primer complementary to the MinK region of the overlapping MinK/MiRP1 forward primer. In a subsequent PCR reaction, the two products were combined, allowed to anneal, and then amplified using the MinK forward primer and the MiRP1 carboxy terminus reverse primer. The resulting product was digested with HindIII and EcoRI, gel purified, and cloned into pSP64T. The MiRP1 and chimera sequences were verified using automated sequencing.

RNA from all constructs was transcribed in vitro from EcoRI-linearized DNA template using Sp6 RNA polymerase, nucleotides, and solutions included in the mMessage machine in vitro transcription kit (Ambion Corp.). RNA size and integrity were evaluated by formaldehyde-agarose gel electrophoresis. RNA concentrations were estimated by comparison with a 0.24–9.5-kb RNA ladder (GIBCO BRL).

Oocyte Preparation and Injection

Oocytes were surgically removed from female *Xenopus laevis*. Oocytes were defolliculated using collagenase (2.0 mg/ml, 2 h) in calcium-free ND-96 (for composition, see below). Stage V–VI oocytes were injected with 46 nl of cRNA (cRNA concentration: KvLQT1, 6 ng/oocyte; MinK constructs, 3 ng/oocyte) and incubated at 18°C in oocyte storage solution (ND-96 plus 50 mg gentamicin and 275 mg pyruvic acid per liter for storage; pH 7.5).

Electrophysiology

Currents were recorded at room temperature 2–5 d after injection using two-microelectrode voltage-clamp technique with an OC-725B amplifier (Warner Instruments Corp.). Pipettes were filled with 3 M KCl and had a 0.5–2 MΩ resistance. Oocytes were bathed in ND-96 containing (mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.5. For experiments in which HERG was used, oocytes were bathed in a solution containing (mM): 96 KCl, 5 NaCl, 0.3 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.5. For Cd^{2+} experiments, a modified ND-96 solution in which NaCl was replaced isotonically with CdCl$_2$ was used. Data were recorded using the PClamp 6 software program (Axon Instruments, Inc.). Data were filtered at 100 Hz and digitized at 1 kHz.

Data Analysis

Data were analyzed and plotted using a combination of pCLAMP and Origin (Microcal Software) software. Normalized isochronal voltage–activation relations were obtained by measuring current at 2 s during depolarizing pulses between −50 and +60 mV from a holding potential of −80 mV. Data were fit with a Boltzmann function of the form: $1/\{1 + \exp[(V - V_{1/2})/k_v]\}$, where $V_{1/2}$ is the half-maximal activation voltage and k_v is the slope factor. Time constants of deactivation were determined by fitting tail currents with a single exponential equation: $A * \exp(-t/t) + C$, where A is an amplitude term, t is time, and C is a constant.

R E S U L T S

MinK modulates KvLQT1 gating by slowing activation and removing inactivation. In addition, MinK changes the rectification of KvLQT1 currents, although the mechanism of this is unclear. MinK also modulates KvLQT1 current amplitude by an unknown mechanism that may or may not be dependent on gating. Fig. 1 illustrates the principle effects of MinK on coexpressed KvLQT1 in *Xenopus* oocytes. Upon depolarization, oocytes expressing KvLQT1 alone exhibit a rapidly activating, slowly deactivating outward potassium current with characteristics similar to previously published data (Fig. 1 A, left; Barhanin et al., 1996; Sanguinetti et al., 1996). KvLQT1 tail currents display a "hook," indicating that KvLQT1 inactivates to some extent (Fig. 1 B, left; Pusch et al., 1998; Tristani-Firouzi and Sanguinetti, 1998). KvLQT1-induced currents exhibit a linear current–voltage relationship from −40 to +60 mV (Fig. 1 C). MinK dramatically modulates KvLQT1 gating, slowing activation (Fig. 1 A, right), removing inactivation (B, right), and shifting the voltage dependence of activation to more positive potentials (D). MinK-modulated currents have three- to fivefold greater current amplitudes than KvLQT1 alone (Fig. 1 C). Understanding how MinK associates with KvLQT1 and identifying subregions involved in gating modulation may provide insight into the mechanism by which MinK modulates KvLQT1.

The MinK Carboxyl Terminus Is Necessary for KvLQT1 Modulation

We used deletion analysis to determine the role of MinK subdomains in mediating its effects on KvLQT1. MinK can be divided into three subdomains: an extracellular NH$_2$ terminus, a transmembrane spanning segment, and an intracellular COOH terminus. Two naturally occurring point mutations in the COOH terminus have been linked to congenital long QT syndrome, suggesting that this subdomain is functionally critical (Splawski et al., 1997). Furthermore, Ben-Efraim et al. (1996) injected synthetic peptide encoding a fragment of the MinK COOH terminus (amino acid residues 67–93) into *Xenopus* oocytes and recorded currents resembling I$_{Ks}$, presumably by modulating an endogenous KvLQT1. To determine whether the COOH terminus is necessary for modulation, we coexpressed a COOH-terminus deleted (MinK ΔCterm) MinK construct (deletion of residues 67–129) and human KvLQT1 in *Xenopus* oocytes. 3 d after cRNA injection, two-microelectrode voltage clamp was used to determine the phenotype of the induced current. Coexpression of MinK ΔCterm and KvLQT1 yielded a potassium current closely resembling that of KvLQT1 alone (Fig. 2 A), although the
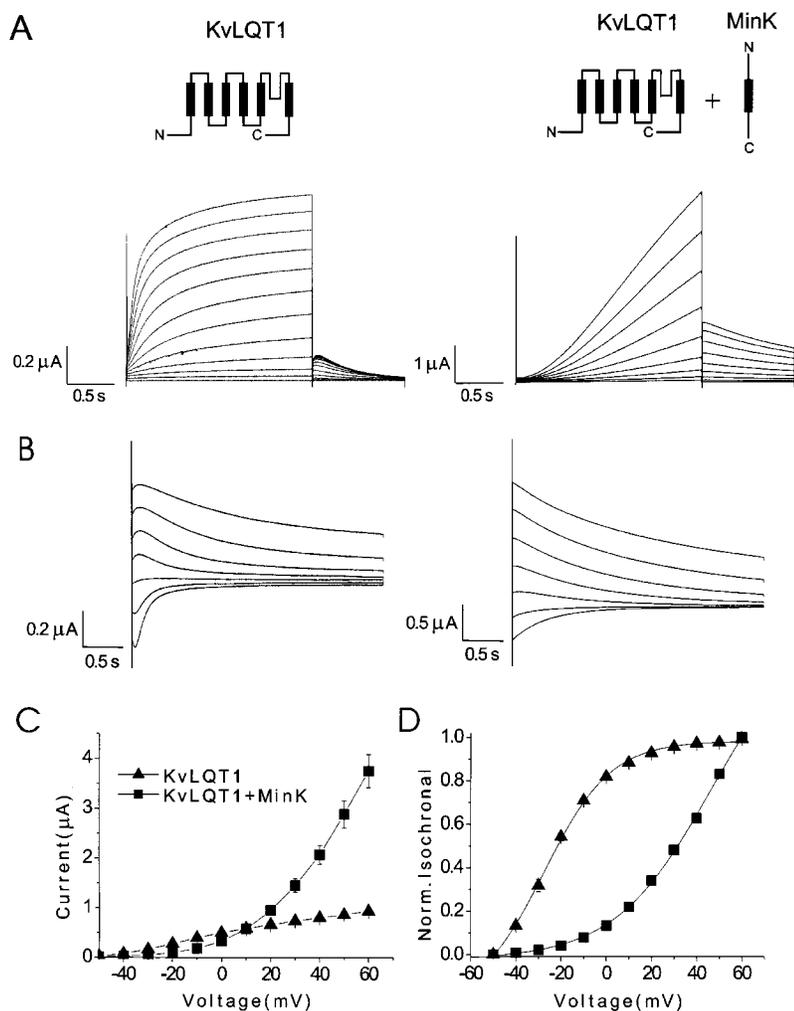
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Figure 1. MinK Modulation of KvLQT1. (A) Two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing either KvLQT1 (left) or KvLQT1+MinK (right). From a holding potential of −80 mV, oocytes were depolarized for 2 s to test potentials between +60 and −50 mV in 10-mV steps, followed by repolarization to −70 mV for 1 s. (B) Tail currents from oocytes expressing either KvLQT1 (left) or KvLQT1+MinK (right). Tail currents were elicited by a 4-s +40-mV prepulse, followed by repolarization to potentials between −40 and −100 mV in 10-mV steps. (C) Current–voltage relationship for KvLQT1 or KvLQT1+MinK. Currents were recorded after 2-s pulses at the given test potential. Error bars represent SEM ($n = 5$). (D) Normalized isochronal ($t = 2$ s) activation curve for five oocytes expressing either KvLQT1 or KvLQT1+MinK. The activation curves were derived from currents elicited by the activation protocol described in A. Experimental data points were fit with the equation $1/[1 + \exp(V - V_{1/2})/k]$, which gave the following apparent $V_{1/2}$ and slope factors: for KvLQT1: $V_{1/2} = -27.4 \pm 1.1$ mV, $k = 14.7 \pm 0.66$; and for KvLQT1+MinK: $V_{1/2\text{app}} = 29.4 \pm 1.7$ mV, $k = 16.4 \pm 1.6$. Error bars represent SEM.

TABLE I

Activation and Deactivation Properties of KvLQT1+MinK Chimera or MinK Mutant Channels

Channel	$V_{1/2\text{app}}$	k	τ_{deact}
	mV		*ms*
KvLQT1	−27.4 ± 1.1*	14.7 ± 0.7	468.9 ± 37.7*
KvLQT1+G55C MinKΔCterm	−16.6 ± 0.5‡	13.6 ± 0.4	279.1 ± 13.4‡
KvLQT1+β1MinK1	−28.9 ± 1.5*	14.3 ± 1.3	380.8 ± 15.2*
KvLQT1+β1MinK2	−27.6 ± 1.4*	16.8 ± 1.1	612.9 ± 48.2*
KvLQT1+β1MinK3	30.6 ± 1.7§	22.2 ± 0.4§	695.8 ± 88.1*
KvLQT1+MinK CΔ79–129	18.4 ± 1.5‡	19.5 ± 1.4	236.5 ± 32.6‡
KvLQT1+MinK CΔ94–129	29.4 ± 1.2§	14.0 ± 1.1	349.4 ± 28.6*
KvLQT1+MiRP1/Cterm-MinK	−33.6 ± 1.9*	16.9 ± 1.6	445.1 ± 13.1*
KvLQT1+MinK/Cterm-MiRP1	17.5 ± 3.8‡	23.4 ± 2.0§	187.3 ± 8.3‡
KvLQT1+MinK	29.4 ± 1.7	16.5 ± 1.6	1252.1 ± 89.11§

*$P < 0.001$ compared with KvLQT1+MinK, ‡$P < 0.001$ compared with KvLQT1 and KvLQT1+MinK, §$P < 0.001$ compared with KvLQT1. $V_{1/2}$ and slope factors were derived as in Fig. 1 D. Time constants of deactivation (τ_{deact}) at −70 mV were obtained as in Fig. 4 D. $n \geq 5$ for each value.

voltage at which half the channels were open was shifted +10 mV and the rate of deactivation was increased (Table I).

To determine whether MinK ΔCterm was associated with KvLQT1 despite the fact that little KvLQT1 modulation was observed, we engineered a functional tag into the MinK ΔCterm transmembrane domain. This tag, a point mutation replacing glycine 55 with cysteine (G55C), has been shown to confer susceptibility to partial block by external Cd^{2+} when coexpressed with KvLQT1 in *Xenopus* oocytes (Tai and Goldstein, 1998). Despite the absence of gating modulation, coexpression of G55C MinK ΔCterm rendered KvLQT1 currents Cd^{2+}sensitive (Fig. 2 B, right). Exposing channels to Cd^{2+} for 15 min resulted in a 35% reduction in current (Fig. 2 D). The time course of the current block fit a monoexponential function, as expected for a simple bimolecular reaction. Furthermore, current block by Cd^{2+} was fully reversible upon washout with ND-96 bath solution (Fig. 2 C). This effect of Cd^{2+} was not due to an interaction with an endogenous cysteine in

KvLQT1, as oocytes expressing KvLQT1 alone were not Cd^{2+} sensitive (Fig. 2 B, left).

Together, these data indicate that (a) the MinK COOH terminus is necessary for KvLQT1 modulation, and (b) an associative interaction between KvLQT1 and either the MinK transmembrane domain or NH_2 terminus can occur in the absence of gating modulation.

Role of the MinK Transmembrane Region in KvLQT1 Modulation

To test which MinK subregions were sufficient for gating modulation, chimeras were constructed between MinK and an unrelated protein, the sodium channel $\beta 1$ subunit. The $\beta 1$ subunit was chosen as the MinK chimeric partner because it has a similar membrane topology and its NH_2 terminus is sufficient for sodium channel modulation providing a functional assay for cell surface expression. To determine whether the MinK COOH terminus alone is sufficient to modulate KvLQT1, a chimera encoding the $\beta 1$ NH_2 terminus and
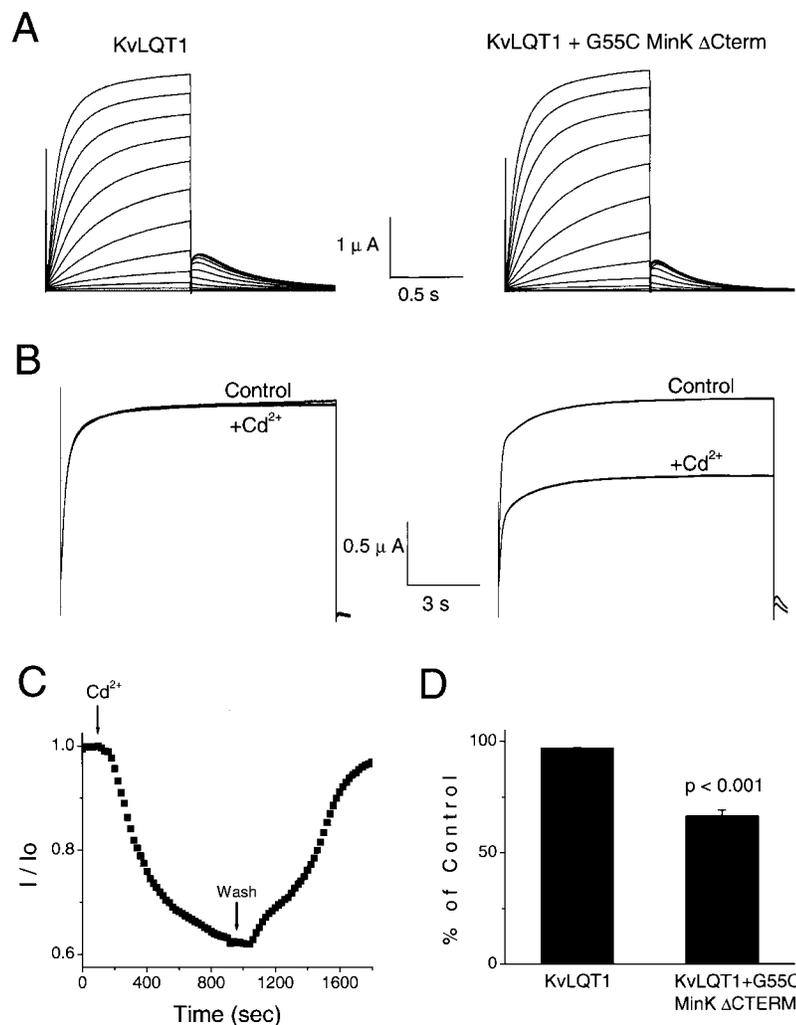

Figure 2. Coexpression of KvLQT1 and G55C MinK ΔCterm. (A) Two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing either KvLQT1 alone (left) or KvLQT1+G55C MinK ΔCterm. Currents were elicited as in Fig. 1 A. (B) Oocytes expressing KvLQT1 (left) or KvLQT1+G55C MinK ΔCterm (right) were exposed externally to 0.5 mM Cd^{2+} in ND-96 bathing solution for 15 min during repetitive 10-s pulsing to +20 mV every 20 s. Representative tracings are from the same oocyte before and after Cd^{2+} exposure. (C) Time of onset and reversibility of Cd^{2+} block. Representative data from a typical experiment, as described in B. Each data point represents steady state current at the end of a 10-s pulse to +20 mV. The oocyte was continuously perfused with Cd^{2+} bathing solution beginning at the time indicated by the arrow. Washout of Cd^{2+} was performed by continuous perfusion of the oocyte with ND-96 beginning at the time indicated by the arrow. (D) Percentage of Cd^{2+}-induced current block. Bar graph representation of the percentage of current remaining after oocytes expressing either KvLQT1 alone (left) or KvLQT1 and G55C MinK ΔCterm (right) were exposed to 0.5 mM Cd^{2+} for 15 min. Data is averaged for five experiments.

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transmembrane domain (amino acid residues 1–182) fused to the MinK COOH terminus (amino acid residues 67–129, β1/MinK1) was coexpressed in *Xenopus* oocytes with KvLQT1. Upon depolarization, currents recorded from cells expressing both KvLQT1 and β1/MinK1 closely resembled those observed in oocytes expressing KvLQT1 alone (Fig. 3 A, left; Table I). In a separate group of oocytes, β1/MinK1 was coexpressed with the human skeletal muscle sodium channel (hSkM1). The resulting sodium currents exhibited more rapid inactivation when compared with hSkM1 alone, demonstrating that β1/MinK1 modulates hSkM1 and indicating that it was expressed at the plasma membrane (Fig. 3 A, right). Together, these results indicate that the MinK COOH terminus is not sufficient for KvLQT1 modulation.

Additional β1/Mink chimeras were used to investigate the role of the MinK transmembrane domain in KvLQT1 modulation. One chimera (β1/MinK2) contained β1 amino acid residues 1–171, encoding the NH_2 terminus and half the transmembrane domain, fused to MinK residues 55–129. Coexpressing β1/MinK2 with KvLQT1 yielded currents that resembled KvLQT1 alone, although activation was slightly slower (Fig. 3 B, left). β1/MinK2 was able to modulate hSkM1, indicating that it was properly targeted to the membrane (Fig. 3 B, right). An additional β1/MinK chimera (β1/MinK3) encoded the β1 NH_2 terminus (amino acid residues 1–160) fused to the MinK transmembrane domain and COOH terminus (amino acid residues 44–129). Coexpression of β1/MinK3 with KvLQT1 resulted in currents nearly identical to I_{Ks}, although the rate of deactivation was increased compared with wild type (Fig. 3 C, left; Table I). This result suggests that the MinK transmembrane domain and COOH terminus together are sufficient to explain the predominant effects of MinK on KvLQT1, while the NH_2 terminus is not necessary for modulation. Coexpression of the chimera with hSkM1 yielded a modulated sodium channel phenotype (Fig. 3 C, right), indicating that the chimera was present at the cell surface and supporting the idea that the β1 amino terminus is sufficient for hSkM1 modulation (Makita et al., 1996; McCormick et al., 1999).

The results of the chimeric analysis indicate that the MinK NH_2 terminus is not necessary for KvLQT1 modulation, while the transmembrane domain is critical. Our results, described above, demonstrating Cd^{2+} sensitivity of currents derived from KvLQT1 and G55C MinK ΔCterm indicate that an associative interaction between KvLQT1 and either the MinK NH_2 terminus or transmembrane domain occurs in the virtual absence of KvLQT1 modulation. This result combined with our chimeric data strongly suggests that an important interaction occurs between KvLQT1 and the MinK transmembrane domain.

MinK Amino Acid Residues 67–93 Are Important for KvLQT1 Gating Modulation

The MinK COOH terminus consists of 63 amino acid residues. Previous data suggests that the ability of MinK to modulate KvLQT1 resides in amino acid residues 67–93 (Takumi et al., 1991; Ben-Efraim et al., 1996). We examined this by coexpressing a MinK deletion mutant lacking amino acid residues 94–129 (MinK CΔ94-129) with KvLQT1. The recorded currents illustrate that MinK CΔ94–129 retains its modulatory effects on KvLQT1 activation (Fig. 4 A, left center). The deletion mutant, like wild-type MinK, also shifted the voltage dependence of KvLQT1 activation to more positive potentials (Fig. 4 B; Table I) and removed KvLQT1 inactivation, as evident by the lack of a hooked tail current (C). It was noted that KvLQT1/MinK CΔ94–129 channels had an increased rate of deactivation when compared with channels formed by KvLQT1 and wild-type MinK (Fig. 4 D; Table I). These results suggest that MinK amino acid residues 94–129 are not necessary for the majority of the MinK effects on KvLQT1 gating.

To further investigate the role of MinK amino acid residues 67–93, an additional MinK deletion mutant missing amino acids 79–129 was engineered (MinK Δ79–129) and coexpressed with KvLQT1 in oocytes. Upon depolarization, MinK Δ79–129 slowed KvLQT1 activation to a lesser extent than wild-type MinK (Fig. 4 A, right center). Additionally, MinK Δ79–129 shifted the voltage dependence of KvLQT1 activation to more positive potentials (Fig. 4 B; Table I), and removed KvLQT1 inactivation (C) implicating the importance of MinK amino acid residues 67–78 in KvLQT1 gating modulation. Interestingly, like MinK Δ94–129, the rate of KvLQT1/MinK Δ79–129 deactivation was also increased compared with KvLQT1/wild-type MinK channels (Fig. 4 D; Table I).

A MinK/MiRP1 Chimera Modulates KvLQT1

MiRP1, like MinK, is a member of a family of small, single transmembrane subunits that modulate voltage-gated potassium channels. MiRP1 and MinK share 27% amino acid identity overall and exhibit several clusters of conserved residues within the transmembrane domain and COOH terminus. Despite these similarities, when coexpressed with KvLQT1, MiRP1 has no effect on gating or current amplitude (Abbott et al., 1999; Fig. 5 B, left). However, MiRP1 does modulate HERG, increasing the rate of deactivation while decreasing current amplitude and shifting the voltage dependence of activation to more positive voltages (Abbott et al., 1999; Fig. 5 B, right). To determine whether MiRP1 associates with KvLQT1, but does not modulate due to differences in the MiRP1 COOH terminus, a chimera containing the MiRP1 NH_2 terminus and transmem-
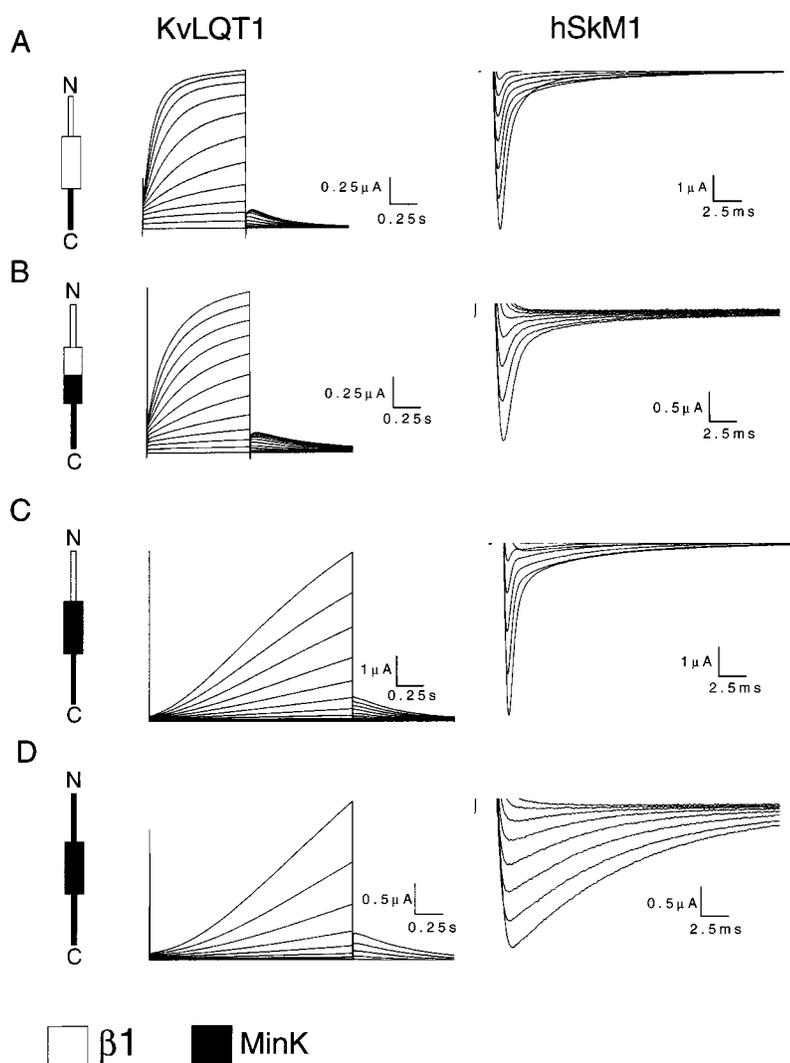
A

KvLQT1

hSkM1

Figure 3. Effects of β/MinK chimeras on KvLQT1 and hSkM1. (A) Two-electrode voltage-clamp recordings from *Xenopus* oocytes expressing either KvLQT1 (left) or hSkM1 (right) and β/MinK1. Currents from oocytes coexpressing the chimera and KvLQT1 were elicited by a 1-s pulse to potentials between +60 and −50 mV in 10-mV steps, followed by repolarization to −70 mV for 1 s. Oocytes were held at −80 mV. Currents recorded from oocytes coexpressing hSkM1 and the chimera were elicited by 30-ms pulses to voltages between −80 and +10 mV from a holding potential of −120 mV. (B) Oocytes coexpressing KvLQT1 or hSkM1 and β/MinK2. The activation protocol used was the same as in A. (C) Oocytes coexpressing KvLQT1 or hSkM1 and β/MinK3. The KvLQT1 activation protocol was the same as the protocol used in A, except depolarizing pulses were 2 s. The hSKM1 activation protocol was the same as in A. (D) Oocytes coexpressing KvLQT1 or hSkM1 and MinK. Activation protocols were the same as in C. Icons represent the proposed membrane topology of MinK and the chimeras. Filled boxes and lines represent MinK regions; open boxes and lines represent β1 regions.

brane domain fused to the MinK COOH terminus (MiRP1/Cterm-MinK) was engineered and coexpressed with KvLQT1 in *Xenopus* oocytes. The recorded currents exhibit a phenotype identical to that of KvLQT1 alone (Fig. 5 C, left; Table I), indicating that MiRP1/Cterm-MinK either does not associate with and modulate KvLQT1 or that it is not expressed at the cell surface. To determine whether MiRP1/Cterm-MinK is targeted to the plasma membrane, we coexpressed MiRP1/Cterm-MinK with HERG. MiRP1/Cterm-MinK modulated HERG much like wild-type MiRP1 (Fig. 5 C, right), suggesting that our chimera is indeed expressed and that it cannot modulate KvLQT1. However, the converse chimera containing the MinK NH$_2$ terminus and transmembrane domain fused to MiRP1 amino acids 73–99 (MinK/Cterm-MiRP1), when coexpressed with KvLQT1 (Fig. 5 D, left), yielded a modulated phenotype, slowing KvLQT1 activation, while also shifting the V$_{1/2}$ and increasing the rate of deactivation (Table I). Based on

the fact that the degree of modulation that occurs with MinK/Cterm-MiRP1 is much more dramatic than the degree of modulation seen with MinK ΔCterm (Fig. 2), and that the COOH terminus of MiRP1 is quite similar to that of MinK, we conclude that a significant degree of the gating modulation seen upon coexpression of the chimera with KvLQT1 is due to the presence of the MiRP1 COOH terminus. Interestingly, coexpression of MinK/Cterm-MiRP1 with HERG yielded currents closely resembling HERG alone (Fig. 5 D, right). Based on our previous results that indicate the MinK COOH terminus can modulate KvLQT1 only when an interaction between KvLQT1 and the MinK transmembrane domain occurs, we conclude that MiRP1 does not modulate KvLQT1; in part because of differences in the transmembrane domain that mediate KvLQT1 association. This conclusion supports the idea that two MinK subregions mediate association and modulation of KvLQT1.
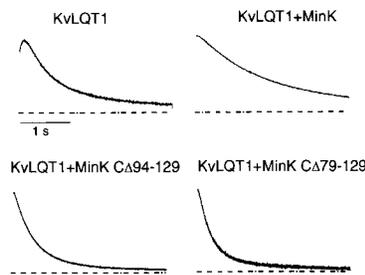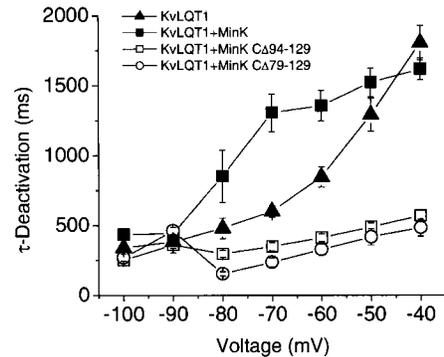
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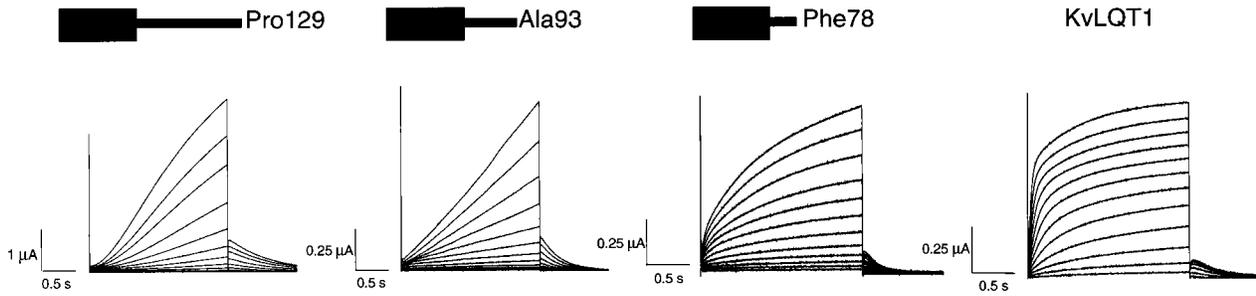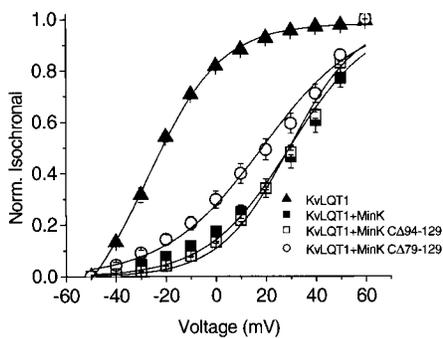
Figure 4. Effects of MinK deletion mutants on KvLQT1. (A) Voltage-clamp recordings of *Xenopus* oocytes expressing (from left to right) KvLQT1+MinK, KvLQT1+MinK CΔ94–129, KvLQT1+MinK CΔ79–129, or KvLQT1 alone. Activation protocol was the same as in Fig. 1. Icons represent MinK with the last amino acid indicated. (B) Normalized isochronal ($t = 2$ s) activation curve for KvLQT1, KvLQT1+MinK, KvLQT1+MinKCΔ94–129, and KvLQT+MinK CΔ79–129. Experimental data points were fit with the function $1/[1 + \exp(V - V_{1/2})/k]$, which gave the following apparent $V_{1/2}$ and slope factors: KvLQT1+MinK: $V_{1/2app} = 29.4 \pm 1.6$ mV, $k = 16.4 \pm 1.6$; KvLQT1+MinK CΔ94–129: $V_{1/2app} = 29.3 \pm 1.2$ mV, $k = 14.0 \pm 1.1$; KvLQT1+MinK CΔ79–129: $V_{1/2app} = 18.4 \pm 1.5$ mV, $k = 19.5 \pm 1.4$ ($n = 5$). Error bars represent SEM. (C) Representative tail current tracings from oocytes expressing KvLQT1 (top, left), KvLQT1+MinK (top right), KvLQT1+MinK CΔ94–129 (bottom left), and KvLQT1+MinK CΔ79–129 (bottom right). Tail currents were elicited by a 4-s +40-mV prepulse, followed by repolarization to −70 mV. (D) Voltage dependence of deactivation time constants for KvLQT1, KvLQT1+MinK, KvLQT1+MinK CΔ94–129, and KvLQT1+MinK CΔ79–129. Time constants were determined from a monoexponential fit of tail currents elicited via the deactivation protocol used in Fig. 1. For KvLQT1+MinK and KvLQT1+MinK deletion mutants, tail currents were fit over a 2-s interval immediately after repolarization. For KvLQT1, tail currents were fit starting at the peak of the tail current hook ($n = 5$). Error bars represent SEM.

DISCUSSION

In this study, we used deletion analysis and chimeras to identify the MinK subdomains involved in KvLQT1 modulation. MinK has multiple effects on KvLQT1 gating, including slowing of activation, shifting the voltage dependence of activation to more positive potentials, and removal of inactivation. MinK increases current amplitude three- to fivefold when coexpressed with KvLQT1. Finally, for modulation to occur, MinK must presumably associate with KvLQT1, but it was previously unknown whether this involves the same or different structures responsible for the gating and current amplitude effects. Here, we present data identifying which Mink subdomains are responsible for association with and gating of KvLQT1.

The results from our deletion analysis and chimeras indicate that the MinK COOH terminus is necessary but not sufficient for KvLQT1 modulation. Our experiments using MinK/β1 chimeras lacking a complete MinK transmembrane domain demonstrate that the MinK COOH terminus does not modulate KvLQT1 in the absence of the MinK transmembrane domain, even though the subdomains reach the plasma membrane. These data indicate that the COOH terminus is not sufficient for modulation and that subunit association requires the MinK transmembrane domain. This associative interaction involving the transmembrane domain may serve to localize the MinK COOH terminus to a region of KvLQT1 that is involved directly or indirectly in the modulation process. In addition to association, the
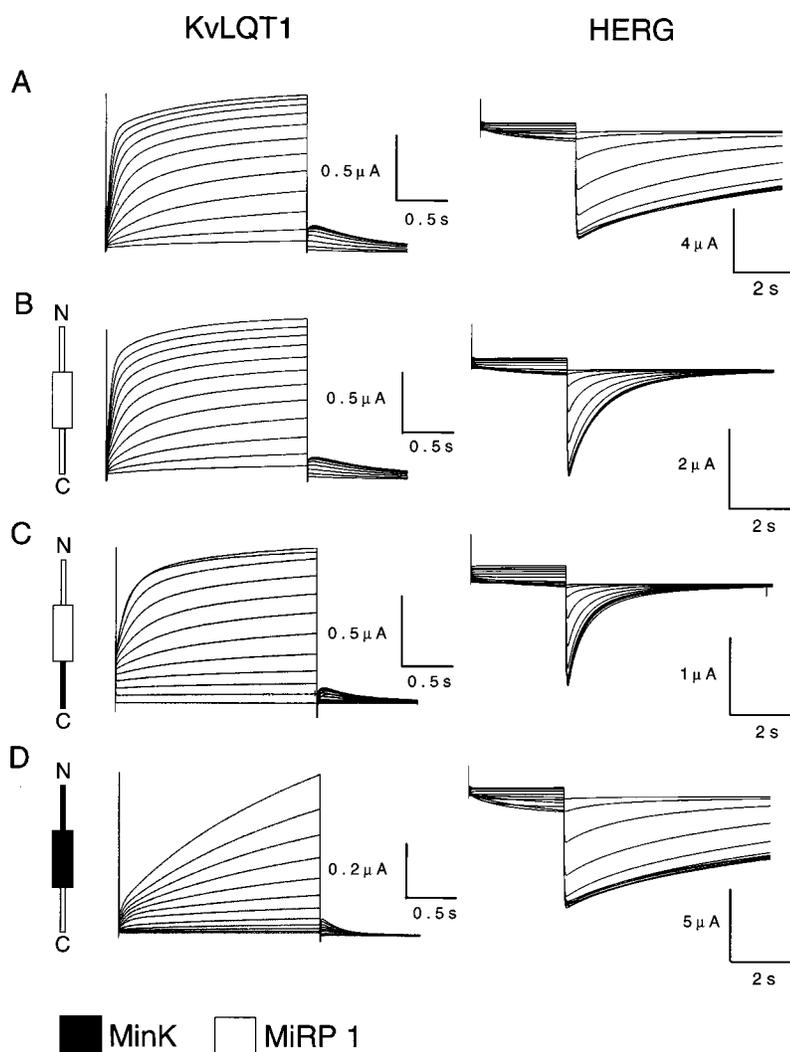
KvLQT1   HERG

Figure 5.   Effects of MinK/MiRP1 chimeras on KvLQT1. (A) Whole-cell voltage-clamp recording of *Xenopus* oocyte expressing either KvLQT1 (left) or HERG (right). For oocytes expressing KvLQT1, currents were elicited by the activation protocol described in Fig. 1 A. Oocytes were bathed in ND-96. For oocytes expressing HERG, currents were elicited by 3-s depolarizing pulses from +40 to −70 mV in 10-mV steps, followed by repolarization to −80 mV for 6 s. For all HERG experiments, oocytes were bathed in 95 mM KCl. (B) Oocytes expressing either KvLQT1 (left) or HERG (right) with MiRP1. Activation protocols were the same as in A. (C) Oocytes expressing either KvLQT1 (left) or HERG (right) with MiRP1/Cterm-MinK. Activation protocols were the same as in A. (D) Oocytes expressing either KvLQT1 (left) or HERG (right) with MinK/Cterm-MiRP1 (right). Activation protocol was the same as in A. Icons represent predicted topology of the chimeras. Filled boxes and lines represent MinK regions; open boxes and lines represent MiRP1 regions.

MinK transmembrane domain may also play a direct role in gating modulation, although this is clearly dependent on the MinK COOH terminus since, in the absence of the COOH terminus, little gating modulation is observed (Fig. 2 A, Table I). Supporting this idea, Takumi et al. (1991), using amino acid substitution, determined that a single point mutation within the MinK transmembrane domain, L52I, altered gating modulation of the KvLQT1 present in *Xenopus* oocytes. In addition, a mutation in the MinK transmembrane domain associated with long QT syndrome has been found to alter MinK-mediated KvLQT1 gating modulation (see below). Whether these two transmembrane domain mutations alter gating modulation because they are directly involved in gating or because the mutations alter protein conformation so that the MinK COOH terminus is not in the proper orientation to modulate KvLQT1 is unknown and requires further study. A recent report indicates that small molecule activators of the I_{Ks} complex, namely stilbenes and fenamates, medi-

ate their pharmacological effects through the MinK NH_2 terminus. Interestingly, the results from our experiments indicate that the MinK NH_2 terminus is not necessary for KvLQT1 modulation, although this does not rule out the possibility that this region is important for pharmacological phenomena.

The mechanism by which MinK modulates KvLQT1 gating is currently unknown. Our MinK COOH-terminal deletion analysis indicates that the critical MinK region involved in gating modulation resides within amino acid residues 67–93. A MinK deletion mutant with amino acids 94–129 deleted was able to slow KvLQT1 activation, shift the voltage dependence of activation to more positive potentials, and remove inactivation. An additional deletion mutant containing only 12 COOH-terminal amino acids (residues 67–78) shifts the voltage dependence of activation to more positive potentials, removes inactivation, and partially slows activation. From these results, we conclude that when associated with KvLQT1 and fused to the MinK trans-

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membrane domain, MinK amino acid residues 67–93 are critical for gating modulation. It will be interesting to determine how this small region of the MinK COOH terminus exerts it effects on KvLQT1.

Eight naturally occurring point mutations in MinK have been discovered and linked to congenital long QT syndrome, five of which have been characterized functionally (Bianchi et al., 1999; Schulze-Bahr et al., 1997; Splawski et al., 1997; Tyson et al., 1997; Duggal et al., 1998). Three of the mutations, S74L, D76N, and W87R are localized to the MinK COOH-terminal region that we have found to be critical for KvLQT1 gating modulation. These three mutants confer altered gating properties to KvLQT1, which is consistent with our results (Splawski et al., 1997; Bianchi et al., 1999). The two other MinK mutants, V47F and L51H, reside in the transmembrane domain. V47F MinK shifts the KvLQT1 voltage dependence of activation to more positive potentials compared with wild-type (Bianchi et al., 1999). In agreement with this finding, our results indicate that the transmembrane domain may play a role in altering KvLQT1 voltage dependence of activation since coexpression of KvLQT1 with MinK ΔCOOH term did shift the KvLQT1 voltage dependence of activation to more positive potentials compared with KvLQT1 alone. Coexpression of L51H MinK with KvLQT1 yields currents closely resembling KvLQT1 alone. Immunohistochemical analysis indicated that this is due to the fact that L51H MinK is not expressed at the cell surface, perhaps because it is misfolded or improperly targeted (Bianchi et al., 1999).

To date, three of five KCNE family members have been functionally characterized. MinK and MiRP2 both coassemble with KvLQT1, while MiRP1 forms channels with HERG (Barhanin et al., 1996; Sanguinetti et al., 1996; Abbott et al., 1999; Schroeder et al., 2000). Interestingly, these three family members share the most similarity within regions that we have identified as important for subunit association and gating modulation. Our chimera experiments indicate that MiRP1 cannot modulate KvLQT1 due to a difference in the transmembrane domain. A chimera containing the MinK COOH terminus in a MiRP1 background does not modulate KvLQT1, presumably because the associative interaction, as well as any interaction important for gating modulation that normally occurs between KvLQT1 and the MinK transmembrane domain, is not present. The converse chimera consisting of the MiRP1 COOH terminus in a MinK background does modulate KvLQT1, suggesting that the MiRP1 COOH terminus can modulate KvLQT1 gating in concert with the MinK transmembrane domain. Together, these results support our conclusion that the transmembrane domain is critical for subunit association and is important for gating modulation when fused to the COOH terminus. In addition, these results provide evidence that other KCNE members may associate with and modulate other K+ channels through analogous subdomains.

In summary, we have identified the MinK subdomains responsible for KvLQT1 gating modulation and subunit association. How and where these subregions interact with KvLQT1 is unknown, but presents an interesting and important question.

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