MinK-KvLQT1 Fusion Proteins, Evidence for Multiple Stoichiometries of the Assembled $I_{\text{sK}}$ Channel*

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$I_K$, a slowly activating delayed rectifier K$^+$ current through channels formed by the assembly of two channel proteins KvLQT1 and MinK, modulates the repolarization of cardiac action potentials. Mutations that map to the KvLQT1 and minK genes account for more than 50% of an inherited cardiac disorder, the Long QT syndrome (Szlacki, I., Tristani-Firouzi, M., Lehmann, M. H., Sanguinetti, M. C., and Keating, M. T. (1997) *Nat. Genet*., 17, 338–340). Despite the importance of these channels to human cardiac function, the molecular basis of their uniquely slow gating properties as well as the stoichiometry and interaction sites of these two subunits are still unclear. We have constructed several fusion channel proteins to begin investigating the stoichiometry of these two subunits and the role of voltage-dependent subunit assembly in channel gating. Functional properties of these constructs were measured using whole cell patch clamp recordings of transiently transfected Chinese hamster ovary cells. The constructs we tested are as follows: MK24 (C terminus of MinK linked to N terminus of KvLQT1); KK40 (a tandem homodimer of KvLQT1); and MKK44 (C terminus of MinK linked to N terminus of KvLQT1). In control experiments (no DNA, control DNA, or only MinK), no time-dependent K$^+$ current was observed. Expression of KvLQT1 or KK40 produced currents that activate and inactivate in a voltage-dependent manner as reported by others for KvLQT1. In contrast, expression of MK24 and MKK44 elicited current with activation kinetics and voltage dependence very similar to native $I_K$ and identical to currents expressed by cells co-transfected with independent MinK and KvLQT1 cDNA. Expression of MK24 plus additional MinK significantly slows current kinetics. Our data raise the possibility 1) of multiple MinK/KvLQT1 stoichiometries and 2) indicate that uniquely slow kinetics of $I_K$ channels is due to voltage-dependent conformational changes of the channel protein and not to assembly of channel subunits.

$I_K$, or MinK, a potassium channel protein sharing little similarity with other cloned potassium channels, induces very slow voltage-dependent K$^+$ channel activity in *Xenopus* oocytes expression system (5). It has only 130 amino acids and a single putative transmembrane domain. The cDNA coding for MinK was first cloned from rat kidney (6) and subsequently from neonatal rat heart (7). These cDNA clones exhibit identical protein sequences, indicating a possible absence of tissue-specific isoforms for this protein. Mouse heart MinK (8) and human heart (9) also show high sequence identity with the rat MinK protein, suggesting evolutionary conservation of biological function.

Expression of MinK in *Xenopus* oocytes or in HEK293 cells can induce a unique slowly activating voltage-dependent K$^+$ selective current that closely resembles native $I_K$, the slowly activating and non-inactivating K$^+$ channel current well characterized in heart (5, 10). Key experiments in *Xenopus* oocytes suggested that the MinK protein was a regulator of expressed channel activity and not sufficient by itself to form functional $I_K$ channels (11, 12). This work has been confirmed based on genetic linkage analysis of an inherited cardiac arrhythmia, the Long QT (LQT1) syndrome and positional cloning strategies (13, 14). Co-expression of KvLQT-1, a cardiac K$^+$ channel linked to LQT1 with MinK, elicits membrane currents with properties of $I_K$ (2, 3).

The interaction of MinK with the KvLQT1 protein changes inactivation and activation properties of the assembled channels (4, 15), and some experiments have suggested that MinK may affect permeation properties of the channel pore (16–19). However, questions remain about the stoichiometry of the MinK/KvLQT1 channels as well as the molecular basis for the uniquely slow gating of the functional $I_K$ channels.

$I_K$ gating kinetics are affected by the amount of MinK cRNA injected into *Xenopus* oocytes (12, 16), suggesting that the characteristically slow time course of $I_K$ is due in part to assembly of MinK and KvLQT1 proteins. Chemical cross-linking of $I_K$ protein also results in alteration in activation and deactivation kinetics (20), supporting the above hypothesis.

Here we report experiments in which we have created tandem multimer channel constructs in which the MinK and KvLQT1 proteins have been fused in different configurations. These fusion proteins were designed to restrict protein interactions and thus contained minimal peptide linkers between the subunits. The purpose of these experiments was to test directly the hypothesis that the slow gating kinetics of expressed $I_K$ channels is due to voltage-dependent assembly of MinK and MinK-free KvLQT1 proteins and to determine whether functional $I_K$ channels are formed by unique MinK/KvLQT1 stoichiometries.

CHO cells were transiently transfected with cDNA encoding fusion proteins of MinK and KvLQT1. Our results show that the kinetics of the MinK-KvLQT1 fusion proteins are identical to those of channels expressed in cells co-transfected with MinK and KvLQT1 cDNAs. This strongly suggests that the unique $I_K$ kinetics are not due to voltage-dependent assembly...
of channel subunits in the cell membrane and are not rate-limited by the amount of MinK-free KvLQT1 subunit in the membrane. Instead, voltage-dependent conformational changes of the MinK-KvLQT1 complex can account for \( I_k \) activation kinetics. However, we additionally find that the channels formed by MinK-KvLQT1 tandem constructs can be further modulated by co-expression with additional monomeric subunits. Thus our data suggest that the MinK subunit may act as an allosteric regulator of \( I_{\text{K}} \) channel kinetics.

**EXPERIMENTAL PROCEDURES**

**Materials**

The restriction enzymes were obtained from New England Biolabs (Beverly, MA) and Promega (Madison, WI). Pfu DNA polymerase (Stratagene (La Jolla, CA), was used in all PCR reactions to ensure sequence fidelity during amplification. Plasmid purification and fragment isolation kits were obtained from Qiagen (Santa Clarita, CA). The human cDNA clone KvLQT1 was a gift from Dr. M. Keating (Department of Human Genetics, University of Utah). The chemicals and reagents in the experiments were obtained from Sigma if not otherwise specified.

**Methods**

**Construction of Fusion Channels**—A two-step PCR strategy was employed to construct human MinK-KvLQT1 fusion channel. All primer sequences are listed in Table I but described here. An oligonucleotide primer WFmK1 overlapping the C terminus of MinK and N terminus of KvLQT1 was paired with another oligonucleotide primer HRKv2 complementary to KvLQT1. These were used as primers in the first round PCR reaction to amplify the chimeric sequence MK-1, composed of 27 base pairs of the C terminus of MinK (stop codon omitted) linked to the N terminus of KvLQT1 in frame. In the second round PCR, an equal molar mixture of MKv-1 and MinK was used as templates that were amplified by two outside primers, HFmNh3 and HRKv2. 10% Me2SO was added to all the reactions to overcome the high annealing temperature caused by higher GC contents in the sequences. The final product was cleaned up by “Wizard PCR Prep” (Promega).

A three-way ligation was performed as the following: pCDNA3.1(-) (Invitrogen) was digested with NheI and BamHI; KvLQT1-pCDNA3 was digested with XhoI and BamHI, and the PCR product was digested with NheI and XhoI. The three fragments were mixed in a molar ratio of 1:3:3 in the ligation reaction. Construction of KvLQT1 into its homo-dimer KK40 was similar to the above approach. The primer pairs for KvLQT1 amplifications were WFKEc5 and HRKBm6, WFkB17 and HKH11, all the PCR-amplified fragments were sequenced by the chain termination method in the DNA Sequencing Facility at Columbia University. KK40 was digested with XhoI, and the 2-kb insert was gel-purified and ligated into the XhoI site of MK24. Ligation was designed to ensure the correct orientation to form MKK44, with one MinK linked in frame to two copies of KvLQT1. The nomenclature used reflects both the relative contribution of each subunit as well as the insert size in pCDNA3.1(-). Thus MK24 reflects MinK-KvLQT1 = 1:1; vector, pCDNA 3.1(-); insert size, 2.4 kb; and MKK44 reflects MinK-KvLQT1 = 1:2; vector, pCDNA 3.1(-); insert size, 4.4 kb. All constructs were sequenced using mutagenesis primers and sequencing primers mk02–mk07, and the sequences were confirmed with the original cDNA sequences.

**TABLE I**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tr>
<td>WFmK1</td>
<td>CACCTTCCCTGAAGCCAAAAGCTTCCCACCATGTGCGGCGGCTCTTCTTCCGCCAGG</td>
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<tr>
<td>HRKv2</td>
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<td>HFmNh3</td>
<td>AAGACTAGCCACATTGCGCGGTGAATCTTGGTCTAC</td>
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<td></td>
<td>HindIII</td>
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**FIG. 1.** Transient expression of cDNA in CHO cells: controls and MinK-KvLQT1 constructs. Membrane currents were elicited by 2-s pulses (−40 to +80 mV, 20-mV intervals) applied from a holding voltage of −80 mV. Pulse rate was 0.1 Hz. Shown are recordings from individual cells (A and B) and averaged traces that had first been normalized to maximal current recorded (+80 mV; C and D). Transient transfection was used as described under “Experimental Procedures” using the following cDNAs: A, green fluorescent protein (GFP); B, MinK; C, KvLQT1 \((n = 7); D, KK40 (n = 5). Note: arrows (C and D) indicate hooks in deactivation tail currents (see text for details). Calibration bars: horizontal, 1 s, all panels; vertical, A and B, 100 pA; C, 770 pA; D, 1000 pA.
CHO cells (ATCC) were cultured in Ham’s F12 medium and transiently transfected using LipofectAMINE with and without LipofectAMINE-PLUS reagents (Life Technologies, Inc.). Cells were passaged the day before transfection at 50–70% confluence in 25-cm² tissue culture flasks. Transfected cells were split and plated directly onto 35-mm plastic Petri dishes after 12–20 h and used for patch clamp analysis 48 h after transfection. Neonatal mouse ventricular cells were prepared and studied as previously reported by us (21).

**Whole Cell Patch Clamp and Data Analysis—**

External solutions contained (in mM) the following: NaCl 132, KCl 4.8, MgCl₂ 1.2, CaCl₂ 1, glucose 5, HEPES 10, pH 7.4. The internal solution contained (in mM) the following: Kₐspartate 110, CaCl₂ 1, HEPES 10, EGTA 11, MgCl₂ 1, K₂ATP 5, pH 7.3 (KOH). Activation of IsK was studied using a series of 2- or 5-s depolarizing pulses from −240 mV to different test potentials in the range of −240 to +80 mV (20-mV increments). Activation curves were determined by measuring time-dependent activating (pulse) or deactivating (tail) currents. Data were collected, stored, and analyzed on IBM-compatible computers interfaced to Axopatch 200A amplifiers driven by pCLAMP software (version 6, Axon Instruments, Burlingame, CA). Graphical and statistical data analyses were carried out using ORIGIN (5.0) software (Microcal, Northampton, MA). Recordings were carried out at room temperature. For comparison of multiple experiments, currents recorded during activation pulse sequences were normalized to the maximal current recorded (at +80 mV) and then averaged for each recording voltage. Where shown, averaged data are noted, and the number of experimental records averaged is indicated.

**RESULTS AND DISCUSSION**

The primary goal of our present study was to investigate the assembly and stoichiometry of the two channel subunits. In control experiments, we transfected CHO cells with no DNA (not illustrated), green fluorescent protein (22) (Fig. 1A, n = 15), and MinK alone (Fig. 1B, n = 14). For each condition, we found no time-dependent K⁺ channel activity. Thus CHO cells do not have detectable endogenous KvLQT1 channel activity and constitute a feasible host system for this study.

In contrast, CHO cells transfected with KvLQT1 cDNA (Fig. 1C) expressed time- and voltage-dependent current with properties similar to IsK as described in the literature (2, 23). We found no time-dependent K⁺ channel activity. Thus CHO cells do not have detectable endogenous KvLQT1 channel activity and constitute a feasible host system for this study. We transfected CHO cells with no DNA (not illustrated), green fluorescent protein (22) (Fig. 1A, n = 15), and MinK alone (Fig. 1B, n = 14). For each condition, we found no time-dependent K⁺ channel activity. Thus CHO cells do not have detectable endogenous KvLQT1 channel activity and constitute a feasible host system for this study.
ramers (25, 26) in which each subunit has six putative α-helical transmembrane segments and a consensus K⁺ pore region. Since KvLQT1 has the above predicted structure (23), it should be possible to express \(IKvLQT1\) by transfecting cells with cDNA encoding KvLQT1 fusion proteins of two or four KvLQT1 subunits. Currents expressed by cells transfected with KK40 were indistinguishable from currents encoded by KvLQT1 (Fig. 1D), consistent with functional channels formed by multiples of the homodimers. These channels retain both the activation and inactivation (tail hook) properties of native KvLQT1 channels (compare Fig. 1, C and D). Thus, not surprisingly, the KvLQT1 channel structure resembles other Shaker K⁺ channels.

We next tested directly the functional properties of KvLQT1 and MinK multimers. First we constructed and studied the functional properties of the fusion channel proteins MK24 and MKK44. A general concern when such a fusion approach is adopted is whether the forced linkage ruptures the natural assembly of the channel, because the forced linkage may impose an unusual arrangement of the folding of and interactions between the domains of the channel and also influence expression of subunits (27). We were thus surprised to find that channels expressed from either MK24 and MKK44 strongly resemble wild type channel activity. Fig. 2 illustrates records for both constructs recorded in response to similar series of 2-s (upper row) and 5-s (lower row) voltage pulses and compares these with wild type MinK/KvLQT1 currents. Fig. 3 summarizes voltage-dependent activation and kinetic properties of the constructs. As is the case for wild type MinK/KvLQT1, currents expressed by both fusion proteins activate slowly, and although the time course of activation becomes faster with depolarization, it never approaches that of the KvLQT1 channels (Fig. 3B). Also consistent with co-expression of MinK and KvLQT1, channel activity expressed by the tandem multimers shows little evidence of inactivation either during voltage pulses or upon return to the holding potential (note lack of hook in deactivating tails).

KK40 and KvLQT1 channels share identical voltage-dependent activation gating properties (Fig. 3A), and co-transfection of MinK and KvLQT1 shifts the voltage dependence of expressed KvLQT1 channel activation to more positive voltages as has been reported by others (23, 28). Furthermore, our results indicate that the voltage dependence of activation and the kinetics of the tandem constructs are the same as that of channels expressed by the MinK/KvLQT-1 monomers (Fig. 3, A and B), i.e. shifted to positive voltages relative to activation of KvLQT1 or KK40. Notice also that even in response to these very prolonged voltage steps, MK24 and MKK44 currents do not reach steady state for any of the channel constructs. This very slow activation process, which is characteristic of native \(I_{Kc}\) channel activity recorded in cardiac cells (29), accounts for
the failure of the 2-s isochronal activation curves for $I_{\text{K}}$ and the tandem multimer constructs to saturate (Fig. 3A). These findings support the view that the forced linkage does not grossly disrupt $I_{\text{K}}$ heteromultimer channel formation. Additionally, the finding that the very slow activation gating kinetics of the expressed channel activity is conserved with the fusion proteins provides strong evidence that this characteristic of wild type $I_{\text{K}}$ is not due to voltage-dependent assembly of MinK with MinK-free KvLQT1 proteins.

The observation that MK24 and MKK44 equally resemble native $I_{\text{K}}$ channels suggests that the stoichiometry of the two proteins may not be fixed in the assembled $I_{\text{K}}$ channel, an atypical scenario for channel subunit interactions in general (30) but one previously suggested by others for $I_{\text{K}}$ (31, 32). Thus, it is highly possible that there is a minimal stoichiometry requirement for incorporation of MinK that modifies KvLQT1 gating such that expressed currents resemble $I_{\text{K}}$. This relationship would be supported by the expressed MKK44 channel activity. In support of this hypothesis is an earlier study in which 2 MinK monomers were suggested to associate with other, then unidentified, non-MinK subunits to form functional $I_{\text{K}}$ channels in Xenopus oocytes (33). How is it thus possible to detect multiple MinK and KvLQT1 stoichiometries?

One approach is to test for changes in multimeric $I_{\text{K}}$ channel activity by co-expression with additional MinK protein. Thus, to investigate whether the fusion proteins MK24 and MKK44 could also be modulated by additional MinK, we co-expressed MinK with the above constructs and characterized the expressed channel activity. If MinK slows the kinetics of the fusion protein, this effect cannot be due to depletion of MinK-free KvLQT1 as previously suggested by others (32, 34) but instead must be due to additional modulation of the assembled fusion protein. Co-transfection with additional MinK slows MK24 activation kinetics (Fig. 4). In order to quantify this difference, we measured the time to attain half-maximal current ($t_{1/2}$) for each construct and each current measurement. Shown in Fig. 4D is a summary of this analysis for the influence of MinK co-transfection on $t_{1/2}$ for MK24 for measurements at +60 mV. Co-transfection with MinK causes a significant ($p < 0.001$) difference in $t_{1/2}$ for MK24 channels. Interestingly,

![Figure 5. MinK increases the amplitude of expressed currents and alters voltage dependence of gating. A, isochronal (2 s) activation curves determined from current tail analysis (under “Experimental Procedures”) for MinK + KvLQT1 (■), MK24 (○), and MK24 + MinK (▲). Activation data obtained from neonatal mouse ventricular cells (○), n = 22. Data are shown as mean ± S.E. B, time to half-maximal pulse current (under “Experimental Procedures”) determined from averaged data presented in Figs. 2 and 4 versus test pulse voltage. The symbols used are: MinK + KvLQT1 (■); MK24 (○); and MinK + MK24 (▲). Data obtained from neonatal mouse ventricular cells are summarized as n = 9 (○). C, mean ± S.E. current measured at the end of 2 s pulses to +60 mV for the constructs indicated along the abscissa. The number of cells studied for all panels was as follows: KvLQT1 (n = 7), KvLQT1 + MinK (KvLQT1 + M, n = 11), Mk24 (n = 8); MK24 + MinK (MK24 + M, n = 6). *, not significantly different; #, significantly different, p < 0.01.](image-url)
deactivation kinetics of MK24 are not affected by MinK co-transfection as is evidenced by deactivation tail records shown in Fig. 4, B and C.

The slowing of MK24 activation kinetics illustrated at one voltage in Fig. 4 occurs over the entire range of activation (Fig. 5B). In addition, co-expression of MinK with MK24 further alters the voltage dependence of activation as evidenced by the MinK-induced changes in the isochronal activation curve illustrated in Fig. 5A. These data indicate that additional MinK protein(s) are able to interact with the MinK-KvLQT1 fusion protein MK24, and this interaction specifically alters channel activation but not deactivation kinetics. Also shown for comparison in Fig. 5 are comparable activation (A) and kinetic (B) data for $I_{K}$ measured in neonatal mouse ventricular cells (21). These data, plotted as open circles in both panels, are remarkably similar to MinK-KvLQT1 and the MK24 data but are distinct from MK24 + MinK data, strongly suggesting that native $I_{K}$ channels in cardiac cells assemble with a minimum MinK-KvLQT1 stoichiometry.

Our results clearly show that the unique functional properties conferred upon KvLQ1 by incorporation of MinK protein are due to differences in voltage-dependent conformational changes of the assembled MinK-KvLQT1 protein complex. Furthermore, we find that MinK can alter the voltage-dependent gating and kinetic properties of tandem multimer complexes strongly suggesting the existence of more than one MinK-KvLQT1 stoichiometry. Work by others has suggested that MinK lines the pore of $I_{K}$ channel, modifying its permeability (18, 35, 36) and single channel properties (32) of the expressed channels. Structural analysis of the potassium channel from Streptomyces lividans, which has sequence similarity to KvLQT1 in the P region, raises the possibility that MinK modulation may arise from interactions outside of the KvLQT1 pore (37). In contrast, channel gating is a dynamic process, the basis for which structural data are not yet available. Our data showing that MinK modulates the MK24 fusion protein raise the interesting possibility that modulation of gating kinetics may involve interaction sites peripheral to the channel pore and that functional changes in channel activity are the result of allosteric modulation of the channel protein. How the MinK protein confers these properties upon the assembled channel and the identification of key interaction sites that underlie these functional changes remain to be determined. Future experiments in which we restrict the linkage to and size of the MinK construct should be very useful in identifying these residues as well as their biophysical roles.

Drugs that target the KvLQT1-MinK complex, blockers of $I_{K}$, have been developed as Class III anti-arrhythmic drugs (38). Recently, the action of these compounds has been found to be dependent on the interactions between KvLQT1 and MinK proteins (39). Hence the fusion proteins developed in the present study should prove to be powerful tools in sorting out the molecular mechanisms of these drugs and modifying their activity by targeting key MinK/KvLQT1 interaction domains.

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