Four and a half LIM protein 1: a partner for KCNA5 in human atrium

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Aims Protein–protein interactions are critical for the normal membrane trafficking, localization, and function of voltage-gated ion channels. In human heart, the Shaker-related voltage-gated K⁺ channel KCNA5 α-subunit forms the major basis of an atrial-specific, ultra-rapid delayed rectifier K⁺ current, I_{Kur}. We sought to identify proteins that interact with KCNA5 in human atrium and investigate their role in the I_{Kur} complex.

Methods and results Using a glutathione-S-transferase (GST)-KCNA5 C-terminal fusion protein and mass spectrometry-based methods, the scaffolding protein four and a half LIM (for Lin-11, Isl-1, and Mec3) protein 1 (FHL1) was identified as a potential protein partner for KCNA5. Immunoprecipitation experiments confirmed a physical interaction of FHL1 with the K⁺ channel complex in human atrium, as well as in Chinese hamster ovary (CHO) cells transfected with both KCNA5 and FHL1. In cotransfected cells, confocal microscopy demonstrated areas of colocalization after immunolabelling both proteins. To investigate the functional effects of this interaction, K⁺ currents were recorded in CHO cells transfected with KCNA5 in the absence and presence of FHL1 coexpression. With coexpression of FHL1, K⁺ current density was markedly increased, compared with cells expressing KCNA5 alone. This effect was associated with a shift in the voltage dependence of K⁺ channel activation to more positive potentials, consistent with findings of I_{Kur} in atrial myocytes. FHL1 also increased the extent and speed of K⁺ current slow inactivation, with additional effects on the voltage dependence and recovery of this process.

Conclusion These results support a role of FHL1 as a key molecular component in the I_{Kur} complex in human atrium, where it likely regulates functional expression of KCNA5.

KEYWORDS K⁺ channel; Ion channels; Membrane currents; Repolarization; Atrial function; FHL1

1. Introduction

In the heart, voltage-gated K⁺ channels play a critical role in myocardial repolarization. Pore-forming K⁺ channel α-subunits coassemble as tetramers and interact with associated accessory subunits to form functional channels. In addition, emerging evidence indicates that channel α-subunits interact with other cellular proteins that regulate expression, intracellular trafficking, plasma membrane localization/anchoring, and channel gating. In general, the nature of these complex interactions in vivo for most channels remains poorly understood.

The ultra-rapid activating delayed rectifier K⁺ current, or I_{Kur}, has generated recent interest as a potential target in the treatment of atrial arrhythmias. Because of its atrial specificity in humans, it is predicted that pharmacologic block of I_{Kur} will be associated with a reduction in the ventricular proarrhythmia that occurs with non-specific K⁺ channel blockers. While the principal molecular component of I_{Kur} is known to be the KCNA5 α-subunit, studies have only recently begun to elucidate the identity and role...
of additional protein partners in the KCNA5 complex. In human atrial cells, \( I_{Kur} \) is regulated by activation of protein kinases and phosphatases, implying that these and other signalling proteins are potential partners in the channel complex.6,7 The accessory subunit KChIP2 has been shown to associate with KCNA5 in mammalian myocardium and regulate cell surface expression.8 The N-terminus of KCNA5 mediates interaction with the cytosolic protein \( \alpha\)-actinin-2, linking the channel subunit to the actin cytoskeleton.9 Additional evidence supports a role for the KCNA5 N-terminus in the regulation of channel function and spatial expression by the anchoring protein SAP97.10

In this study, we tested the hypothesis that the KCNA5 C-terminus can also serve to mediate protein–protein interactions in the \( I_{Kur} \) complex. In the analysis of macromolecular complexes in vivo, mass spectrometry-based techniques provide the opportunity to identify associated protein components in an unbiased manner.11,12 Utilizing mass spectrometry-based methods, we identified four and a half LIM protein 1 (FHL1) as a potential protein partner for KCNA5 in human atrium. LIM domains (for Lin-11, Isi-l, and Mec3) are a double zinc finger protein–protein interaction motif, and LIM proteins can function as scaffolding proteins for sarcomeric and signalling complexes.13 In this report, we present evidence that FHL1 coassembles in the \( I_{Kur} \) complex in vivo, where it likely plays a role to regulate KCNA5 current density and channel function.

2. Methods

2.1 Materials

Cell culture reagents were obtained from Gibco, with F-12 Nutrient Mixture (Ham) complete medium from Invitrogen. Analytical grade reagents and quinidine sulphate were obtained from Sigma. An affinity-purified, polyclonal rabbit antibody directed against the N-terminus (amino acids 1–248) of KCNA5 was kindly supplied by Merck Research Laboratory. The affinity-purified polyclonal rabbit antibody directed against a unique peptide in the fourth LIM domain (amino acids 261–272) of human FHL1 has been characterized previously.13,14 Additional antibodies for immunolocalization studies included a monoclonal antibody directed against the hae-magglutinin (HA) epitope (Sigma), and Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse secondary antibodies (Molecular Probes).

2.2 Heart extract preparation

Human atrial and ventricular tissue was obtained from cardiomypathic hearts explanted at the time of cardiac transplantation that was stored in a tissue repository approved by the Vanderbilt University Institutional Review Board. This investigation conforms to the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–4). Atrium or ventricle was ground with a mortar and pestle under liquid nitrogen prior to homogenization, resuspended in 10 volumes of homogenization buffer (20 mM HEPES, pH 7.5, 200 mM sucrose, 2 mM EDTA, 2 mM EGTA, 1 mM PMSE, 1 mM 4-[2-aminoethyl] benzenesulphonyl fluoride hydrochloride [AEBSF], 10 \( \mu \)g/mL leupeptin, 10 \( \mu \)g/mL pepstatin), and homogenized with a glass/Teflon homogenizer. Debris was pelleted by centrifugation at 1000 \( \times \) \( g \) for 10 min at 4 °C. The resulting supernatant was saved, and pellet was re-suspended, homogenized, and centrifuged as above. The two supernatants were combined. Cytosolic and membrane fractions were generated by centrifugation at 100 000 \( \times \) \( g \) for 1 h at 4 °C (supernatant is cytosolic fraction).

2.3 Preparation of Affi-Gel glutathione-S-transferase, glutathione-S-transferase-KCNA5 affinity matrix

To generate the KCNA5 C-terminal glutathione-S-transferase (GST) fusion protein (GST-KCNA5), cDNA encoding the C-terminus (amino acids 517–612) was cloned in frame to the 5′ end of GST in the bacterial expression vector pGEX-2T-KG (Amersham). BL21 Escherichia coli expressing either GST, or GST-KCNA5 fusion protein, were resuspended in 10 mL phosphate-buffered saline (PBS) plus protease inhibitors (Complete Mini, Roche) and lysed with three freeze-thaw cycles, followed by sonication. The lysate was centrifuged at 15 000 \( \times \) \( g \) for 15 min at 4 °C. The resulting supernatant plus 1% Triton X-100 was added to 1 mL of packed beads (Glutathione Sepharose 4B, GE Healthcare) and incubated rocking overnight at 4 °C. Beads were pelleted, transferred to a 5 mL column in 3 mL wash buffer (1× PBS, 1% Triton X-100, protease inhibitors [Complete Mini]), and washed with 100 mL wash buffer to remove any unbound GST fusion protein. Beads were then resuspended in 1 mL elution buffer (10 mM glutathione in 50 mM HEPES, pH 7.5) and allowed to incubate overnight at 4 °C. Eluted proteins were then used to prepare affinity columns. Purified fusion protein (2.5 mg, GST or GST-KCNA5) was incubated with 1 mL of Affi-Gel 10 (BioRad) for 4 h rocking at 4 °C. Beads were pelleted, fusion protein was removed and beads were washed 3× with wash buffer.

2.4 Affinity chromatography for silver staining, mass spectrometry

Four mL of a 1 mg/mL atrial cytosolic fraction was prepared in wash buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, protease inhibitors [Complete Mini]). Control affinity matrix (GST-Affi-Gel 10, 200 \( \mu \)L) was added to the heart fraction and incubated rocking at 4 °C for 30 min. Beads were pelleted, supernatant was moved to a fresh tube, and an additional 200 \( \mu \)L of control matrix was added to the supernatant for further pre-clearing (with incubation rocking at 4 °C for 30 min). The sample was centrifuged, and supernatant was divided into two (2 mL) fractions. To one fraction, 100 \( \mu \)L of control matrix was added, and 100 \( \mu \)L of experimental affinity matrix (GST-KCNA5 Affi-Gel 10) was added to the second fraction. Fractions were incubated overnight rocking at 4 °C. Beads were pelleted, re-suspended in 1 mL of wash buffer, and transferred to a 2 mL column. The column was washed with five volumes of wash buffer, and 500 \( \mu \)L elution buffer (500 mM NaCl, 100 mM glycine, pH 4.0) was added (with 1 h at 4 °C before elution). Eluted proteins were immediately precipitated with methanol. Dried proteins were resuspended in 1× sample buffer and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) or submitted for mass spectrometry analysis. Gels were silver stained with PlusOne Silver Staining Kit (GE Healthcare).

2.5 High-pressure liquid chromatography and tandem mass spectrometry

Eluted proteins in sample buffer were heated at 70 °C for 10 min, and then applied to a 10% SDS–PAGE gel with no stacker. Electrophoresis was continued until the dye-front had migrated into the gel ~1–2 cm. The gel was then stained with Gel Code Blue (Pierce), and the protein band was excised from the gel, minced, and washed. The proteins were subjected to in-gel trypsin digestion and the resulting peptides were eluted from the gel and submitted for analysis using high-pressure liquid chromatography (HPLC) coupled with tandem mass spectrometry (LC-MS-MS). This was performed using a Thermo Finnigan LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 1.4 instrument control as previously described.16 The MS/MS spectra of the peptides were performed using data-dependent scanning in
which one full MS spectrum, using a full mass range of 400–2000 amu, was followed by 3 MS/MS spectra. Proteins were identified using the TurboSEQUEST v.27 (rev. 12) algorithm (Thermo Electron, San Jose, CA, USA)\textsuperscript{17} on a high speed, multiprocessor Linux cluster in the Advanced Computing Center for Research & Education at Vanderbilt University using the human subset of the Uniref 100 database (www.uniprot.org). The database was concatenated with the reverse protein sequences so that false discovery rates could be calculated. Protein matches were preliminarily filtered to remove false positive results as previously described.\textsuperscript{16} Eluted proteins common to both control (GST) and experimental (GST-KCN5) columns, as well as common contaminants (e.g. keratin isoforms), were subtracted from the results as non-specific binding proteins.

2.6 K⁺ channel expression
cDNA encoding full-length KCN5 was cloned into the mammalian expression plasmid pBK-CMV.\textsuperscript{18} FHL1 cDNA was cloned previously into the pcDNA vector, generating FHL1 protein having an N-terminal HA tag.\textsuperscript{14} To monitor FHL1 expression, cDNA for FHL1-HA was cloned into a bicistronic plasmid (FHL1-IRES-GFP) that also encoded enhanced green fluorescent protein. Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells were transiently transfected with pBK-CMV-KCN5 (2 μg) combined with either 1 μg (1% gel) or FHL1-IRES-GFP (2 μg) using FuGene6 (Roche Diagnostics) according to the manufacturer’s instructions (with minor modifications).

2.7 Co-immunoprecipitation
Human atrial cytosol (700 μg) was precleared for 1 h at 4°C with 20 μL of a 50%/50% mixture of Protein A/G beads (Sigma), with the beads pelleted. KCN5 antibody (2.5 μg) or pre-immune sera (2.5 μg, negative control) was incubated with 30 μL of Protein A/G beads and 500 μL 1 × PBS for 1 h at 4°C. Antibody/pre-immune sera-bound beads were pelleted, washed three times with solubilization buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.32M sucrose, 5 mM EDTA, 2.5 mM EGTA, 1.0% Triton X-100, protease inhibitors [1 mM PMSF, 1 mM AEBSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin]), and resuspended in 100 μL of solubilization buffer. The pre-cleared cytosolic fraction and protease inhibitors (Complete Mini) were added to the antibody-bound beads in solubilization buffer, with incubation rocking at 4°C overnight. The beads were pelleted, washed four times with RIPA buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 10% glucose, 0.1% SDS, 0.5% Na-deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin), followed by two washes with solubilization buffer. Sample buffer (5 μL, 4 × ) was added, and samples were heated at 60°C for 20 min with vortexing. Proteins were separated by SDS-PAGE using a 4–12% gel, followed by western transfer onto a PVDF membrane. Membranes were blocked with 5% non-fat dry milk (NFDM) in TBST (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% [v/v] Tween-20) at room temperature for 2 h, washed 3 × 15 min in TBST, and placed in primary antibody (anti-FHL1, 1:6) in 5% NFDM/TBST at 4°C overnight. They were washed 3 × 15 min in TBST, placed in secondary antibody (goat anti-rabbit, 1:10,000 Jackson Laboratories) in 5% NFDM/TBST at room temperature for 2 h, washed 3 × 15 min in TBST, and developed with ECL western blotting analysis system (GE Healthcare). For identification of KCN5 in the immunoprecipitate, proteins were separated using an 8–15% SDS polyacrylamide gel, with a primary antibody dilution of 1:8000.

CHO cells were transfected with KCN5 in the absence and presence of FHL1-IRES-GFP. After 48 h, transfected (and untransfected) cells were washed three times with cold 1 × PBS, scraped, resuspended in harvest buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 1% Triton X-100, protease inhibitors [Complete Mini]), and frozen at −20°C. Cells were thawed and PMSF was added to a final concentration of 1 mM (generating the lysis buffer).

The cells were homogenized, with centrifugation at 14 000 rpm for 20 min at 4°C to pellet debris. The supernatant (whole cell lysate) was collected for immunoprecipitation. Antibody-conjugated beads were prepared by incubating the anti-KCN5 antibody (2.5 μg) with 30 μL of Protein A/G beads and 500 μL 1 × PBS for 1 h at 4°C. Antibody-bound beads were pelleted, washed three times (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% Triton X-100), and resuspended in 100 μL of lysis buffer. Whole cell lysate (700 μg) was pre-cleared for 1 h at 4°C with 20 μL of Protein A/G beads with 1/100 vol of protease inhibitor cocktail (Sigma), and the beads pelleted. The pre-cleared cell lysate was added to the antibody-conjugated beads in lysis buffer, and incubated rocking overnight at 4°C. Beads were pelleted and washed four times with RIPA buffer, followed by two washes with 1 × PBS. LDS sample buffer (20 μL, 4 × , Invitrogen) was added to achieve a final concentration of 2 × , and samples were heated at 60°C for 20 min with vortexing. Proteins were separated by SDS–PAGE (10% gel, Invitrogen) and transferred onto a PVDF membrane. Membranes were blocked with 5% NFDM in TBST at room temperature for 2 h, washed 3 × 15 min in TBST, and incubated overnight with primary antibody (anti-FHL-1, 1:6) in 5% NFDM/TBST at 4°C. Membranes were washed 3 × 15 min in TBST, incubated at room temperature with secondary antibody (goat anti-rabbit, 1:10 000, Jackson Laboratories) in 5% NFDM/TBST for 2 h, washed 3 × 15 min in TBST, and developed with ECL.

2.8 Immunolabelling and imaging
CHO cells were transfected as described and grown in 35 mm MatTek dishes (MatTek Corporation). After 48 h, cells were fixed with 2% paraformaldehyde for 10 min followed by neutralization with 100 mM glycine buffer for 10 min. They were washed with PBS for 10 min and permeabilized with 0.1% Triton X for 10 min, followed by washing with PBS. Blocking was performed by incubation with 10% horse serum for 1 h. Cells were labelled for 3 h at room temperature with antibodies specific for KCN5 (Merk; 1:1000) and FHL1 (monoclonal anti-HA, Sigma; 1:1000). Secondary antibodies were Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse (Molecular Probes; 1:1000, 1 h at room temperature). Cells were washed three times with PBS. Images were acquired using a confocal LSM 510 microscope (Carl Zeiss, Inc.) and prepared using the LSM510 software.

2.9 Electrophysiologic recordings and data analysis
Cells exhibiting green fluorescence were selected for patch clamping. K⁺ currents were recorded using the whole-cell patch-clamp technique as described previously.\textsuperscript{19,20} Specific voltage-clamp protocols are depicted in each figure, and the cycle time between pulses was 5 s. The pipette (intracellular) solution contained 120 mM K-β-aspartate, 25 mM KCl, 1 mM MgCl₂, 4 mM NaATP, 2 mM NaGTP, 5 mM HEPEs, 2 mM Phosphocreatine-Na₂ and 0.1 mM KBAPTA, pH 7.2 (adjusted with KOH, yielding a final intracellular K⁺ concentration of 145 mM). The bath solution contained 145 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPEs, pH 7.35 (adjusted with NaOH). K⁺ currents were recorded using an Axopatch 200 amplifier (Axon Instruments), with data acquisition and command potentials controlled using pCLAMP 9.2 (Axon Instruments). Electrode resistance was 1.5–2.5 MΩ, with whole-cell capacitance and series resistance ~90% compensated (uncompensated access resistance was 3.7–5.7 MΩ, with a voltage error <5 mV). All experiments were conducted at room temperature (22 ± 2°C).

Analysis of data was performed using pCLAMP 8.2. Activation curves were constructed from deactivating tail currents and fitted with a Boltzmann equation. Results are presented as mean ± standard error of the mean. Statistical significance was assumed for P < 0.05.
3. Results

3.1 Identification of four and a half LIM protein 1 as a potential KCNA5 partner

To search for potential protein partners of KCNA5, a GST fusion protein of the KCNA5 C-terminus was generated, coupled to Affi-Gel matrix, and incubated with cytosolic fraction of human atrium. Bound proteins were eluted, separated by SDS–PAGE, and subjected to silver staining. In the eluate, a ~32 kDa band was detected that was absent from control (Figure 1A). Analysis of eluted proteins was performed using chromatographic separation of tryptic peptides and mass-spectrometry-based sequencing. Peptide hits covering ~10% of the amino acid sequence identified FHL1 in association with the KCNA5 C-terminal fusion protein (Figure 1B). The band migrating at 32 kDa was excised from the gel and directly sequenced using mass spectrometry. The identification of FHL1 was confirmed, proving its existence in the KCNA5 C-terminal complex that was isolated.

3.2 Interaction of four and a half LIM protein 1 and KCNA5 in vivo

To confirm an interaction between FHL1 and KCNA5 in heart, immunoprecipitation experiments were performed with human atrium. Using an antibody directed against the N-terminus of the K+ channel α-subunit, KCNA5 could be immunoprecipitated from both left and right atrial tissue (Figure 2A). As illustrated in Figure 2B, FHL1 was also identified in the immunoprecipitate generated using the anti-KCNA5 antibody (but not pre-immune serum, as a negative control). The occasional detection of FHL1 as a doublet (Figure 2B and D) is consistent with previous studies using this isoform-specific antibody21–23 (likely representing splice variants, at least one of which has been characterized previously24). These findings demonstrate that FHL1 is present in the KCNA5 complex in human atrium.

HEK 293 and Chinese hamster ovary (CHO) cells were cotransfected with KCNA5 and FHL1 (with expression verified by western blotting, Figure 2D and E). Because endogenous FHL1 was readily identified in HEK cells, with minimal expression in non-transfected CHO cells, subsequent experiments to examine the physical and functional interaction of FHL1 and KCNA5 were conducted using CHO cells. In cells cotransfected with KCNA5 and FHL1 (but not KCNA5 alone), western analysis following immunoprecipitation using the anti-KCNA5 antibody revealed that FHL1 was also communoprecipitated (Figure 2C; when the blot was stripped and reprobed with the anti-KCNA5 antibody, KCNA5 was identified in lanes 1, 2, and 4, indicating successful pulldown of the channel subunit protein). These results provide additional confirmation that FHL1 physically interacts with the KCNA5 complex.

To further examine this interaction, cotransfected CHO cells were fixed, labelled with antibodies specific for KCNA5 and FHL1, and imaged using confocal microscopy. As shown in Figure 3, the general pattern of distribution for the two proteins differed considerably; this result is not surprising, given the known function of the two proteins. However, areas of colocalization were evident at the membrane region, providing additional support for the presence of FHL1 and KCNA5 in the same complex.
3.3 Functional effects of four and a half LIM protein 1 on KCN5A channels

To further explore the functional effects of this interaction, K^+ currents were recorded from CHO cells expressing KCNA5 in the absence and presence of FHL1. With coexpression of FHL1, KCNA5 currents were markedly increased, compared with currents generated by expression of KCNA5 alone [Figure 4; steady-state K^+ current density (after 250 ms) at +50 mV was 173 ± 52 pA/pF for KCNA5 alone, and 713 ± 112 pA/pF for KCNA5 + FHL1; n = 13 each; P < 0.01]. To investigate the effects of FHL1 on the voltage dependence of K^+ channel activation, deactivating tail currents were plotted as a function of test potential. Coexpression with FHL1 caused a significant shift in K^+ channel opening to more positive potentials (Figure 5; midpoint or V_1/2 was −18 ± 1 mV and −3 ± 1 mV, for KCNA5 alone and KCNA5 + FHL1, respectively; n = 5 each; P < 0.01). A two-pulse protocol was used to examine recovery from slow inactivation, which could be fitted by a two-exponential function. For K^+ currents generated by coexpression of KCNA5 and FHL1, the slow component was faster compared with KCNA5 alone (Figure 6; t2 was 58 ± 9 s and 39 ± 6 s, for KCNA5 alone and KCNA5 + FHL1, respectively; n = 4 each; P < 0.01), while no difference was observed in the fast component of recovery (t1 was 2.0 ± 0.2 s and 1.9 ± 0.2 s, for KCNA5 alone and KCNA5 + FHL1, respectively).

Antiarrhythmic drugs that block KCNA5 bind preferentially to the inner pore of the channel, with a critical role for the S6 segment in this process. Given the proximity of the drug-binding site to the C-terminus, we hypothesized that
interaction of FHL1 with the channel at this region might alter properties of antiarrhythmic drug binding. Therefore, the effect of FHL1 coexpression on drug block of KCNA5 by quinidine was examined. However, our results demonstrated that the drug–channel interaction was not obviously altered by the presence FHL1 (IC50 was 8.4 ± 0.5 and 8.6 ± 2.1 μM, for KCNA5 alone and KCNA5 + FHL1, respectively; n = 4; P > 0.05; data not shown).

4. Discussion

In this investigation, we identified FHL1 as a potential protein partner of KCNA5 using the channel C-terminus. In co-immunoprecipitation experiments, we demonstrated a physical interaction between FHL1 and the KCNA5 complex in vivo in human atrial tissue and cotransfected CHO cells. Consistent with these findings, immunolabelling studies revealed areas of colocalization in the region of the cell membrane. Evidence for a functional effect of FHL1 to modulate KCNA5 current was also established, as coexpression of the LIM protein increased K+ current density, altered channel gating, and enhanced slow inactivation. Taken together, these data provide strong evidence that FHL1 modulates KCNA5 activity to promote α-subunit cell surface expression and to regulate channel function in the IKur complex. A major advantage of the mass spectrometry-based approach that we employed is the ability to identify novel proteins present in a macromolecular complex in an unbiased manner, without first purifying the protein component to homogeneity.11 A similar strategy was recently employed to identify proteins that interact with the C-terminus of inward rectifier K+ channel (Kir2.X) isoforms in heart and brain.12

Previous studies have demonstrated differences between IKur and K+ current derived from heterologous expression of KCNA5, implying that additional components are required in the channel complex to recapitulate the native current phenotype. For channel opening, the voltage dependence that we observed for KCNA5 alone (V1/2 = −9 ± 1 mV) is in agreement with prior studies using CHO cells (V1/2 = −6 to −19 mV).30 On the other hand for IKur, K+ current activation typically occurs at more positive potentials (V1/2 = −4 to 12 mV).29 Thus, the positive shift associated with FHL1 coexpression (V1/2 = 11 ± 2 mV) resulted in K+ currents that more closely resembled IKur. Characterization of slow inactivation for IKur using the experimental conditions and pulse protocols that we employed has not been previously undertaken. However, available data indicate that IKur inactivation during a 5 s-sustained depolarization typically exceeds 50%, which is analogous to the effects of FHL1 coexpression.31 These findings, coupled with enhanced KCNA5 expression at the cell surface, strongly support a functional role for FHL1 in the IKur complex in human atrium.

Our results provide additional evidence that LIM proteins are critical for normal cardiac function. LIM domains are highly conserved, cysteine/histidine-rich, double zinc finger motifs known to mediate protein–protein interactions.13,14,21 In many LIM proteins, multiple LIM domains (or additional protein binding modules) are present, enabling them to function as scaffolds for the coordinated assembly of multiple proteins. LIM domain-mediated complexes have been shown to regulate gene transcription in the nucleus, and to participate in the generation of signal-ling and cytoskeletal/sarcomeric protein networks in the cytoplasm. Considerable evidence indicates that multiple LIM proteins participate in cardiac development and function, and that they also play a role in human cardiomyopathies.14,12,33 Muscle-specific LIM protein (MLP) is highly expressed in developing and adult myocardium,12 and elimination of MLP in null mice causes dilated cardiomyopathy and heart failure.34 MLP mutations have been identified in human dilated and hypertrophic cardiomyopathy, with reduced expression in failing hearts.32 Mice lacking α-actinin-associated LIM protein (ALP) develop a right ventricular cardiomyopathy.35

The FHL family consists of LIM-only proteins that contain four complete LIM domains preceded by an N-terminal half-LIM motif. The FHL2 isofrom is highly expressed in heart, and it was recently shown to bind the KCNE1 K+ channel subunit as a prerequisite to generate IKur current in

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**Figure 5** Four and a half LIM protein 1 (FHL1) alters voltage-dependent activation of KCNA5. Analysis of deactivating tail currents demonstrates that coexpression of FHL1 shifts the activation curve for KCNA5 to more positive membrane potentials.

**Figure 6** Four and a half LIM protein 1 (FHL1) modulates KCNA5 slow inactivation. Using prolonged (5 s) depolarizing pulses for the voltage clamp protocol described for Figure 3, it is evident that FHL1 coexpression enhances slow inactivation of KCNA5 current.
a heterologous cell expression system. FHL1 demonstrates moderate expression in mammalian heart, and it has been strongly implicated in the pathogenesis of human cardiomyopathy. Selective downregulation of FHL1 (but not FHL2 or FHL3) mRNA and protein was demonstrated in dilated cardiomyopathy, while increased expression was observed in the MLP-null model and human hypertrophic cardiomyopathy.

Immunolocalization studies have shown that in cardiac myocytes, KCNA5 channels are concentrated at the intercalated disc, although protein can also be detected at the Z-discs. At the Z-disc region, overlapping actin thin filaments are cross-linked by tight interactions with α-actinin. Z-discs represent a unique region of the cardiomyocyte, positioned at the interface of the sarcomere, cytoskeleton, and sarcolemma. Not surprisingly, FHL1 has been shown to colocalize with α-actinin at the Z-disc in skeletal muscle. Collectively, these data suggest that KCNA5, FHL1, and α-actinin coassemble in a multimeric protein complex at the complex Z-disc region.

Previous studies have reported down-regulation of \( I_{Kur} \) in atrial myocytes from patients with atrial fibrillation, although these results have been controversial.

In some cases, a reduction in KCNA5 protein was observed, although mRNA levels remained unchanged. Altered expression of FHL1, or another regulatory protein in the KCNA5 complex, in the presence of atrial fibrillation could offer a potential explanation for this apparent post-transcriptional regulation of channel expression.

In conclusion, FHL1 is a newly identified protein partner in the \( I_{Kur} \) complex, where it modulates functional expression of KCNA5. Based upon evidence to date, FHL1 dysfunction in humans would likely have serious clinical consequences, and this protein should be considered a potential candidate to cause genetically determined arrhythmia and/or cardiomyopathy syndromes.

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