Kv11.1 (ERG1) K+ Channels Localize in Cholesterol and Sphingolipid Enriched Membranes and Are Modulated by Membrane Cholesterol

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ABSTRACT

The localization of ion channels to specific membrane microdomains can impact the functional properties of channels and their role in cellular physiology. We determined the membrane localization of human Kv11.1 (hERG1) α-subunit protein, which underlies the rapidly activating, delayed rectifier K+ current (I_K) in the heart. Immunocytochemistry and membrane fractionation using discontinuous sucrose density gradients of adult canine ventricular tissue showed that Kv11.1 channel protein localized to both the cell surface and T-tubular sarcolemma. Furthermore, density gradient membrane fractionation using detergent (Triton X-100) and non-detergent (OptiPrep) methods from canine ventricular myocytes or HEK293 cells demonstrated that Kv11.1 protein, along with MiRP1 and Kv7.1 (KCNQ1) proteins, localize in cholesterol and sphingolipid enriched membrane fractions. In HEK293 cells, Kv11.1 channels, but not long QT-associated mutant G601S-Kv11.1 channels, also localized to cholesterol and sphingolipid enriched membrane fractions. Depletion of membrane cholesterol from HEK293 cells expressing Kv11.1 channels using methyl-β-cyclodextrin (MβCD) caused a positive shift of the voltage dependence of activation and an acceleration of deactivation kinetics of Kv11.1 current (I_{Kv11.1}). Cholesterol loading of HEK293 cells reduced the steep voltage dependence of I_{Kv11.1} activation and accelerated the inactivation kinetics of I_{Kv11.1}. Incubation of neonatal mouse myocytes in MβCD also accelerated the deactivation kinetics of I_K. We conclude that Kv11.1 protein localizes in cholesterol and sphingolipid enriched membranes and that membrane cholesterol can modulate I_{Kv11.1} and I_K.

ABBREVIATIONS

hERG1, human-ether-a-go-go related gene 1; Kv, voltage activated K+ channels; I_K, rapidly activating delayed rectifier K+ current; LQTS, Long QT Syndrome; I_{Kv11.1}, Kv11.1 current; HEK293, human embryonic kidney 293; WGA, wheat germ agglutinin; MβCD, methyl-β-cyclodextrin; MiRP1, MinK-related peptide 1; β1-AR, β1-adrenergic receptor; GPI, glycosylphosphatidyl inositol.

INTRODUCTION

The human-ether-a-go-go related gene (hERG1 or KCNH2) encodes the voltage gated K+ channel α-subunit 11.1 protein (Kv11.1), which underlies the rapidly activating delayed rectifier K+ current (I_K) in the heart. I_K has emerged as an important human K+ current involved in normal cardiac repolarization. Mutations in hERG1 and many drugs that block Kv11.1 current (I_{Kv11.1}) and I_K have been linked to congenital and acquired arrhythmia syndromes, such as Long QT Syndrome (LQTS). Hundreds of studies are dedicated to understanding the unusual biophysical properties of I_{Kv11.1}, identifying drugs that block it, defining putative drug binding domains in Kv11.1, characterizing mutant Kv11.1 channels linked to arrhythmia syndromes, and understanding the processes that regulate the Kv11.1 transport or trafficking to the cell surface membrane. However, little is known about the localization of Kv11.1 within the sarcosomal lipid environment.

The cell surface membrane is a heterogeneous mixture of proteins, cholesterol, glycerol-, phospho- and sphingolipids. Cholesterol and sphingolipids are postulated to laterally associate with one another and form liquid-ordered microdomains (lipid rafts) in a glycerolipid liquid-disordered membrane. Multiple types of liquid-ordered microdomains have been proposed based on different protein markers, morphological features,

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and their relative cholesterol to sphingolipid content. Cholesterol and sphingolipid enriched membranes are believed to coordinate multiple cellular processes, which include second messenger regulation, membrane recycling of proteins, and ion channel function. Using a comprehensive array of biochemical and electrophysiological techniques, we tested the hypotheses that the Kv11.1 channels localize in cholesterol and sphingolipid enriched membranes and that membrane cholesterol can modulate Kv11.1 channel function. These are the first data to show that Kv11.1 localizes in cholesterol and sphingolipid enriched membrane fractions, and that conditions that modify cell membrane cholesterol modulate the electrophysiological properties of $I_{K_{\text{slo}}}$ and $I_{K_{\text{Cq}}}$. These data suggest that alterations in membrane cholesterol may modify $I_{K_{\text{slo}}}$ function, which in turn may alter susceptibility to arrhythmia.

**MATERIALS AND METHODS**

**cDNA preparation, cell culture, tissue and myocyte isolation.** Wild-type human Kv11.1 (WT-Kv11.1) or the glycine-to-serine mutation at residue 601 (G601S-Kv11.1) channels were expressed in Human Embryonic Kidney 293 (HEK293) cells (as previously described Refs. 8-11). The HEK293 cells were maintained in modified MEM (GIBCO/Invitrogen, Carlsbad, CA) and cultured at 37°C in 5% CO₂. Adult canine left ventricular tissue samples, adult canine myocytes, and neonatal mouse myocytes were isolated (as described previously Refs. 12, 13).

**Immunofluorescence.** Wheat germ agglutinin (WGA), which recognizes surface membrane glycoproteins, was used to visualize the surface and T-tubular sarcolemma of canine myocytes. 11,15 Non-permeabilized intact, isolated canine myocytes were incubated with 100 µg/ml of WGA coupled to Alexa488 (Molecular Probes Inc., Eugene, OR) for 30 min. The myocytes were rinsed three times with Tyrode's solution, fixed with 2% buffered paraformaldehyde for 10 min, permeabilized with Triton X-100 (0.1%) for 10 min and then quenched for aldehyde groups in 0.75% glycin buffer for 10 min. After being washed twice for 10 min with Tris-buffered saline (TBS), myocytes were incubated with 1 ml blocking solution (2% bovine serum albumin, 2% normal goat serum, and 0.05% NaN₃ in TBS) for 2 hr at 4°C with gentle agitation. Subsequently, the aliquots of myocytes were incubated overnight with anti-Kv11.1 antibody in blocking solution at 4°C. Myocytes were washed with blocking solution and then incubated overnight with the secondary antibody. Alexa568 goat anti-rabbit immunoglobulin (IgG) (H+L) (Molecular Probes Inc.) diluted 1:200 in blocking solution. Finally, the myocytes were washed three times (2 hr each) with the blocking solution, resuspended in the blocking solution, and mounted onto a cover slip for imaging using a confocal microscope.

**Membrane fractionation and isolation of cholesterol and sphingolipid enriched fractions.** In some experiments membrane fractionation of canine ventricular tissue was performed using the discontinuous sucrose gradient fractionation technique to obtain the surface sarcolemmal-enriched fraction (FI), T-tubular sarcolemmal-enriched fraction (FII), and junctional complex-enriched fraction (FIII), (previously described in ref. 12). In other experiments, cholesterol and sphingolipid enriched membrane fractions were prepared from canine ventricular myocytes or HEK293 cell membranes using continuous sucrose density gradient centrifugation of Triton X-100 insoluble membranes, (previously described in refs. 16 and 17). Isolated canine ventricular myocytes (2.0 x 10⁷ cells) or HEK293 cells stably expressing either WT- or G601S-Kv11.1 were grown to confluence in six 10 cm culture dishes. Cells were suspended in 5 ml of ice-cold homogenization buffer (150 mM NaCl, 20 mM Na₂HPO₄, 2 mM NaH₂PO₄, 20% (w/v) glycerol plus protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 50 µg/ml benzamidine, 50 µg/ml leupeptin, 5 µM pepstatin A) (Sigma/Aldrich, St. Louis, MO), and homogenized with a Dounce Homogenizer (20 strokes) on ice. The homogenate was sonicated (Microson Ultrasonic Cell Disruptor XL2000, Misonix Inc, Farmingdale, NY) three times at 30 s pulses and centrifuged using a Sorvall 34 rotor at 10,000 rpm for 10 min at 4°C to sediment nuclei and debris. The supernatant was then centrifuged at 35,000 rpm for 1 hr at 4°C to obtain a membrane pellet. The membrane pellet was resuspended in ice-cold solubilization buffer (25 mM Mes pH 6.5, 150 mM NaCl, 1% Triton X-100 plus protease inhibitors) and incubated for 30 min. The solubilized membrane fraction (2 ml) was then adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose in Mes-Buffered Saline (MBS; 25 mM Mes pH 6.5, 150 mM NaCl plus protease inhibitors) and placed at the bottom of an ultracentrifuge tube. A 5–30% continuous sucrose (4 ml of 5% sucrose/4 ml of 35% sucrose) gradient was formed and layered on top of the sample in the ultracentrifuge tube. Samples were centrifuged at 100,000 x g for 12–16 hr at 4°C (SW41 rotor, Beckman Instruments, Palo Alto, CA). From each gradient, a total of 12 one ml fractions were collected and the pellet was washed extensively with PBS before being solubilized in MBS with 2% SDS. Sucrose density in each of the gradient fractions was estimated using a handheld Brik refractometer. All the procedures were performed at 4°C. Each experiment was repeated 3–4 times.

We also performed membrane fractionation using an OptiPrep protocol adapted from the previously reported method by Smart et al. HEK293 cells stably expressing WT-Kv11.1 channels were grown to confluence in six 10 cm culture dishes and washed twice with PBS, scraped and pelleted by centrifugation at 1500 x g for 5 min at 4°C. The pellet was resuspended in ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8 plus the protease inhibitors), and homogenized with a loose-fitting Dounce homogenizer on ice (20 strokes). The homogenate was centrifuged at 1000 x g for 10 min at 4°C. The above step was repeated, and the postnuclear supernatants were combined and assayed for protein content. Four milligrams of protein were then overlaid onto 8 ml 30% percoll (diluted in homogenization buffer) and centrifuged at 84,000 x g for 30 min at 4°C (70.1 Ti fixed angle rotor, Beckman). After centrifugation, the plasma membrane fraction, which was visible as an opaque band, was extracted (~2 ml) through the sidewall of the tube. An aliquot was saved on ice (for cholesterol estimation and for positive control) and the rest combined with 0.16 ml homogenization buffer and 1.84 ml 50% OptiPrep (Sigma/Aldrich) diluted in homogenization buffer. A linear 10–20% density gradient was achieved by the addition of 4 ml of 10% OptiPrep (OptiPrep diluted in homogenization buffer), followed by 4 ml of 20% OptiPrep and then centrifuged at 52,000 x g for 90 min at 4°C (SW40Ti rotor, Beckman). The light fractions (top 6 ml) were removed, mixed with 4 ml 50% OptiPrep, and overlaid with 2 ml 5% OptiPrep (OptiPrep diluted in homogenization buffer). After a final centrifugation at 52,000 x g for 90 min at 4°C (SW40Ti rotor, Beckman), 12 one-ml fractions were collected. An aliquot from each fraction was saved for protein and cholesterol measurements, and the remaining samples...
were precipitated with 10% TCA and boiled for 5 min in reducing SDS loading buffer for immunoblotting.

Western blot and densitometric analysis. Sucrose density gradient fractions were separated by SDS-PAGE (4–15%) and transferred to PVDF membranes. Nonspecific binding sites were blocked by immersing membranes in phosphate buffered saline (PBS) with 0.1% Tween-20 and 5% (w/v) dried skim milk. Membranes were subsequently probed with anti-Kv11.1 antibody (rabbit polyclonal, 1:10,000, directed against the C terminus),\textsuperscript{11} anti-Kv7.1 antibody (1:500, Alomone Labs, Jerusalem, Israel), anti-MiRP1 antibody (rabbit polyclonal, 1:200, Alomone Labs), anti-T-Cadherin antibody (rabbit polyclonal, 1:1000, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA.), anti-caveolin 3 antibody (mouse monoclonal, 1:5000, BD Biosciences), anti-CD59 antibody (mouse monoclonal, 1:1000, BD Biosciences), and anti-β₁ adrenergic receptor antibody (rabbit polyclonal, 1:200, Santa Cruz Biotechnologies, Inc.). Goat anti-mouse IgG conjugated to horseradish peroxidase (1:25,000) detected bound antibodies for caveolin 3 (Cav3) and CD59, and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:25,000) detected bound antibodies for Kv11.1, Kv7.1, MiRP1, β₁ adrenergic receptor (β₁-AR), and T-cadherin (Bio-Rad Laboratories Hercules, CA). Immunoreactivity was visualized using a peroxidase-based chemiluminescent detection system, ECL (Amersham Biosciences, Piscataway, NJ). Quantitation of anti-Kv11.1 immunoblots was accomplished using a Bio-Rad GS-700 Image Densitometer. Multiple exposure times of the autoradiograms were performed to optimize linearity and avoid signal saturation. Linearity of the signal as a function of the amount of protein loaded (10 μg) was assured by establishing the relationship between antigen concentration and signal. For quantification of Kv11.1 signal in immunoblots performed on the sucrose density gradient fractions from canine ventricular myocytes, the signal density was normalized to protein band of 40% sucrose (fraction 12). For quantification of Kv11.1 band density in HEK293 cells, signal was normalized to band intensity of 10 μg of HEK293 cell lysates expressing Kv11.1 protein.

Dot blot analysis. Ganglioside GM₁₇ is a sphingolipid present in the cholesterol and sphingolipid enriched membrane fractions. For detection of GM₁₇, we used B subunit of cholera toxin that interacts with GM₁₇.\textsuperscript{19} OptiPrep gradient fractions were dotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were then treated as described for Western blot analysis by probing with HRP conjugated cholera toxin β subunit (Sigma/Aldrich) and visualized using the enhanced chemiluminescence method.

Immunoprecipitation. Canine ventricular myocytes were homogenized in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 1% NP-40, and protease inhibitors using a polytron homogenizer and nuclei and debris were removed by centrifugation at 1500 × g for 10 min at 4°C. The supernatant was solubilized in buffer containing 25 mM Tris-HCl, pH 8, 150 mM NaCl, 60 mM N-acyt glycoside, 1% Triton X-100 and protease inhibitors. The solubilized homogenate (1 mg total protein) was pre-cleared using protein A-sepharose (Repligen, Waltham, MA) followed by incubation for 1 hr at 4°C with anti-Kv11.1 antibody or control antibodies in a total of 450 μl. A 50 μl of 1:1 slurry of protein A-sepharose beads was added to the mix and incubated for 4 hr at 4°C on a rocking platform. The beads were washed four times with the solubilization buffer and bound proteins were eluted with 50 μl of SDS-PAGE sample buffer and boiled for 5 min. Samples were separated using 4–15% gradient gels (Bio-Rad). Western blot analyses were carried out using antibodies specific to Kv11.1, and Cav3 as described above. The same blot was stripped (62.5 mM Tris-HCL, pH 6.8, 10% SDS, and 20 mM dithiothreitol for 30 min at 55°C) and reprobed with anti-MiRP1.

Cholesterol modifying agents and cholesterol assay. Methyl-β-cyclodextrin (MβCD) or MβCD loaded with cholesterol (cholesterol + MβCD) (Sigma/Aldrich) was added to the culture media to deplete or load the cell membranes with cholesterol, respectively, (previously described in refs. 14 and 21–24). Cyclodextrins are cyclic oligomers of glucose that sequester lipophiles in their hydrophobic core, thereby enhancing cholesterol solubility in aqueous solution.\textsuperscript{25–27} In cell culture systems MβCD has been shown to remove cholesterol from cell membranes,\textsuperscript{14,20,21,27} whereas cholesterol + MβCD loads cell membranes,\textsuperscript{20,23,24,28,29} thus cyclodextrins can function as efficient acceptors and donors of cholesterol. For cholesterol depletion a final concentration of 10 mM MβCD was used, and for the cholesterol loading of cells, cholesterol + MβCD with a molar ratio of cholesterol/MβCD of 1/5 at a final concentration of 10 mM MβCD was used. Initial experiments established that a 15 min incubation period with MβCD was sufficient to alter membrane cholesterol content (Supplemental Fig. 1). Thus we incubated HEK293 cells in MβCD or cholesterol + MβCD for 15 min.

Cholesterol assay. Cholesterol was determined enzymatically on aliquots of sucrose gradient fractions, using a commercial assay kit (Calbiochem, San Diego, CA) according to manufacturer’s recommendations. Absorbance was measured at 570 nm using a plate reader (ELx808, Bio Tek Instruments, Winooski, VT). Results are expressed as μg cholesterol/ml for each fraction. In the sucrose gradients, cholesterol recovery relative to total cholesterol present in the initial crude lysate was 71 ± 4% (n = 3) for control and 67 ± 7% (n = 3) for gradients prepared after treatment with MβCD. In the OptiPrep gradients, the cholesterol recovery relative to total cholesterol present in the plasma membrane was 76 ± 6% for control (n = 3), 68 ± 8% for MβCD (n = 3), and 82 ± 6% for cholesterol + MβCD (n = 3).

Electrophysiology. I<sub>Kv11.1</sub> and I<sub>Kr</sub> were measured from HEK293 cells and myocytes, respectively, using the whole-cell patch clamp technique (previously described in refs. 30 and 31). The extracellular bath solution contained (in mM) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH), and the intracellular pipette solution contained (in mM) 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2 with KOH). The holding potential was -50 or -80 mV, and zero current is indicated as a dashed line in the figures. The voltage dependence of I<sub>Kv11.1</sub> activation, deactivation, inactivation, and I<sub>Kv11.1</sub> density were measured (previously described in refs. 8 and 32). E-4031 (Alomone Labs) was used to selectively block I<sub>Kr</sub>.\textsuperscript{33} In myocytes, current traces were averaged for 50 pulses. All voltage clamp experiments were performed at 22–23°C within 1 hr of removing the cells from their culture conditions.

Statistics. Data are presented as the mean ± standard error (S.E.). Student’s t-test is used for statistical analysis with p < 0.05 considered significant.

RESULTS

Kv11.1 channels localize to the cholesterol and sphingolipid enriched sarcloemal membranes in canine ventricular myocytes. We first verified that I<sub>Kr</sub> was present in our isolated canine ventricular
myocytes. I_Kv11.1 was measured by depolarizing myocytes from a holding potential of -50 to 20 mV for 5 s, followed by a test pulse to -40 mV for 3 s to record tail current, before and after the addition of 5 μM E-4031. The mean peak tail amplitude of the E-4031-sensitive current measured at -40 mV was 0.08 ± 0.02 pA/pF (n = 4 myocytes, data not shown). We next investigated the subcellular distribution of Kv11.1 protein. Figure 1A shows Kv11.1 immunofluorescence (top panel) in a permeabilized canine ventricular myocyte, showing staining at the surface sarcolemma as well as a punctate pattern for T-tubular sarcolemma. The same myocyte was initially labeled with WGA-Alexa488 (middle panel), which recognizes glycosylated membrane proteins, to outline the surface and T-tubule sarcolemma. Both probes resulted in prominent staining of surface and T-tubule sarcolemmal membrane, and the merged image (bottom panel) shows a high degree of overlap. Similar confocal images were obtained in 10 different canine myocytes examined from three different preparations. Previous reports have shown expression of Kv11.1 protein in both surface and T-tubular sarcolemma, or predominantly in the T-tubular sarcolemma. To examine further surface and T-tubular membrane localization, we performed membrane fractionation to evaluate Kv11.1 immunoreactivity in surface sarcolemma-enriched fractions (FI), T-tubule-enriched fractions (FII), junctional complex-enriched fractions (FIII) and the tissue homogenate (H). The Kv11.1 antibody detected a single protein band of approximately 165 kDa consistent with the complexly-glycosylated mature canine Kv11.1 (cERG1a) in all three fractions with more intense staining in the surface and T-tubular sarcolemmal fractions (Fig. 1B). This pattern was observed in four different experiments. Together the immunofluorescence labeling of canine ventricular myocytes and membrane fractionation studies demonstrate that Kv11.1 channels are present in both surface and T-tubular sarcolemma.

To identify potential sarcolemmal microdomain localization of Kv11.1 channels, we isolated cholesterol and sphingolipid enriched membrane fractions. Canine ventricular myocytes were lysed in the presence of 1% Triton X-100 and the detergent-insoluble membrane fractions were separated using the continuous sucrose density gradient (5–40% sucrose) centrifugation technique. Overnight centrifugation of the lysates in the sucrose gradient yielded 12 membrane fractions, with the cholesterol and sphingolipid enriched fractions located between 15 to 20% sucrose. Figure 2A shows the total protein content of each fraction (n = 4), with most protein present in the denser sucrose gradient fractions. Figure 2B shows representative Western blot analyses of the sucrose gradient membrane fractions probed for different proteins (n = 4). A single Kv11.1 protein band at 165 kDa was detected in the cholesterol and sphingolipid enriched fractions (15 to 20% sucrose, fractions 4–6) as well as in the densest fractions containing insoluble proteins. Figure 2A shows the densitometric analysis of Kv11.1 protein with it enriched in the 15–20% sucrose fractions (n = 4). Other delayed rectifier Kv channels, Kv7.1 and the accessory subunit, MiRP1, have been reported to associate with Kv11.1. Figure 2B shows that these proteins also localized in the cholesterol and sphingolipid enriched membrane fractions (fractions 4–6). In addition, Figure 2B confirms that other proteins known to localize into cholesterol and sphingolipid enriched membrane microdomains (T-cadherin, the glycosylphosphatidylinositol (GPI)-anchored protein CD 59, and Cav3) were likewise enriched in fractions 4–6. It has been previously shown that the β3AR does not selectively localize in cholesterol and sphingolipid enriched membrane fractions and it was present in all membrane fractions of ≥ 15% sucrose.

Caveolin 3 does not interact with Kv11.1 channel protein. We tested if we could detect an interaction between the Kv11.1 channel protein and muscle specific caveolin, Cav3, in canine ventricular myocytes using immunoprecipitation experiments. Myocyte lysate was immunoprecipitated using the anti-Kv11.1 antibody. The Kv11.1 protein band at 165 kDa and MiRP1 protein band at 25 kDa were detected in the myocyte lysate and the immunoprecipitate lanes (Fig. 2C, n = 3). In distinction, we detected Cav3 protein (18 kDa band) only in the myocyte lysate but not in the immunoprecipitate using the anti-Kv11.1 antibody. These results suggest that in canine ventricular myocytes Kv11.1 and MiRP1 interact with one another, but Cav3 does not.

Heterologously expressed Kv11.1 channels also localize in cholesterol and sphingolipid enriched membranes. We next determined if Kv11.1 channels also localize in cholesterol and sphingolipid enriched membranes studied in HEK293 cells stably overexpressing human WT-Kv11.1. The fractionation procedure was the same as that used in Figure 2B. Figure 3A (top row) shows a representative Western blot analysis (n = 3) of the sucrose gradient membrane fractions isolated from cells expressing WT-Kv11.1 channels. In this overexpression model, both the complexly- and core-glycosylated (-155 kDa and -135 kDa, respectively) protein
bands of WT-Kv11.1 were detected in the cholesterol and sphingolipid enriched membrane fractions. As a control experiment, we also studied the congenital long QT syndrome trafficking-deficient G601S-Kv11.1 mutant channel, which is retained in the endoplasmic reticulum as a core-glycosylated protein. As shown in Figure 3A, G601S-Kv11.1 protein was detected as only a 135 kDa protein in gradient fractions of 23% or greater sucrose (n = 2) and it was not present in the cholesterol and sphingolipid enriched membrane fractions. These findings agree with known differences in the protein processing of WT- and G601S-Kv11.1 channels. Furthermore, the absence of G601S-Kv11.1 protein in fractions 4–6 provides evidence that the presence of WT-Kv11.1 protein in these fractions is not an artifact of membrane fractionation technique.

We studied the effect of MβCD, which has been widely used as a membrane cholesterol depleting agent, on the localization of WT-Kv11.1 protein in the different sucrose gradient membrane fractions. HEK293 cells over-expressing WT-Kv11.1 channels were treated with and without 10 mM MβCD for 15 min and sucrose gradient membrane fractions were isolated, and we measured the cholesterol and protein concentrations in each sucrose gradient fraction. Figure 3B shows that for control conditions, fractions 4–6 were maximally enriched in cholesterol (n = 3) and following incubation of cells with MβCD, cholesterol content is significantly reduced (n = 3). Figure 3C shows that incubation of cells with MβCD slightly shifted the distribution of total protein to higher density sucrose gradient fractions (n = 3 each). We next performed Western blot and densitometric analysis on the Kv11.1 protein from these sucrose gradient fractions. Figure 3D shows representative Western blot analysis of sucrose gradient fractions isolated from cells expressing WT-Kv11.1 channels or β1AR before and after incubation of cells with MβCD (n = 3). MβCD treatment resulted in the appearance of the core- and complexly-glycosylated WT-Kv11.1 protein in denser membrane fractions compared to control data, but did not alter β1AR localization which does not localize to the cholesterol and sphingolipid enriched membrane fractions. Densitometric analysis for the relative distribution of the Kv11.1 protein is shown in Figure 3E, and confirms that after MβCD treatment there is a shift in the Kv11.1 protein density to higher gradient fractions.

To confirm our membrane fractionation results we also used an OptiPrep fractionation technique. This method purifies cholesterol and sphingolipid enriched plasma membrane fractions by density gradient centrifugation without the use of the Triton X-100 detergent. Cholesterol concentration measured in the density gradient membrane fractions from control WT-Kv11.1 HEK293 cells showed a distribution pattern similar to that found in the sucrose density gradient fractions; fractions 4, 5 and 6 were highly enriched in cholesterol (Fig. 4A, left panel, n = 3). The total membrane cholesterol in the gradient fractions was 5.3 ± 0.9 μg/mg of membrane protein. We also probed for the presence of GM1, a sphingolipid that localizes to the cholesterol and sphingolipid enriched membrane fractions. In the OptiPrep density gradient membrane fractions GM1 was detected in the cholesterol and sphingolipid enriched membrane fractions (Fig. 4A, right panel). Immunoblot analysis for WT-Kv11.1 showed both the complexly- and core-glycosylated (~155 kDa and ~135 kDa, respectively) protein bands in the cholesterol and sphingolipid enriched membrane fractions (Fig. 4A, right panel). Incubation of cells with 10 mM MβCD significantly reduced the cholesterol content (3.0 ± 1.6 μg/mg of membrane protein) relative...
Kv11.1 Channel Is Modulated by Cholesterol

The amount of protein in gradient membrane fractions and shifted the cholesterol distribution to higher density membrane fractions (Fig. 4B, left panel, n = 3). Similarly, the distribution of GM, sphingolipid and Kv11.1 protein were redistributed into higher density gradient fraction (Fig. 4B, right panel). We also measured the effect of cholesterol + MβCD incubation on these cells. Incubation of cells with 10 mM cholesterol + MβCD resulted a slight increase in the total membrane cholesterol in density gradient membrane fractions (6.8 ± 1.5 μg/mg of membrane protein) and a slight shift of cholesterol and Kv11.1 to lower density gradient fractions (Fig. 4C, left and right panels, n = 3).

**Cholesterol depletion and loading alter I_{Kv11.1}**. We determined whether I_{Kv11.1} was modulated by MβCD, or cholesterol + MβCD. HEK293 cells expressing WT-Kv11.1 channels were incubated in MβCD (10 mM for 15 min) or cholesterol + MβCD (10 mM for 15 min), and current was recorded within 1 hr. Figure 5A shows double exponential process to the decay following the prepulse to 70 mV. The mean fast ($\tau_{fast}$) and slow ($\tau_{slow}$) deactivation rates of I_{Kv11.1} were much faster in cells incubated in MβCD (260 ± 40 ms and 1495 ± 254 ms) when compared to cells in control conditions (632 ± 30 ms and 4140 ± 164 ms, p < 0.05) or cells incubated in cholesterol + MβCD (501 ± 20 ms and 3866 ± 139 ms, p < 0.05). The relative amplitude of $A_{fast}$ ($A_{fast}/A_{fast}+A_{slow}$) was also larger in cells incubated in MβCD (0.53 ± 0.03) when compared to control cells (0.39 ± 0.01, p < 0.05) and cells incubated in cholesterol + MβCD (0.36 ± 0.02, p < 0.05). There was no difference in the $\tau_{fast}$ of $A_{slow}$ or the relative amplitude of $A_{slow}$ measured from cells incubated in cholesterol + MβCD or control conditions (p > 0.05). These data suggest cholesterol depletion, but not cholesterol loading, accelerates the fraction of channels that deactivate rapidly.

We also determined whether we could reverse the functional changes of I_{Kv11.1} associated with cholesterol depletion by...
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Cholesterol-sphingolipid enriched membranes isolated using OptiPrep gradient centrifugation

Figure 4. Characterization of cholesterol and sphingolipid membranes isolated from WT-Kv11.1 over expressing HEK293 cells. HEK293 cells were subjected to gradient membrane fractionation by using the OptiPrep method. Twelve one ml fractions were collected from the top of the gradient and characterized cholesterol content, expression of ganglioside GM1 and Kv11.1 protein. Cholesterol (●) and protein (○) concentrations [left panel, n = 3, mean ± S.E.] is shown for control (A), incubation of cells with MβCD (B), and incubation of cells with cholesterol + MβCD (C). Dot blot analysis of GM1 and immunoblot for Kv11.1 protein is shown on right.

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Kv11.1 Channel Is Modulated by Cholesterol

Cholesterol depletion modulates native ventricular I Kr. We determined if cholesterol depletion with MβCD modulates the

subsequently loading cells with cholesterol. We used the same protocol shown in Figure 5A to measure I_{Kv11.1} from cells first incubated in MβCD (10 mM for 15 min) followed by incubation in cholesterol + MβCD (10 mM for 15 min) (n = 5). The peak I_{Kv11.1} measured during the test-pulse was plotted as a function of the prepulse potential and the data were fit with a Boltzmann equation to calculate the V_{1/2} and k. The mean V_{1/2} was 2.4 ± 2.6 mV (p < 0.05 compared to control) and the k was 9.0 ± 0.8 mV/e-fold change (p < 0.05 compared to control). However, the deactivation rates of I_{Kv11.1} measured during the test-pulse (τ_{fast} 558 ± 10 ms and τ_{slow} 3946 ± 129 ms) were not different compared to control cells. These functional changes are similar to cells incubated in cholesterol + MβCD alone (Fig. 5A), and demonstrate that the acceleration in the deactivation rate of I_{Kv11.1} caused by cholesterol depletion with MβCD (10 mM for 15 min) can be reversed by subsequent cholesterol loading with cholesterol + MβCD (10 mM for 15 min).

The effect on the voltage dependencies of the deactivation τ_{fast} and τ_{slow} were measured from cells in control conditions and cells incubated in MβCD by applying a 2 s prepulse to 50 mV and then applying a test pulse of -120 to -40 mV in 10 mV increments. Figure 5C shows the corresponding families of I_{Kv11.1} for cells in control conditions and cells after they were incubated in MβCD for 15 min. Figure 5D shows the calculated mean τ_{fast} and τ_{slow} plotted as a function of the test-pulse voltage (n = 5–6 cells per group), with τ_{fast} and τ_{slow} not calculated for test-pulse voltages around the I_{Kv11.1} reversal potential. These data demonstrate that incubating cells in MβCD resulted in acceleration of the voltage-dependent deactivation rates of τ_{fast} and τ_{slow} for I_{Kv11.1} (p < 0.05).

The kinetics of I_{Kv11.1} inactivation was measured using a 3-pulse protocol. Cells were depolarized to 50 mV for 1.5 s, hyperpolarized to -100 mV for 2.5 ms to open most channels, and then depolarized to test-pulses between 0 and 90 mV in 10 mV increments for 1.5 s. The I_{Kv11.1} decay during the test-pulse is a measure of channel transitions from open to inactivated states, and was fit as a single-exponential process to calculate a time constant for channel inactivation (τ_{inact}). Figure 5E shows families of I_{Kv11.1} traces recorded during test-pulses from cells in control conditions, cells incubated in MβCD, or cells incubated in cholesterol + MβCD. Figure 5F shows the mean τ_{inact} (n = 4–5 cells per group) plotted as a function of the test-pulse voltage for cells of each of these conditions. The τ_{inact} calculated from cells incubated in cholesterol + MβCD was faster than the τ_{inact} recorded from cells in control conditions or from cells incubated in MβCD alone (p < 0.05 for all voltages). Incubation in MβCD appeared to slow slightly τ_{inact} compared to control conditions for all voltages tested. These data show that cholesterol loading of the cell membrane accelerated τ_{inact}, whereas, cholesterol depletion had a minimal effect on τ_{inact}.

Cholesterol depletion modulates native ventricular I Kr. We determined if cholesterol depletion with MβCD modulates the
deactivation rate of $I_{Kr}$ in neonatal mouse cardiac myocytes. The amplitude of $I_{Kr}$ from neonatal mouse myocytes is relatively large when compared to adult mouse myocytes. The myocytes were depolarized from a holding potential of -50 to 20 mV for 5 s, followed by a test pulse to -50 mV to record tail current. Addition of 5 μM E-4031 completely blocked the tail current consistent with $I_{Kr}$ (peak tail $I_{Kr} = 2.0 \pm 0.3 \text{pA/pF}$, $n = 4$, data not shown). Figure 6A shows superimposed traces of $I_{Kr}$ measured from myocytes cultured in control conditions or with 10 mM MβCD added for 15 min (similar to protocol used in Fig. 5). The deactivation rate of $I_{Kr}$ was faster in myocytes incubated in MβCD. The deactivation rate was fit as a double exponential process, and similar to $I_{Kv11.1}$. Figure 6B shows that the mean $\tau_{fast}$ and $\tau_{slow}$ of $I_{Kr}$ were faster in myocytes incubated in MβCD compared to control ($n = 4$ myocytes per group, $p < 0.05$). The effect of MβCD on activation or inactivation properties of $I_{Kr}$ could not be reliably measured because of overlapping currents present in the myocytes.

**DISCUSSION**

Cholesterol and sphingolipid enriched membrane microdomains are detergent insoluble and float to the lower sucrose density membrane fractions during centrifugation. We used this approach to show that Kv11.1 channels are localized in cholesterol and sphingolipid enriched membranes isolated from canine ventricular myocytes and transfected HEK293 cells. We found that the LQTS-associated mutation, G6015-Kv11.1, which is retained in the endoplasmic reticulum as a trafficking-deficient protein, did not localize into the cholesterol and sphingolipid membrane fractions. We confirmed the localization of WT-Kv11.1 in cholesterol and sphingolipid enriched membrane fractions, isolated from HEK293 cells, using the OptiPrep density gradient fractionation technique. We also show that Kv11.1 channels do not associate with the muscle-specific Cav3 in canine ventricular myocytes, suggesting that Kv11.1 localizes to non-caveolar cholesterol and sphingolipid enriched membranes. These are new findings for the Kv11.1 channel.

Our findings show that HEK293 cells treated with MβCD to deplete cholesterol resulted in a shift of WT-Kv11.1 channels to denser gradient membrane fractions, caused a positive shift in the voltage dependence of activation of $I_{Kv11.1}$, accelerated the deactivation rate, and slightly slowed the inactivation rate. MβCD treatment likewise accelerated the deactivation rate of $I_{Kr}$ in native myocytes. Incubation of HEK293 cells expressing Kv11.1 channels with MβCD saturated with cholesterol caused a positive shift in the voltage dependence of activation of $I_{Kv11.1}$, decreased the steep voltage dependence of activation, and accelerated the rate of inactivation. The functional changes of $I_{Kv11.1}$ depended on whether or not MβCD was balanced with cholesterol, suggesting that it is the depleting or loading of membrane cholesterol that modulated $I_{Kv11.1}$. One explanation for these findings is that altering cholesterol content alters the microenvironment for the Kv11.1 channel in the plasma membrane.

Three Kv11.1 mRNA variants are expressed in the heart (variants 1–3). Heterologous expression of the full-length Kv11.1 channel ERG1a (variant 1) results in $I_{Kv11.1}$ with electrophysiological and pharmacological properties similar to $I_{Kr}^{11}$. The Kv11.1 antibody used in this study recognizes variant 1 and 3, but not variant 2. Kv11.1 ERG1b (variant 3) has been shown to associate with Kv11.1 variant 1, but the functional significance of this
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interaction is not known. In canine ventricular myocytes Kv11.1 variant 1 has a molecular weight of ~165 kDa and Kv11.1 variant 3 is ~95 kDa.36,48 Figure 1B shows a Western blot analysis of Kv11.1 protein in different membrane fractions and the homogenate. Kv11.1 variant 1 protein was present in all membrane fractions. We did not detect a band for Kv11.1 variant 3 in our Western analyses, but were able to detect Kv11.1 variant 3 mRNA (NCBI accession number AY627685). Thus, Kv11.1 variant 3 was not detected in adult canine ventricular myocytes, which agrees with previous work by Pond and colleagues.35

Several studies have shown that different Kv channels selectively localize to cholesterol and sphingolipid enriched microdomains. Kv1.5 localize to cholesterol and sphingolipid microdomains containing the scaffolding protein caveolin, Kv2.1 localize into cholesterol and sphingolipid microdomains that do not contain caveolin, and Kv4.2 do not localize to cholesterol and sphingolipid enriched microdomains.49,50 Consistent with this, incubation in cycloexodrin alters the function of Kv1.5 and Kv2.1, but not Kv4.2. In addition, the functional changes in the voltage dependent gating of Kv1.5 and Kv2.1 following cycloexodrin treatment are different than that observed for Kv11.1. Together, these data suggest that cycloexodrin treatment does not modify Kv channel gating in a non-specific manner. Other ion channels and exchangers have been shown to localize to caveolar microdomains in cardiac myocytes including the L-type Ca\(^{2+}\) channels, the HCN4 pacemaker channel, the Na\(^{+}/\)Ca\(^{2+}\) exchanger, and Na\(^{+}\) channel,14,51-53 and their function can be altered by cholesterol modifying agents.

Kv11.1 is an important ion channel for the control of cardiac repolarization in humans and it has been associated with specific arrhythmia syndromes. Mutations or drugs that decrease I\(_{\text{Kv11.1}}\) are known to increase the risk of LQTS whereas, increasing I\(_{\text{Kv11.1}}\) is one mechanism causing short QT syndrome. Our findings that cholesterol depletion with M\(_{\text{βCD}}\) and cholesterol loading with cholesterol loaded M\(_{\text{βCD}}\) can modulate I\(_{\text{Kv11.1}}\) and I\(_{\text{Kr}}\) provide evidence to suggest that alteration of the lipid environment can affect WT-Kv11.1 channel function. The specific mechanism(s) for this are not certain, although cholesterol is known to alter structural and functional properties of phospholipid bilayers and native plasma membranes, modify membrane microdomain composition, and interact with multiple membrane proteins.54-57 Although speculative, our data provide support for the evolving idea that alteration of membrane cholesterol could be a mechanism for modulating arrhythmia susceptibility in the heart.

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