Caveolin Regulates Kv1.5 Trafficking to Cholesterol-Rich Membrane Microdomains

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ABSTRACT

The targeting of ion channels to cholesterol-rich membrane microdomains has emerged as a novel mechanism of ion channel localization. Previously, we reported that Kv1.5, a prominent cardiovascular K+ channel α-subunit, localizes to caveolar microdomains. However, the mechanisms regulating Kv1.5 targeting and the functional significance of this localization are largely unknown. In this study, we demonstrate a role for caveolin in the trafficking of Kv1.5 to lipid raft microdomains where cholesterol modulates channel function. In cells lacking endogenous caveolin-1 or -3, the association of Kv1.5 with low-density, detergent-resistant membrane fractions requires coexpression with exogenous caveolin, which can form channel-caveolin complexes. Caveolin is not required for cell surface expression, however, and caveolin-trafficking mutants sequester Kv1.5, but not Kv2.1, in intracellular compartments, resulting in a loss of functional cell surface channel. Coexpression with wild type caveolin-1 does not alter Kv1.5 current density; rather, it induces depolarizing shifts in steady-state activation and inactivation. These shifts are analogous to those produced by elevation of membrane cholesterol. Together, these results show that caveolin modulates channel function by regulating trafficking to cholesterol-rich membrane microdomains.

Voltage-dependent K+ (Kv) channels play an essential role in the complex electrical responses of the cardiovascular system (Armstrong and Hille, 1998). These channels, which open and close in response to a change in membrane voltage, are critical for numerous cellular functions, including control of resting membrane potential and the shaping of action potentials (Rolf et al., 2000). Both the level of cell surface expression and the proper plasma membrane localization are important for Kv channel function. Kv1.5 is a prominent cardiovascular K+ channel that is vital for atrial repolarization in the human heart and the regulation of vascular tone in multiple peripheral vascular beds. Genetic variations of Kv1.5 have been identified in patients with idiopathic pulmonary arterial hypertension (Remillard et al., 2007), whereas mutations cause human atrial fibrillation (Olson et al., 2006). Alterations in the cell surface expression of functional Kv1.5 contribute to the pathophysiology of paroxysmal and persistent atrial fibrillation (Van Wagoner et al., 1997; Tanabe et al., 2006) as well as chronic hypoxic pulmonary hypertension (Michelakis and Weir, 2001; Moudgil et al., 2006). Despite the

clear links between changes in Kv1.5 surface expression and cardiovascular disease, relatively little is known regarding the mechanisms controlling its plasma membrane targeting or localization.

Advances in the study of cell membrane organization have demonstrated that microdomains exist within the fluid bilayer of the plasma membrane. One form of microdomain, termed a lipid raft, is a dynamic structure rich in tightly packed sphingolipids and cholesterol (Simons and Ikonen, 1997). The rafts, which are present in both excitable and nonexcitable cells, localize a number of membrane proteins, including multiple signal transduction molecules but exclude others (Harder and Simons, 1997). It is likely that these rafts serve as scaffolding regions in which signal transduction pathways interface (Schlegel et al., 1998). Caveolae are specialized lipid rafts sharing a similar lipid profile but also containing the scaffolding protein caveolin (Cheng et al., 2006; Parton and Simons, 2007). Numerous ion channels in multiple cell types have been described to localize to lipid raft domains and or caveolae (Martens et al., 2004; O'Connell et al., 2004; Maguy et al., 2006). However, the mechanisms controlling their association and the physiological significance of this localization are unclear. For monotopic proteins, post-translational acylation and/or specific transmembrane sequences may determine their affinity for lipid rafts (Zhang et al., 1998; Melkonian et al., 1999). Much less is known

ABBREVIATIONS: Kv, voltage-dependent K+ channel; CFP, cyan fluorescent protein; PAGE, polyacrylamide gel electrophoresis; FRT, Fisher rat thyroid.

about the molecular mechanisms of targeting polytopic proteins, such as ion channels, to lipid rafts. Recent reports have indicated a role for caveolin-1 and -3 in trafficking polytopic proteins to the plasma membrane (Brazer et al., 2003; Wyse et al., 2003). Furthermore, they have both been shown to bind and even functionally regulate proteins in lipid-rich microdomains (Anderson, 1998; Okamoto et al., 1998). We have reported that Kv1.5 can localize to caveolar microdomains (Martens et al., 2000, 2001). Others have described this interaction in both heart and vascular smooth muscle (Cogolludo et al., 2006; Abi-Char et al., 2007). However, a role for caveolin in Kv1.5 function and membrane localization has not been elucidated. Therefore, in this work, we tested the hypothesis that caveolin regulates the trafficking of Kv1.5 to lipid raft microdomains and that there is a functional consequence of this localization.

Materials and Methods

DNA Constructs. Full-length rat caveolin-1 cDNA was generated by PCR as described previously (Uittenbogaard and Smart, 2000). The three amino acid sequence TFT, corresponding to amino acids 91 to 93 of caveolin-1, were removed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create the dominant-negative caveolin-1-ΔTFT construct. To generate the caveolin-1-CFP fusion constructs, the caveolin-1 cDNA was subcloned into the EcoRI site of pECFP-C1 (Clontech, Mountain View, CA). Full-length caveolin-3 was a generous gift of Dr. Jeffrey Pessin (SUNY Stony Brook, Stony Brook, NY) and was originally purchased from American Type Culture Collection (Manassas, VA). The caveolin-3-CFP fusion protein was created by cloning caveolin-3 into pECFP-C1 using EcoRI and XbaI restriction enzymes. All constructs were verified for sequence integrity and in-frame fusion with CFP by sequence analysis. cDNA encoding human Kv1.5 and Kv1.5-GFP have been described previously (Martens et al., 2001; McEwen et al., 2007; Zhang et al., 2007).

Lipid Raft Isolation. Isolation of low-density, Triton X-100–insoluble complexes was performed as described previously (Martens et al., 2000) with minor modifications. In brief, cells or tissue were homogenized in 1% Triton X-100, and sucrose added to a final concentration of 40%. A 5%-to-30% discontinuous sucrose gradient was layered on top of this detergent extract followed by ultracentrifugation (54,000 rpm) for 18 to 24 h at 4°C. Gradient fractions were analyzed by SDS-PAGE gel electrophoresis on a NuPAGE Novex 4 to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose and probed with the indicated primary antibody for 1 h at room temperature. Blots were then incubated with secondary antibodies conjugated to horseradish peroxidase (1:5000; Zymed, South San Francisco, CA) and visualized using the Western Lightning enhanced chemiluminescent reagent according to manufacturer's protocol (PerkinElmer Life and Analytical Sciences, Waltham, MA). Images were captured using the EpiChemi3 Darkroom (UVP, Inc., Upland, CA).

Immunostaining. Immunofluorescent labeling of Kv1.5 was performed essentially as described previously (Martens et al., 2000). Cells stably expressing Kv1.5 with CFP-tagged wild-type and mutant caveolin constructs were fixed and permeabilized before incubation with anti-Kv1.5 antibodies (1:1000; Mays et al., 1995; Martens et al., 2001). After incubation in biotinylated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), the staining was visualized with Cy3-conjugated streptavidin (1:500; Jackson ImmunoResearch Laboratories, Inc.). Imaging was performed on an Axiovert 200 M microscope (Carl Zeiss Inc., Thornwood, NY) equipped with standard epifluorescence and a Photometrics Cool Snap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ).

Electrophysiology. Whole-cell voltage-clamp experiments were performed as described previously (Benson et al., 2007; Zhang et al., 2007). In brief, cells were plated in 35-mm plates and transiently transfected with the indicated cDNAs. Patch pipettes (1–2 MΩ) were pulled from borosilicate glass capillaries. The intracellular pipette solution contained 110 mM KCl, 10 mM HEPES, 5 mM K_2BAPTA, 5 mM K_2ATP, and 1 mM MgCl_2; it was adjusted to pH 7.2 with KOH. The bath solution contained 110 mM NaCl, 4 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2, 10 mM HEPES, and 1.8 mM glucose; it was adjusted to pH 7.35 with NaOH. Cells were maintained at a holding potential of -80 mV. The current-voltage relationships and activation curves were measured by using 250-ms voltage-clamp pulses applied in 10-mV steps between -110 mV and $+40$ mV. Tail current amplitude immediately after the capacitive transient was measured to obtain the voltage dependence of activation curves. Steady-state inactivation was measured by using a 5-s conditioning pulse applied in 10-mV steps from -110 to $+40$ mV followed by a 500-ms test pulse at $+30$ mV. Current was measured at the end of the 500-ms test pulse. For both activation and inactivation recordings, cells were allowed to recover for 15 s between traces. Normalized activation and inactivation curves were fitted to the Boltzmann equation, and results are expressed as mean ± S.E.M. For each condition tested, all potassium currents were stable throughout the length of the recording period.

Immunoprecipitation. The immunoprecipitation technique used is based on the protocol originally described by Drisdel and Green (Dubreuil et al., 1996). Fisher rat thyroid (FRT) cells were washed twice with potassium-free phosphate-buffered saline, scraped, and collected at 1000g for 4 min. The pellet was then resuspended and solubilized in solubilization buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.02% NaN_3, supplemented with protease inhibitors) for 10 min at room temperature. The solubilized membranes were centrifuged in a microcentrifuge at 10,000g for 20 min at 4°C. The supernatant was collected and precleared with 10 μl of protein A (anti-caveolin; BD Biosciences, San Jose, CA) or protein G (anti-V5; Invitrogen, Carlsbad, CA) beads (Sigma) for 1 h at 4°C with gentle mixing. The beads were removed by centrifugation at 13,000 rpm for 5 min at 4°C. The sample was then incubated with polyclonal anti-caveolin (BD Biosciences) or monoclonal anti-V5 (Invitrogen, immunoprecipitating Kv1.5) antibody and 10 μl of protein A or protein G beads overnight at 4°C with gentle mixing. The next day the beads with the bound antibody-antigen complex were removed by centrifugation at 13,000 rpm for 5 min at 4°C and washed twice for 5 min at RT in wash buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.02% NaN_3). After the second wash, the beads were removed by centrifugation at 10,000g at 4°C and resuspended in 50 μl of SDS sample buffer. The proteins were eluted from the beads at 100°C for 5 min, spun down, and the supernatant was collected and analyzed using SDS-PAGE and Western blotting as described above.

Cholesterol Treatment and Quantitation. Cholesterol supplementation was achieved by incubating cells for 1 h in 2% methyl-β-cyclodextrin precomplexed with 1 mM cholesterol in serum-free media at 37°C, with shaking every 5 min. Serum-free medium plus 0.01% ethanol was used as a vehicle control. Cholesterol levels were assayed using the Amplex Red Cholesterol kit from Invitrogen (Carlsbad, CA) Cells were homogenized and lysed in reaction buffer containing 5 mM sodium cholate, and 0.1% Triton X-100 in phosphate-buffered saline at pH 7.4 for 30 min on ice and then sonicated. Cholesterol was then measured according to manufacturer's protocol. Fluorescence was measured using a PerkinElmer Victor^2 microplate reader with an excitation wavelength of 545 nm and emission wavelength of 590 nm.

Results

Caveolin Was Necessary for the Association of Kv1.5 with Lipid Rafts in Transfected FRT Cells. The mecha-

nism responsible for the targeting of Kv1.5 to lipid raft microdomains is unknown. To examine the role of caveolin in this specialized localization, we used a caveolin-null cell line, FRT cells. In FRT cells, which express no endogenous caveolin-1 or -3, Kv1.5 was found in the high-density, detergent-soluble fractions, similar to the non–raft-associated transferrin receptor (Fig. 1A). Coexpression of caveolin-1 with Kv1.5, however, resulted in the shift of Kv1.5 to the lower density, detergent-resistant, lipid raft fractions (Fig. 1B, top) where it comigrated with caveolin (Fig. 1B, bottom). Under these conditions, a population of Kv1.5 remained in the detergent-soluble fractions, most likely because of overexpression of the channel, as has been described previously (Martens et al., 2001). As a negative control, the transferrin receptor remained localized in the higher-density nonraft fractions (Fig. 1B, center).

Next, we tested whether Kv1.5 interacts in complex with caveolin proteins. FRT cells were stably transfected with Kv1.5 and either caveolin-1 or -3. Immunoprecipitation with an anti-caveolin antibody shows that both caveolin-3 and -1 are in complex with Kv1.5, from which both the glycosylated and nonglycosylated forms of the channel were detected (Fig. 1C). These data were confirmed with the reverse immunoprecipitation, where an anti-Kv1.5 antibody pulled down caveolin from transfected FRT cells (Fig. 1D, third panel). All blots were stripped and reprobed with the respective immunoprecipitating antibody to ensure efficient pull-down (Fig. 1, C and D). Immunoprecipitation with nonimmune serum revealed no significant signal (Fig. 1, C and D). Together, these results indicate that Kv1.5 can associate in complex with multiple isoforms of caveolin.

Disruption of Caveolin Trafficking Sequestered Kv1.5 Intracellularly. We next tested whether the interaction between caveolin and Kv1.5 was responsible for trafficking of Kv1.5 to the cell surface. In FRT cells, Kv1.5 was able to traffic to the plasma membrane in the absence of caveolin as indicated by the staining around the cell periphery (Fig. 2, A–C). This indicates that caveolin is not required for cell surface expression of Kv1.5, but does not address its behavior in the presence of caveolin.

It has been suggested that no single caveolin antibody or fixation technique can successfully detect all caveolin pools within a cell (Bush et al., 2006). Thus, CFP-tagged caveolins were used to accurately determine their subcellular localization. When we coexpressed CFP-tagged caveolin-1 with Kv1.5, the channel trafficked to the plasma membrane, where the two proteins colocalized at the cell periphery (Fig. 2, D–F). A three-amino-acid deletion in the scaffolding domain of caveolin-3 (ΔTFT) is associated with diseases such as limb-girdle muscular dystrophy and is characterized by accumulation in the Golgi complex and a reduction of surface levels of both caveolin and certain binding partners (Galbiati et al., 1999, 2000, 2001). To establish whether caveolin was indeed involved in trafficking Kv1.5 to the plasma membrane, we expressed a CFP-caveolin-1 ΔTFT-mutant together with Kv1.5 in FRT cells. Coexpression of Kv1.5 with the caveolin-1 ΔTFT-mutant resulted in the intracellular accumulation of Kv1.5 with a concomitant loss in peripheral cell-surface staining (Fig. 2, G–I).

We showed previously that potassium channel localization to lipid raft microdomains is isoform-specific; Kv1.5 localizes to caveolae whereas Kv2.1 is found in noncaveolar lipid rafts

(Martens et al., 2001). Therefore, coexpression of Kv2.1 with the caveolin-1 ΔTFT-mutant should not disrupt its trafficking to the plasma membrane. Indeed, as seen in Fig. 2K,
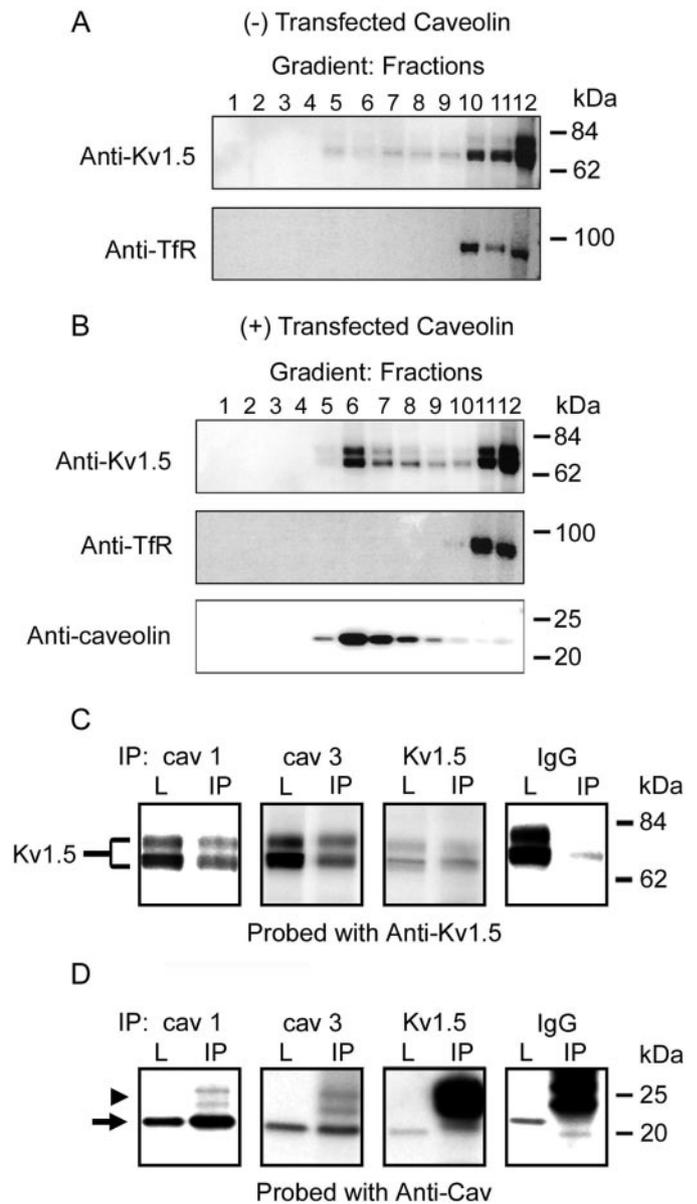

Fig. 1. Kv1.5 requires caveolin for localization in lipid raft microdomains. Cell membranes from FRT cells stably transfected with human Kv1.5 and caveolin were subject to SDS-PAGE. A, sucrose density gradient centrifugation of 1% Triton X-100 solubilized extracts from FRT cells stably expressing Kv1.5 alone were prepared as described under *Materials and Methods*. Western blots, probed with anti-Kv1.5 (top), demonstrate that the channel is not present in detergent-insoluble lipid raft fractions. Western blots probed with anti-transferrin (bottom) were used as a negative control to maintain that the cells are completely solubilized. B, sucrose density gradient centrifugation in FRT cells stably expressing Kv1.5 and caveolin were prepared as described under *Materials and Methods*. Western blots, probed with anti-Kv1.5 (top) demonstrate that the channel is present in detergent-insoluble, low-density lipid raft fractions upon association with caveolin (bottom). Western blots probed with anti-transferrin (middle) were used as a negative control to maintain that the cells are completely solubilized. C–D, solubilized membranes from FRT cells stably transfected with caveolin and/or Kv1.5 were immunoprecipitated with anti-caveolin or anti-Kv1.5. Western blots of the immunoprecipitates were probed with anti-Kv1.5 (C) or anti-caveolin (D). Channel is shown to precipitate with caveolin 3, itself, and caveolin 1. An immunoprecipitation with a nonimmune antibody (C and D) was used as a negative control (C and D). IP, immunoprecipitate; L, FRT cell lysate.

Kv2.1 maintains the ability to traffic to the plasma membrane in the presence of the caveolin-1 ΔTFT-mutant, as indicated by the peripheral staining of channel similar to Fig. 2B (Fig. 2, J–L). Next, we tested whether exchanging domains of the Kv2.1 channel with those from Kv1.5 could confer caveolin sensitivity to the Kv2.1 channel. We found that exchanging the amino terminus of Kv2.1 with that from Kv1.5 conferred sensitivity to the caveolin-1 ΔTFT-mutant, resulting in a significant intracellular accumulation (Fig. 2, M–O). Therefore, the amino terminus of Kv1.5 is critical for caveolin-mediated trafficking of Kv1.5 to the plasma membrane.

Coexpression of Kv1.5 with the Caveolin ΔTFT-Mutant Abolished Potassium Channel Currents in FRT Cells, Which Were Not Recovered with the Addition of Exogenous Cholesterol. Coexpression of Kv1.5 with the caveolin-1 ΔTFT-mutant resulted in mislocalization of the

channel away from the cell periphery to intracellular compartments. To directly measure cell surface levels, we used two complementary approaches, including electrophysiological measurements and live cell labeling of channels on the plasma membrane. To determine whether any functional channels remained at the cell surface, FRT cells expressing Kv1.5 alone, Kv1.5 with wild-type caveolin-1, or Kv1.5 with the caveolin-1 ΔTFT-mutant were examined by whole-cell voltage clamp. As shown in Fig. 3A, current traces for Kv1.5 + caveolin-1 are similar to those for channel alone, whereas Kv1.5 current was markedly reduced in the presence of the caveolin-1 ΔTFT-mutant. This decrease in current was statistically significant across a wide range of voltages (Fig. 3B). These results were confirmed with immunohistochemistry experiments using an extracellular-tagged Kv1.5-GFP construct, allowing us to discriminate surface from intracellular channels, as described previously (McEwen et al., 2007). Kv1.5 trafficked efficiently to the cell surface in the presence or absence of caveolin-1 (Fig. 3C). In contrast, coexpression of Kv1.5 with the mutant caveolin-1 resulted in the abolishment of cell surface channel (Fig. 3C), despite efficient expression (Fig. 3C, inset).

Lipid rafts are specialized domains of the plasma membrane that are enriched in cholesterol. Caveolin is a cholesterol-binding molecule (Murata et al., 1995), and its mislocalization may sequester intracellular cholesterol and indirectly affect Kv1.5 channel trafficking. Therefore, we tested whether the addition of exogenous cholesterol could overcome the effects of the caveolin-1 ΔTFT-mutant. Despite efficient incorporation of cholesterol in the plasma membrane (Fig. 4B), addition of exogenous cholesterol was not sufficient to recover cell surface potassium current (Fig. 4A, compare ▼ with ○), indicating that the caveolin-1 ΔTFT-
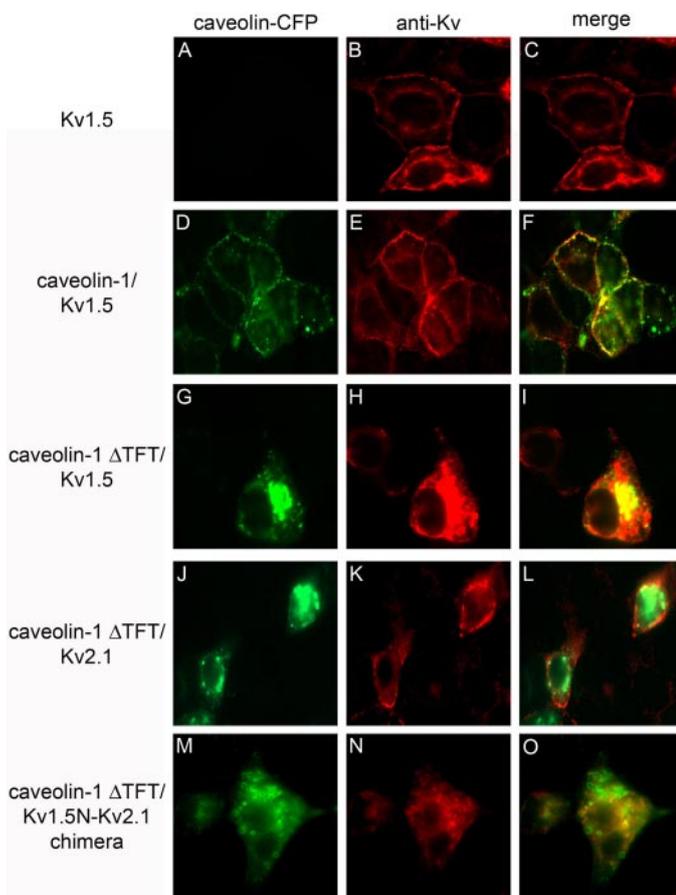

Fig. 2. Caveolin traffics Kv1.5 to the plasma membrane. A–C, Kv1.5 (red) is able to traffic to the plasma membrane in the absence of caveolin, as indicated by the ring around the cell periphery (A). D, immunofluorescence localization of caveolin-1 and Kv1.5 (E) in FRT cells stably expressing channel and caveolin-1-CFP protein shows punctate cell surface distributions, as indicated by the merged image (F). G–I, Kv1.5 cell surface distribution is altered with the addition of a mutant form of caveolin-1-CFP, caveolin-1-ΔTFT. Cells were immunostained with anti-Kv1.5 antibody (1:1000) and detected using a Cy3-conjugated fluorochrome. J–L, FRT cells stably expressing Kv2.1 and the mutant form of caveolin-1-CFP were immunostained with an anti-Kv2.1 antibody to show that the mutant form of caveolin does not alter the expression of Kv2.1. M–O, FRT cells stably expressing a Kv1.5N-Kv2.1 chimera (N) show normal cell surface distribution that is altered with the addition of the mutant form of caveolin-CFP (M). In all panels, caveolin-1-CFP fluorescence is green, whereas Kv1.5 staining is red. Colocalization of caveolin-1 with Kv1.5 in merged images is indicated by yellow color.
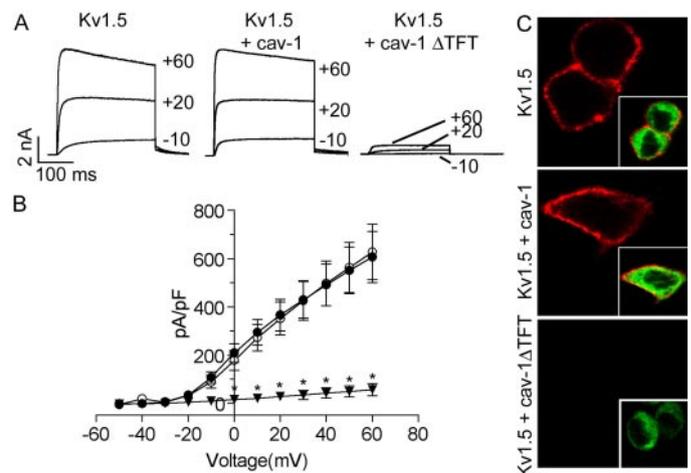

Fig. 3. Coexpression of a dominant-negative caveolin mutant with Kv1.5 results in the loss of functional cell surface channel. A, representative current traces at −10, +20, and +60 mV from FRT cells expressing Kv1.5 alone (left), Kv1.5 + caveolin-1 (middle), or Kv1.5 + dominant negative caveolin-1 (right). B, whole-cell, patch-clamp recordings from stably transfected FRT cells expressing Kv1.5 alone (●, $n = 20$), Kv1.5 + caveolin-1 (○, $n = 19$), or Kv1.5 + dominant-negative caveolin-1 (▼, $n = 10$). Currents were normalized to cell capacitance. C, immunocytochemistry of FRT cells expressing Kv1.5-GFP alone (top), Kv1.5-GFP + caveolin-1 (middle), or Kv1.5-GFP + dominant negative caveolin-1 (bottom) surface staining for channel with an anti-GFP antibody (1:500). Images represent a single confocal image through a medial plane of each representative cell. Insets show overlay of total Kv1.5-GFP expression (green) with anti-GFP surface labeling (red).

mutant was directly inhibiting trafficking of Kv1.5 to the plasma membrane compared with wild-type caveolin (Fig. 4B).

Caveolin and Cholesterol Cause a Depolarizing Shift in the Steady-State Activation and Inactivation Properties of Kv1.5. In Fig. 3A, we showed that coexpression of caveolin-1 with Kv1.5 had no effect of potassium current density in FRT cells. When we looked at the steady-state kinetics of the channel, however, the presence of caveolin-1 shifted both the half-voltage ($V_{1/2}$) of activation (Fig. 5A; Kv1.5 = -12.80 ± 1.19, Kv1.5 + caveolin-1 = -5.40 ± 1.48) and the $V_{1/2}$ of inactivation (Fig. 5B; Kv1.5 = -12.04 ± 1.24, Kv1.5 + caveolin-1 = -3.59 ± 0.94) in the depolarizing direction by approximately 10 mV. Results from Fig. 1, showing that caveolin targets Kv1.5 to detergent-insoluble raft fractions, suggest that, in the presence of caveolin, the local cholesterol environment of Kv1.5 may contribute to these functional changes. To further investigate this possibility, we treated FRT cells expressing Kv1.5 alone with exogenous cholesterol. As seen in Fig. 6A, treatment with exogenous cholesterol resulted in an increase in the whole-cell cholesterol/protein ratio compared with untreated cells, indicating that exogenous cholesterol incorporates into the plasma membrane. It is noteworthy that treatment with cholesterol resulted in a similar 10- to 20-mV shift in the $V_{1/2}$ of both steady-state activation (Kv1.5 = -13.08 ± 0.65, Kv1.5 + cholesterol = 2.89 ± 0.71) and inactivation (Kv1.5 = -12.04 ± 1.24, Kv1.5 + cholesterol = 5.40 ± 1.00) compared with untreated FRT cells (Fig. 6, B and C, respectively). These effects are analogous to results obtained with coexpression of caveolin. Together, these results indicate that association with caveolin traffics Kv1.5 to lipid raft microdomains, where interactions with cholesterol alter the steady-state kinetics of Kv1.5 channel function.

Discussion

In this study, we show for the first time that caveolin is necessary for the targeting of Kv1.5 channels to lipid raft microdomains. Naturally occurring hypomorphic mutants of caveolin, which mislocalize to the Golgi complex (Galbiati et al., 1999, 2000, 2001), sequester Kv1.5 intracellularly. This mistargeting completely suppresses Kv1.5-mediated current in a cholesterol-independent manner. Furthermore, these results reveal a functional consequence of raft microdomain localization by demonstrating that the cholesterol composition of the membrane microenvironment is critical for regulation of channel steady-state properties.

One important finding of our study is that caveolin can act as a protein chaperone for Kv1.5 targeting to lipid raft microdomains. This is consistent with previous reports for other polytopic membrane proteins, such as the angiotensin II type 1 receptor and transient receptor potential canonical 1 (TRPC1) (Brazer et al., 2003; Wyse et al., 2003). Recent work in atrial myocytes, using immunohistochemical methods, suggests that Kv1.5 and caveolin-3 do not associate at the plasma membrane (Eldstrom et al., 2006; Maguy et al., 2006; Abi-Char et al., 2007). In other studies from native tissue, however, Kv1.5 has been shown by Western blot analysis to interact in complex with both caveolin-1 and -3 (Folco et al., 2004; Cogolludo et al., 2006) in cardiac and vascular myocytes. These differences may result from the mode of detection. It is now clear that immunohistochemical labeling of nondenatured protein can detect different subpopulations of caveolin within a single cell and is dependent on the method of fixation and the specific caveolin antibody used (Bush et al., 2006). Therefore, certain populations of caveolin may go undetected, potentially explaining the lack of detectable surface colocalization of Kv1.5 with caveolin in cardiac cells. It is noteworthy that in pulmonary arterial smooth muscle cells,
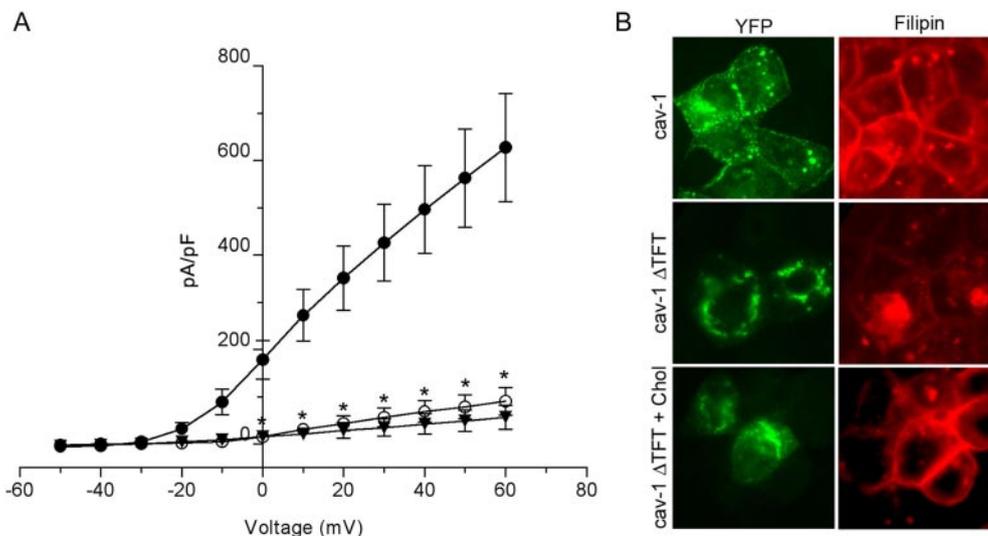
Fig. 4. Supplementing with exogenous cholesterol does not restore Kv1.5 currents in the presence of the caveolin-ΔTFT mutant. A, current density traces of whole-cell patch clamp electrophysiological recordings from FRT cells stably expressing Kv1.5 + wild-type caveolin-1 (●, n = 19), Kv1.5 + caveolin-1-ΔTFT mutant (▲, n = 10), or Kv1.5 + caveolin-1-ΔTFT mutant with cholesterol enrichment (○, n = 6). Cholesterol supplementation does not restore Kv1.5 currents. B, immunocytochemistry of FRT cells expressing Kv1.5 + wild-type caveolin-1 (top), Kv1.5 + caveolin-1-ΔTFT mutant (middle), or Kv1.5 + caveolin-1-ΔTFT mutant with cholesterol enrichment (bottom) stained for Kv1.5 (green) and cholesterol (red). In the presence of CFP-tagged wild-type caveolin-1, Kv1.5 traffics to the plasma membrane (top), whereas coexpression with the caveolin-ΔTFT mutant sequesters Kv1.5 in a subcellular perinuclear compartment (middle). Addition of exogenous cholesterol does not restore Kv1.5 trafficking to the plasma membrane in the presence of the caveolin-ΔTFT mutant (bottom).

caveolin-1 was only weakly associated with Kv1.5 under basal conditions, and only after serotonin stimulation and channel internalization was the interaction augmented (Cogolludo et al., 2006). Thus, caveolin may interact with Kv1.5 transiently during the trafficking of the channel into and out of the plasma membrane, as has been shown for the angiotensin II receptor (Wyse et al., 2003). In agreement with this, our data show that caveolin is not required for cell surface expression but is essential for its localization to raft microdomains, which functionally regulate steady-state channel properties. This may explain, in part, the lack of overt cardiovascular phenotypes in caveolin-null mice (Hnasko and Lisanti, 2003). Therefore, the functional changes in steady-state properties that occur in the absence of caveolin may not manifest themselves under normal conditions but may appear under periods of stress. This suggests that, unlike the knockout of caveolin, alterations in Kv1.5 function and/or expression may be more apparent in disease states in which caveolin is genetically mutated or its surface trafficking impaired.

Results of our study show that disruption of normal caveolin trafficking traps Kv1.5 in intracellular, perinuclear com-partments and prevents functional channel expression on the cell surface. This interaction occurs through the NH$_2$ terminus of Kv1.5, because replacement of the NH$_2$ terminus of Kv2.1, a potassium channel that does not interact with caveolin, with that of Kv1.5 sequesters the chimera in the Golgi complex (Fig. 2). These data are consistent with a previous report showing Kv1.5 interacts with caveolin-3, as part of a complex with SAP97 in the heart, through a region located at the NH$_2$ terminus (Folco et al., 2004). However, in contrast to other reports (Roy et al., 1999), our data indicate that the dominant-negative effects of mutant caveolin on channel surface levels are cholesterol-independent.

The caveolin deletion-mutant used in our studies lacks three amino acids (TFT) in the scaffolding domain and has been shown to result in the mislocalization and reduced cell-surface expression of caveolin and its plasma-membrane binding partners (McNally et al., 1998; Galbiati et al., 1999; Vorgerd et al., 2001; Woodman et al., 2004). Furthermore,
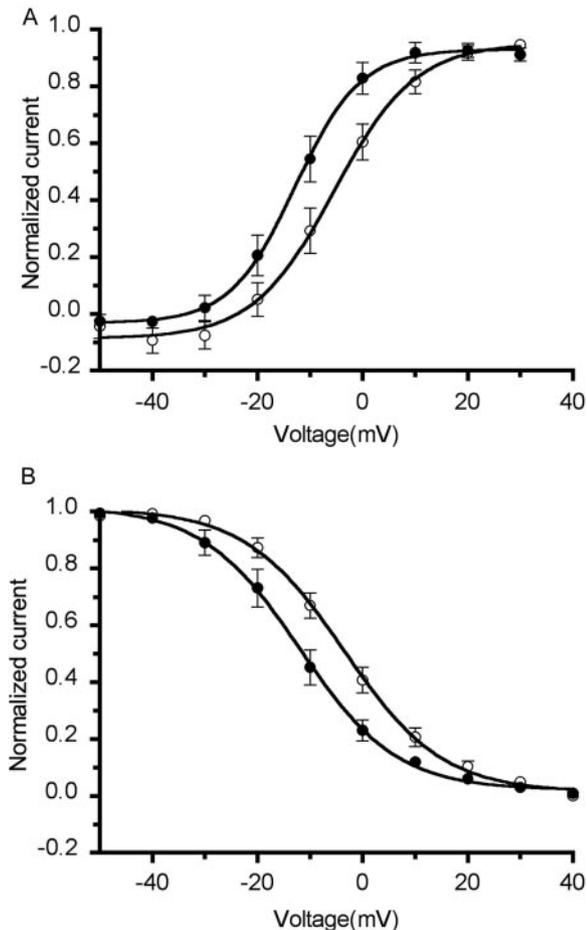

Fig. 5. Coexpression of caveolin-1 with Kv1.5 causes depolarizing shifts in both steady-state activation and inactivation. A and B, whole-cell patch clamp recordings from FRT cells stably expressing Kv1.5 alone (●, $n = 14$) or Kv1.5 + caveolin-1 (○, $n = 15$). The presence of caveolin-1 causes a depolarizing shift in the $V_{1/2}$ of activation (Kv1.5 = -12.80 ± 1.19, Kv1.5 + caveolin-1 = -5.40 ± 1.48) (A) and inactivation (Kv1.5 = -12.04 ± 1.24, -3.59 ± 0.94, Kv1.5 + caveolin-1 = -3.59 ± 0.94) (B) of the Kv1.5 channel. The $V_{1/2}$ of both activation and inactivation of Kv1.5 are significantly shifted in the presence of caveolin, as determined by an unpaired t test ($p < 0.0001$).
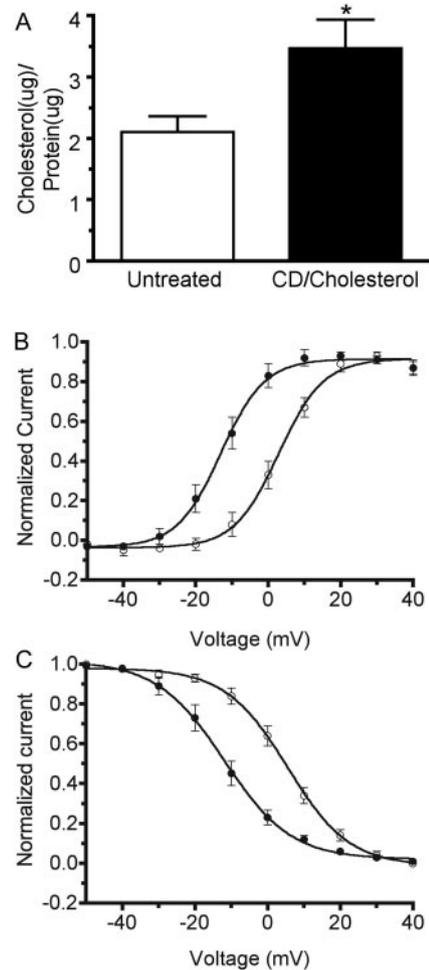

Fig. 6. Cholesterol supplementation results in depolarizing shifts in steady-state activation and inactivation. A, supplementing with exogenous cholesterol resulted in an increase in cholesterol incorporation in the treated cells (asterix indicates $p < 0.05$). B and C, whole-cell, patch-clamp recordings from FRT cells expressing Kv1.5 that were either untreated (●, $n = 13$) or supplemented with cholesterol (○, $n = 11$). Supplementing with cholesterol shifted the $V_{1/2}$ of activation (Kv1.5 = -13.08 ± 0.65, Kv1.5 + cholesterol = 2.89 ± 0.71) (B) and inactivation (Kv1.5 = -12.04 ± 1.24, Kv1.5 + cholesterol = 5.40 ± 1.00) (C) in the depolarizing direction. The $V_{1/2}$ of both activation and inactivation of Kv1.5 are significantly shifted with the addition of exogenous cholesterol, as determined by an unpaired t test ($p < 0.0001$).

this mutation in caveolin-3 has been shown to be involved in limb-girdle muscular dystrophy (Galbiati et al., 1999, 2000, 2001). Although alterations in cardiac excitability or peripheral vascular tone were not examined, limb-girdle muscular dystrophy patients with the TFT deletion in caveolin-3 do not seem to exhibit an overt cardiac deficit (Bönnemann and Finkel, 2002). Evidence does suggest, however, that the penetrance of caveolin mutations is different in skeletal and cardiac muscle, and the mechanisms regulating caveolin surface expression in the two tissues may be divergent (Cagliani et al., 2003). It is noteworthy that a mutation in caveolin-3 analogous to ΔTFT (T63S) was identified in a patient with familial hypertrophic cardiomyopathy (Hayashi et al., 2004; Woodman et al., 2004). Given our results and the increasing number of ion channels reported to localize to lipid rafts/caveolae, this issue warrants future study. Nevertheless, our results clearly show that Kv1.5 and caveolin, when expressed together, can interact early in the exocytic pathway to regulate cell surface localization.

Perhaps the most significant finding of this work is that it provides a direct link between functional properties of Kv1.5 and lipid raft localization. In the absence of caveolin, the channel targets to detergent-soluble, nonraft domains, where its steady-state activation and inactivation properties are different from in the presence of caveolin, where the channel localizes to lipid rafts (Figs. 1 and 5). Previous work, including our own (Martens et al., 2001, 2004), has used cholesterol-depleting agents such as β-methyl cyclodextrin to disrupt microdomain organization and alter channel function while extrapolating these effects to functional consequence of lipid raft localization (Hnasko and Lisanti, 2003; Barbuti et al., 2004; Davies et al., 2006; Maguy et al., 2006; Pottosin et al., 2007). However, increased awareness to the plurality of their effects, including cytoskeletal disruption, complicates the interpretation of data using these agents (Kwik et al., 2003). Nevertheless, our data do indicate that the cholesterol content of the membrane microenvironment is critical for channel regulation. Both the addition of exogenous cholesterol and coexpression with caveolin shifted the $V_{1/2}$ of Kv1.5 steady-state activation and inactivation to more depolarizing potentials. We have shown previously that depletion of cholesterol results in a shift in the steady-state activation and inactivation kinetics of Kv1.5 in the hyperpolarizing direction, opposite that measured with cholesterol enrichment (Martens et al., 2001). It is interesting that most other reports of cholesterol modulation of ion channel activity also find effects on the activation and/or inactivation properties (Barbuti et al., 2004; Martens et al., 2004; Maguy et al., 2006; Pottosin et al., 2007). Together, these data emphasize the importance of membrane cholesterol levels and microdomain localization in regulating the voltage-sensitivity of Kv1.5. We believe this work provides new insight into the mechanisms of ion channel trafficking and membrane localization and emphasizes the importance of caveolin-mediated compartmentalization.

References

Abi-Char J, Maguy A, Coulombe A, Balse E, Ratajczak P, Samuel JL, Nattel S and Hatem SN (2007) Membrane cholesterol modulates Kv1.5 potassium channel distribution and function in rat cardiomyocytes. *J Physiol* **582:**1205–1217.

Anderson RG (1998) The caveolae membrane system. *Annu Rev Biochem* **67:**199–225.

Armstrong CM and Hille B (1998) Voltage-gated ion channels and electrical excitability. *Neuron* **20:**371–380.

Barbuti A, Gravante B, Riolfo M, Milanesi R, Terragni B, and DiFrancesco D (2004) Localization of pacemaker channels in lipid rafts regulates channel kinetics. *Circ Res* **94:**1325–1331.

Benson MD, Li QJ, Kieckhafer K, Dudek D, Whorton MR, Sunahara RK, Iniguez-Lluhi JA, and Martens JR (2007) SUMO modification regulates inactivation of the voltage-gated potassium channel Kv1.5. *Proc Natl Acad Sci U S A* **104:**1805–1810.

Bönnemann CG and Finkel RS (2002) Sarcolemmal proteins and the spectrum of limb-girdle muscular dystrophies. *Semin Pediatr Neurol* **9:**81–99.

Brazer SC, Singh BB, Liu X, Swaim W, and Ambudkar IS (2003) Caveolin-1 contributes to assembly of store-operated Ca^{2+} influx channels by regulating plasma membrane localization of TRPC1. *J Biol Chem* **278:**27208–27215.

Bush WS, Ihrke G, Robinson JM, and Kenworthy AK (2006) Antibody-specific detection of caveolin-1 in subapical compartments of MDCK cells. *Histochem Cell Biol* **126:**27–34.

Cagliani R, Bresolin N, Prelle A, Gallanti A, Fortunato F, Sironi M, Ciscato P, Fagiolari G, Bonato S, Galbiati S, et al. (2003) A CAV3 microdeletion differentially affects skeletal muscle and myocardium. *Neurology* **61:**1513–1519.

Cheng ZJ, Singh RD, Marks DL, and Pagano RE (2006) Membrane microdomains, caveolae, and caveolar endocytosis of sphingolipids. *Mol Membr Biol* **23:**101–110.

Cogolludo A, Moreno L, Lodi F, Frazziano G, Cobeno L, Tamargo J, and Perez-Vizcaino F (2006) Serotonin inhibits voltage-gated K^+ currents in pulmonary artery smooth muscle cells: role of 5-HT2A receptors, caveolin-1, and KV1.5 channel internalization. *Circ Res* **98:**931–938.

Davies A, Douglas L, Hendrich J, Wratten J, Tran Van Minh A, Foucault I, Koch D, Pratt WS, Saibil HR, and Dolphin AC (2006) The calcium channel alpha2delta-2 subunit partitions with CaV2.1 into lipid rafts in cerebellum: implications for localization and function. *J Neurosci* **26:**8748–8757.

Dubreuil RR, MacVicar G, Dissanayake S, Liu C, Homer D, and Hortsch M (1996) Neuroglian-mediated cell adhesion induces assembly of the membrane skeleton at cell contact sites. *J Cell Biol* **133:**647–655.

Eldstrom J, Van Wagoner DR, Moore ED and Fedida D (2006) Localization of Kv1.5 channels in rat and canine myocyte sarcolemma. *FEBS Lett* **580:**6039–6046.

Folco EJ, Liu GX, and Koren G (2004) Caveolin-3 and SAP97 form a scaffolding protein complex that regulates the voltage-gated potassium channel Kv1.5. *Am J Physiol Heart Circ Physiol* **287:**H681–H690.

Galbiati F, Razani B, and Lisanti MP (2001) Caveolae and caveolin-3 in muscular dystrophy. *Trends Mol Med* **7:**435–441.

Galbiati F, Volonte D, Minetti C, Bregman DB, and Lisanti MP (2000) Limb-girdle muscular dystrophy (LGMD-1C) mutants of caveolin-3 undergo ubiquitination and proteasomal degradation. Treatment with proteasomal inhibitors blocks the dominant negative effect of LGMD-1C mutants and rescues wild-type caveolin-3. *J Biol Chem* **275:**37702–37711.

Galbiati F, Volonte D, Minetti C, Chu JB, and Lisanti MP (1999) Phenotypic behavior of caveolin-3 mutations that cause autosomal dominant limb girdle muscular dystrophy (LGMD-1C). Retention of LGMD-1C caveolin-3 mutants within the Golgi complex. *J Biol Chem* **274:**25632–25641.

Harder T and Simons K (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr Opin Cell Biol* **9:**534–542.

Hayashi T, Arimura T, Ueda K, Shibata H, Hohda S, Takahashi M, Hori H, Koga Y, Oka N, Imaizumi T, et al. (2004) Identification and functional analysis of a caveolin-3 mutation associated with familial hypertrophic cardiomyopathy. *Biochem Biophys Res Commun* **313:**178–184.

Hnasko R and Lisanti MP (2003) The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol Interv* **3:**445–464.

Kwik J, Boyle S, Fooksman D, Margolis L, Sheetz MP, and Edidin M (2003) Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proc Natl Acad Sci U S A* **100:**13964–13969.

Maguy A, Hebert TE, and Nattel S (2006) Involvement of lipid rafts and caveolae in cardiac ion channel function. *Cardiovasc Res* **69:**798–807.

Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, and Tamkun MM (2000) Differential targeting of Shaker-like potassium channels to lipid rafts. *J Biol Chem* **275:**7443–7446.

Martens JR, O'Connell K, and Tamkun M (2004) Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts. *Trends Pharmacol Sci* **25:**16–21.

Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, and Tamkun MM (2001) Isoform-specific localization of voltage-gated K^+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. *J Biol Chem* **276:**8409–8414.

Mays DJ, Foose JM, Philipson LH, and Tamkun MM (1995) Localization of the Kv1.5 K^+ channel protein in explanted cardiac tissue. *J Clin Invest* **96:**282–292.

McEwen DP, Schumacher SM, Li Q, Benson MD, Iniguez-Lluhi JA, Van Genderen KM, and Martens JR (2007) Rab-GTPase-dependent endocytic recycling of KV1.5 in atrial myocytes. *J Biol Chem* **282:**29612–29620.

McNally EM, de Sa Moreira E, Duggan DJ, Bonnemann CG, Lisanti MP, Lidov HG, Vainzof M, Passos-Bueno MR, Hoffman EP, Zatz M, et al. (1998) Caveolin-3 in muscular dystrophy. *Hum Mol Genet* **7:**871–877.

Melkonian KA, Ostermeyer AG, Chen JZ, Roth MG, and Brown DA (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *J Biol Chem* **274:**3910–3917.

Michelakis ED and Weir EK (2001) The pathobiology of pulmonary hypertension. Smooth muscle cells and ion channels. *Clin Chest Med* **22:**419–432.

Moudgil R, Michelakis ED, and Archer SL (2006) The role of K^+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. *Microcirculation* **13:**615–632.

Murata M, Peranen J, Schreiner R, Wieland F, Kurzchalia TV, and Simons K (1995) VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci U S A* **92:**10339–10343.

O'Connell KM, Martens JR, and Tamkun MM (2004) Localization of ion channels to

lipid Raft domains within the cardiovascular system. *Trends Cardiovasc Med* **14:**37–42.

Okamoto T, Schlegel A, Scherer PE, and Lisanti MP (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem* **273:**5419–5422.

Olson TM, Alekseev AE, Liu XK, Park S, Zingman LV, Bienengraeber M, Sattiraju S, Ballew JD, Jahangir A, and Terzic A (2006) Kv1.5 channelopathy due to KCNA5 loss-of-function mutation causes human atrial fibrillation. *Hum Mol Genet* **15:**2185–2191.

Parton RG and Simons K (2007) The multiple faces of caveolae. *Nat Rev Mol Cell Biol* **8:**185–194.

Pottosin II, Valencia-Cruz G, Bonales-Alatorre E, Shabala SN, and Dobrovinskaya OR (2007) Methyl-beta-cyclodextrin reversibly alters the gating of lipid rafts-associated Kv1.3 channels in Jurkat T lymphocytes. *Pflugers Arch* **454:**235–244.

Remillard CV, Tigno DD, Platoshyn O, Burg ED, Brevnova EE, Conger D, Nicholson A, Rana BK, Channick RN, Rubin LJ, et al. (2007) Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. *Am J Physiol Cell Physiol* **292:**C1837–C1853.

Rolf S, Haverkamp W, Borggrefe M, Musshoff U, Eckardt L, Mergenthaler J, Snyders DJ, Pongs O, Speckmann EJ, Breithardt G, et al. (2000) Effects of antiarrhythmic drugs on cloned cardiac voltage-gated potassium channels expressed in *Xenopus* oocytes. *Naunyn Schmiedebergs Arch Pharmacol* **362:**22–31.

Roy S, Luetterforst R, Harding A, Apolloni A, Etheridge M, Stang E, Rolls B, Hancock JF, and Parton RG (1999) Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. *Nat Cell Biol* **1:**98–105.

Schlegel A, Volonte D, Engelman JA, Galbiati F, Mehta P, Zhang XL, Scherer PE, and Lisanti MP (1998) Crowded little caves: structure and function of caveolae. *Cell Signal* **10:**457–463.

Simons K and Ikonen E (1997) Functional rafts in cell membranes. *Nature* **387:**569–572.

Tanabe Y, Hatada K, Naito N, Aizawa Y, Chinushi M, and Nawa H (2006) Overexpression of Kv1.5 in rat cardiomyocytes extremely shortens the duration of the action potential and causes rapid excitation. *Biochem Biophys Res Commun* **345:**1116–1121.

Uittenbogaard A and Smart EJ (2000) Palmitoylation of caveolin-1 is required for cholesterol binding, chaperone complex formation, and rapid transport of cholesterol to caveolae. *J Biol Chem* **275:**25595–25599.

Van Wagoner DR, Pond AL, McCarthy PM, Trimmer JS, and Nerbonne JM (1997) Outward K$^+$ current densities and Kv1.5 expression are reduced in chronic human atrial fibrillation. *Circ Res* **80:**772–781.

Vorgerd M, Ricker K, Ziemssen F, Kress W, Goebel HH, Nix WA, Kubisch C, Schoser BG, and Mortier W (2001) A sporadic case of rippling muscle disease caused by a de novo caveolin-3 mutation. *Neurology* **57:**2273–2277.

Woodman SE, Sotgia F, Galbiati F, Minetti C, and Lisanti MP (2004) Caveolinopathies: mutations in caveolin-3 cause four distinct autosomal dominant muscle diseases. *Neurology* **62:**538–543.

Wyse BD, Prior IA, Qian H, Morrow IC, Nixon S, Muncke C, Kurzchalia TV, Thomas WG, Parton RG, and Hancock JF (2003) Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. *J Biol Chem* **278:**23738–23746.

Zhang L, Foster K, Li Q, and Martens JR (2007) S-acylation regulates Kv1.5 channel surface expression. *Am J Physiol Cell Physiol* **293:**C152–C161.

Zhang W, Trible RP, and Samelson LE (1998) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* **9:**239–246.

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