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CAVEOLIN REGULATES Kv1.5 TRAFFICKING TO CHOLESTEROL-RICH MEMBRANE MICRODOMAINS

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Running Title Page

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Abbreviations used: CFP, Cyan Fluorescent Protein; FRT, Fisher Rat Thyroid cells; Kv, voltage dependent potassium channel.

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 caveolin-3, the association of Kv1.5 with low-density, detergent-resistant membrane fractions requires co-expression 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. Co-expression with wild type caveolin-1 does not alter Kv1.5 current density, but rather 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) while 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 (Tanabe et al., 2006; Van Wagoner et al., 1997) 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 non-excitable cells, localize a number of membrane proteins, including multiple signal transduction molecules, while excluding others (Harder and

Simons, 1997). It is likely that these rafts serve as scaffolding regions where 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 (Maguy et al., 2006; Martens et al., 2004; O'Connell et al., 2004). 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 (Melkonian et al., 1999; Zhang et al., 1998). Much less is known 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 caveolin-3 in trafficking polytopic proteins to the plasma membrane (Brazer et al., 2003; Wyse et al., 2003). Further, they have both been shown to bind and even functionally regulate proteins in lipid-rich microdomains (Anderson, 1998; Okamoto et al., 1998). Previously, we reported that Kv1.5 can localize to caveolar microdomains (Martens et al., 2000; Martens et al., 2001). Others have described this interaction in both heart and vascular smooth muscle (Abi-Char et al., 2007: Cogolludo et al., 2006). 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 91-93 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) 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., 2007a).

Lipid Raft Isolation - Isolation of low density, Triton X-100 insoluble complexes was performed as described (Martens et al., 2000) with minor modifications. Briefly, cells or tissue were homogenized in 1% Triton X-100 and sucrose added to a final concentration of 40%. A 5-30% discontinuous sucrose gradient was layered on top of this detergent extract followed by ultracentrifugation (54,000 rpm) for 18-24 h at 4°C. Gradient fractions were analyzed by SDS-PAGE gel electrophoresis on a NuPAGE® Novex 4-12% 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, San Francisco, CA) and visualized using the Western Lightning enhanced chemiluminescent reagent according to manufacturer's protocol (Perkin Elmer Life Sciences, Wellesley, 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 CFPtagged wild type and mutant caveolin constructs were fixed and permeabilized before incubation with anti-Kv1.5 antibodies (1:1000; (Martens et al., 2001; Mays et al., 1995)). 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 a Zeiss Axiovert 200 M microscope 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 previously described (Benson et al., 2007; Zhang et al., 2007b). Briefly, cells were plated in 35-mm plates and transiently transfected with the indicated cDNAs. Patch pipettes (1-2 M Ω) were pulled from borosilicate glass capillaries. The intracellular pipette solution contained (in mM): KCl 110, HEPES 10, K₂BAPTA 5, K₂ATP 5, and MgCl₂ 1; it was adjusted to pH 7.2 with KOH. The bath solution contained (in mM): NaCl 110, KCl 4, MgCl₂ 1, CaCl₂ 1.8, HEPES 10, and glucose 1.8; 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-

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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 mV 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 ± standard error of the mean. 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). FRT cells were washed 2x with potassium-free PBS, scraped, and collected at 1,000 x g 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% NaN₃, supplemented with protease inhibitors) for 10 min at room temperature. The solubilized membranes were centrifuged in a microcentrifuge at 10,000 x g for 20 min at 4 °C. The supernatant was collected and pre-cleared 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 hr 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

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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% NaN₃). After the second wash the beads were removed by centrifugation at 10,000 x g at 4 °C and resuspended in 50 μ L 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. Cells were homogenized and lysed in reaction buffer containing 5mM sodium cholate and 0.1% Triton X-100 in PBS at pH 7.4 for 30 minutes on ice and then sonicated. Cholesterol was then measured according to manufacturer's protocol. Fluorescence was measured using a Perkin-Elmer Victor² microplate reader with an excitation wavelength of 545nm and emission wavelength of 590nm.

Results

Caveolin is necessary for the association of Kv1.5 with lipid rafts in transfected *FRT cells* – The mechanism responsible for the targeting of Kv1.5 to lipid raft microdomains is unknown. To examine the role of caveolin in this specialized localization, we utilized a caveolin-null cell line, Fisher Rat Thyroid (FRT) cells. In FRT cells, which express no endogenous caveolin-1 or -3, Kv1.5 is found in the high-density, detergent-soluble fractions, similar to the non-raft associated transferrin receptor (Figure 1A). Co-expression of caveolin-1 with Kv1.5, however, resulted in the shift of Kv1.5 to the lower density, detergent-resistant, lipid raft fractions (Figure 1B, upper panel) where it co-migrated with caveolin (Figure 1B, lower panel). Under these conditions, a population of Kv1.5 remained in the detergent-soluble fractions most likely due to 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 non-raft fractions (Figure 1B, middle panel).

Next, we tested if Kv1.5 interacts in complex with caveolin proteins. FRT cells transfected with Kv1.5 either stably and caveolin-1 caveolin-3. were or Immunoprecipitation with an anti-caveolin antibody shows that both caveolin-3 and caveolin-1 are in complex with Kv1.5, from which both the glycosylated and nonglycosylated forms of the channel were detected (Figure 1C). These data were confirmed with the reverse immunoprecipitation, where an anti-Kv1.5 antibody pulled down caveolin from transfected FRT cells (Figure 1D, third panel). All blots were stripped and re-probed with the respective immunoprecipitating antibody to ensure efficient pull-down (Figure 1C and D). Immunoprecipitation with non-immune serum

revealed no significant signal (Figure 1C and D). Together, these results indicate that Kv1.5 can associate in complex with multiple isoforms of caveolin.

Disruption of caveolin trafficking sequesters Kv1.5 intracellularly – We next tested if 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 (Figure 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 co-expressed CFP-tagged caveolin-1 with Kv1.5, the channel trafficked to the plasma membrane, where the two proteins co-localized at the cell periphery (Figure 2, D-F). A three amino acid deletion in the scaffolding domain of caveolin-3 (Δ TFT) 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., 2001; Galbiati et al., 2000; Galbiati et al., 1999). To establish whether caveolin was indeed involved in trafficking Kv1.5 to the plasma membrane, we expressed a CFP-caveolin-1 Δ TFT-mutant together with Kv1.5 in FRT cells. Co-expression of Kv1.5 with the caveolin-1 Δ TFT-mutant resulted in the intracellular accumulation of Kv1.5 with a concomitant loss in peripheral, cell-surface staining (Figure 2, G-I).

Previously, we showed that potassium channel localization to lipid raft

microdomains is isoform specific, where Kv1.5 localizes to caveolae while Kv2.1 is found in non-caveolar lipid rafts (Martens et al., 2001). Therefore, co-expression of Kv2.1 with the caveolin-1 Δ TFT-mutant should not disrupt its trafficking to the plasma membrane. Indeed, as seen in Figure 2H, Kv2.1 maintains the ability to traffic to the plasma membrane in the presence of the caveolin-1 Δ TFT-mutant, as indicated by the peripheral staining of channel similar to Figure 2B (Figure 2, J-L). Next, we tested if 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 Δ TFT-mutant, resulting in a significant intracellular accumulation (Figure 2, M-O). Therefore, the amino-terminus of Kv1.5 is critical for caveolin-mediated trafficking of Kv1.5 to the plasma membrane.

Co-expression of Kv1.5 with the caveolin Δ TFT-mutant abolishes potassium channel currents in FRT cells, which are not recovered with the addition of exogenous cholesterol – Co-expression of Kv1.5 with the caveolin-1 Δ TFT-mutant resulted in mislocalization of the channel away from the cell periphery to intracellular compartments. To directly measure cell surface levels, we used two complimentary approaches including electrophysiological measurements and live cell labeling of channels on the plasma membrane. To determine if 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 Δ TFT-mutant were examined by whole-cell voltage clamp. As shown in Figure 3A, current traces for Kv1.5 + caveolin-1 are similar to those for channel alone, while Kv1.5 current was markedly reduced in the presence of the caveolin-1 Δ TFT-mutant. This decrease in current is statistically significant across a

wide range of voltages (Figure 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 previously described (McEwen et al., 2007). Kv1.5 trafficked efficiently to the cell surface in the presence or absence of caveolin-1 (Figure 3C). In contrast, co-expression of Kv1.5 with the mutant caveolin-1 resulted in the abolishment of cell surface channel (Figure 3C), despite efficient expression (Figure 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 if the addition of exogenous cholesterol could overcome the effects of the caveolin-1 Δ TFT-mutant. Despite efficient incorporation of cholesterol in the plasma membrane (Figure 4B), addition of exogenous cholesterol was not sufficient to recover cell surface potassium current (Figure 4A, compare triangles with open circles), indicating that the caveolin-1 Δ TFT-mutant is directly inhibiting trafficking of Kv1.5 to the plasma membrane compared to wild type caveolin (Figure 4B).

Caveolin and cholesterol cause a depolarizing shift in the steady state activation and inactivation properties of Kv1.5 – In Figure 3A, we showed that co-expression 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 (Figure 5A; Kv1.5 = -12.80 ± 1.19, Kv1.5 + caveolin-1 = -5.40 ± 1.48) and the V_{1/2} of inactivation (Figure 5B; Kv1.5 = -12.04 ±

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 $1.24, -3.59 \pm 0.94$, Kv1.5 + caveolin-1 = -3.59 \pm 0.94) in the depolarizing direction by approximately 10 mV. Results from Figure 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. То further investigate this possibility, we treated FRT cells expressing Kv1.5 alone with exogenous cholesterol. As seen in Figure 6A, treatment with exogenous cholesterol resulted in an increase in the whole-cell cholesterol:protein ratio compared to untreated cells, indicating that exogenous cholesterol incorporates into the plasma membrane. Interestingly, treatment with cholesterol resulted in a similar 10-20 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 to untreated FRT cells (Figure 6B and 6C, respectively). These effects are analogous to results obtained with co-expression 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., 2001; Galbiati et al., 2000; Galbiati et al., 1999), sequester Kv1.5 intracellularly. This mistargeting completely suppresses Kv1.5-mediated current in a cholesterol-independent manner. Further, 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 TRPC1 (Brazer et al., 2003; Wyse et al., 2003). Recently, work in atrial myocytes, using immunohistochemical methods, suggests that Kv1.5 and caveolin-3 do not associate at the plasma membrane (Abi-Char et al., 2007; Eldstrom et al., 2006; Maguy et al., 2006). 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 caveolin-3 (Cogolludo et al., 2006; Folco et al., 2004) in cardiac and vascular myocytes. These differences may result from the mode of detection. It is now clear that immunohistochemical labeling of non-denatured protein can detect different subpopulations of caveolin within a single cell and is dependant 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 co-localization of Kv1.5 with caveolin in cardiac cells. Interestinaly, in pulmonary arterial smooth muscle cells, caveolin-1 was only weakly associated with Kv1.5 under basal conditions, and only following 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 rather is essential for its localization to raft microdomains, which functionally regulate steady-state channel properties. This may, in part, explain 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 compartments and prevents functional channel expression on the cell surface. This interaction occurs through the NH₂ -terminus of Kv1.5, since replacement of the NH₂-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 (Figure 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 NH₂-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 (Galbiati et al., 1999; McNally et al., 1998; Vorgerd et al., 2001; Woodman et al., 2004). Further, this mutation in caveolin-3 has been shown to be involved in limb-girdle muscular dystrophy (Galbiati et al., 2001; Galbiati et al., 2000; Galbiati et al., 1999). While 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 (Bonnemann and Finkel, 2002). Evidence, however, does suggest 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). Interestingly, 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 when expressed together, Kv1.5 and caveolin 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, non-raft domains, where its steady-

state activation and inactivation properties are different than in the presence of caveolin, where the channel localizes to lipid rafts (Figs. 1 & 5). Previous work, including our own (Martens et al., 2004; Martens et al., 2001), 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 (Barbuti et al., 2004; Davies et al., 2006; Hnasko and Lisanti, 2003; 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 to 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; Maguy et al., 2006; Martens et al., 2004; 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 to the mechanisms of ion channel trafficking and membrane localization and emphasizes the importance of caveolin-mediated compartmentalization.

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Footnotes

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Figure Legends

Kv1.5 requires caveolin for localization in lipid raft microdomains. Cell Fig. 1. 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 Western blots, probed with anti-Kv1.5 (top panel), described in the "Methods". demonstrate that the channel is not present in detergent-insoluble, low-density lipid raft fractions. Western blots probed with anti-transferrin (bottom panel) 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 in the "Methods". Western blots probed with anti-Kv1.5 (top panel) demonstrate that the channel is present in detergent-insoluble, low-density lipid raft fractions upon association with caveolin (bottom panel). Western blots probed with anti-transferrin (middle panel) 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 non-immune antibody (C and D) was used as a negative control (C and D). IP = immunoprecipitate, L = FRT cell lysate.

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

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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- Δ TFT. 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 (panel N) show normal cell surface distribution that is altered with the addition of the mutant form of caveolin-CFP fluorescence is green while Kv1.5 staining is red. Co-localization of caveolin-1 with Kv1.5 in merged images is indicated by yellow color.

Fig. 3. Co-expression 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 (filled circles, n=20), Kv1.5 + caveolin-1 (open circles, n=19), or Kv1.5 + dominant negative caveolin-1 (closed triangles, n=10). Currents normalized to cell capacitance. were (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).

Fig. 4. Supplementing with exogenous cholesterol does not restore Kv1.5 currents in the presence of the caveolin- Δ TFT mutant. (A) Current density traces of whole-cell patch clamp electrophysiological recordings from FRT cells stably expressing Kv1.5 + wild-type caveolin-1 (closed circles, n=19), Kv1.5 + caveolin-1-∆TFT mutant (closed triangles, n=10), or Kv1.5 + caveolin-1- Δ TFT mutant with cholesterol enrichment (open Cholesterol supplementation does not restore Kv1.5 currents. circles. n=6). (B) Immunocytochemistry of FRT cells expressing Kv1.5 + wild-type caveolin-1 (top), Kv1.5 + caveolin-1- Δ TFT mutant (middle), or Kv1.5 + caveolin-1- Δ TFT 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), while coexpression with the caveolin- Δ TFT mutant sequesters Kv1.5 in a sub-cellular perinuclear compartment (middle). Addition of exogenous cholesterol does not restore Kv1.5 trafficking to the plasma membrane in the presence of the caveolin- Δ TFT mutant (bottom).

<u>Fig. 5.</u> Co-expression 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 (closed circles, n=14) or Kv1.5 + caveolin-1 (open circles, 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).

<u>Fig. 6.</u> 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 (closed circles, n=13) or supplemented with cholesterol (open circles, 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).

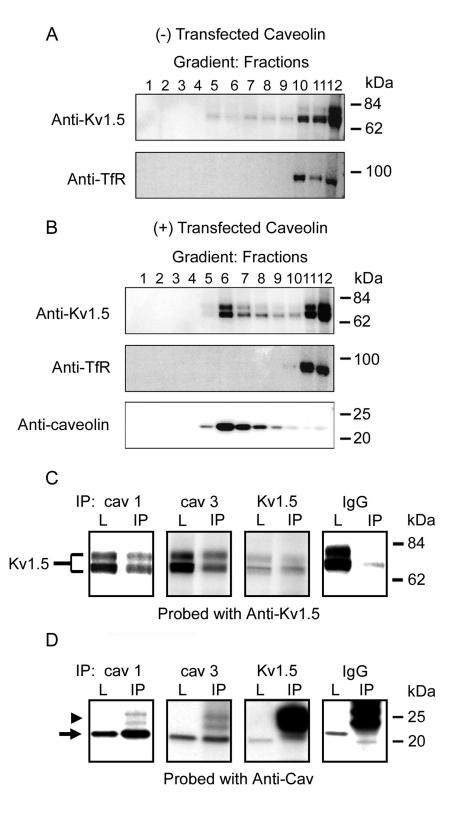


Figure 1

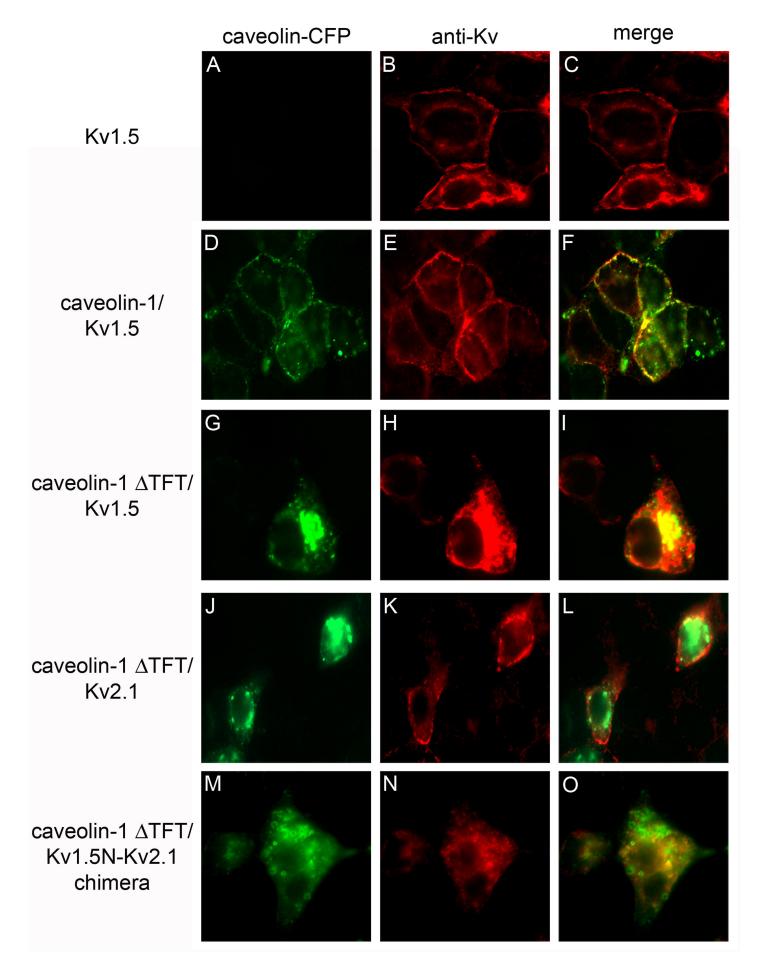


Figure 2

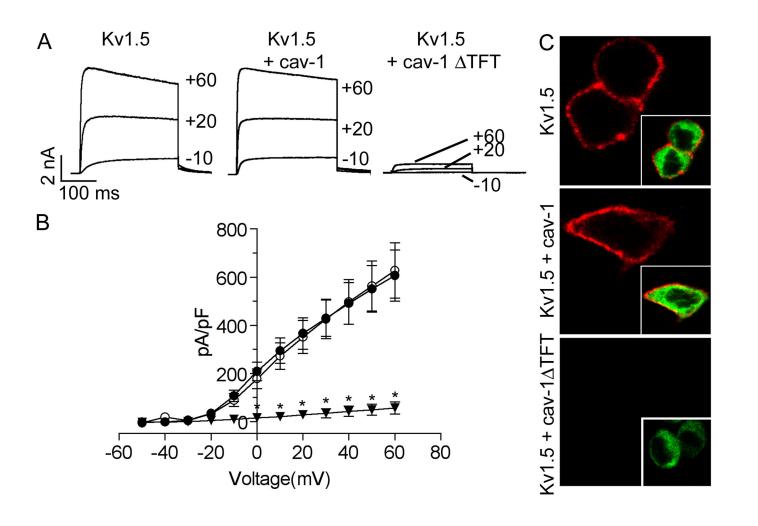


Figure 3

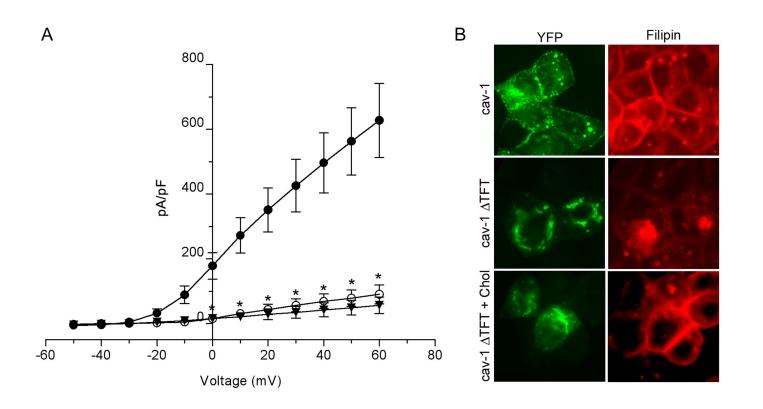


Figure 4

