The number of ion channels expressed on the cell surface shapes the complex electrical response of excitable cells. Maintaining a balance between anterograde and retrograde trafficking of channel proteins is vital in regulating steady-state cell surface expression. Kv1.5 is an important voltage-gated K⁺ channel in the cardiovascular system underlying the ultra-rapid rectifying potassium current (I_{Kur}), a major repolarizing current in atrial myocytes, and regulating the resting membrane potential and excitability of smooth muscle cells. Defects in the expression of Kv1.5 are associated with pathological states such as chronic atrial fibrillation and hypoxic pulmonary hypertension. There is, thus, substantial interest in understanding the mechanisms regulating cell surface channel levels. Here, we investigated the internalization and recycling of Kv1.5 in the HL-1 immortalized mouse atrial myocytes. Kinetic studies indicate that Kv1.5 is rapidly internalized to a perinuclear region where it co-localizes with the early endosomal marker, EEA1. Importantly, we identified that a population of Kv1.5, originating on the cell surface, internalized and recycled back to the plasma membrane. Notably, Kv1.5 recycling processes are driven by specific Rab-dependent endosomal compartments. Thus, co-expression of GDP-locked Rab4S22N and Rab11S25N dominant-negative mutants decreased the steady-state Kv1.5 surface levels, whereas GTPase-deficient Rab4Q67L and Rab11Q70L mutants increased steady-state Kv1.5 surface levels. These data reveal an unexpected dynamic trafficking of Kv1.5 at the myocyte plasma membrane and demonstrate a role for recycling in the maintenance of steady-state ion channel surface levels.

The complex electrical response of excitable cells in the cardiovascular system is dependent on the expression and regulation of ion channels (1–3). Voltage-dependent K⁺ (Kv)² channels, which open and close in response to a change in membrane voltage, are essential for the control of resting membrane potential and the shaping of action potentials (4). In the human atrium, Kv1.5 underlies the ultra-rapid rectifying current (I_{Kur}) and is responsible for myocyte repolarization (5, 6), whereas in pulmonary arterial smooth muscle cells, Kv1.5 is critical for the oxygen sensitive regulation of arterial tone (7–9).

Mounting evidence indicates that steady-state expression levels of Kv1.5 in cardiovascular tissues modulate cellular excitability. Overexpression of Kv1.5 in rat cardiomyocytes dramatically shortens action potential duration producing a phenotype similar to that observed in short QT syndrome (10). Conversely, a reduction in Kv1.5 current has been implicated in the pathophysiology of cardiovascular disease. In patients with persistent or paroxysmal atrial fibrillation, reduced outward potassium current is observed (11, 12). This reduction in current is due, in part, to a diminished Kv1.5 protein expression, whereas other potassium channels, such as Kv2.1, remain unaffected (11). Interestingly, Kv1.5 mRNA levels are unchanged (12), indicating that alterations in steady-state protein levels may be responsible for the aberrant phenotype.

The balance between the anterograde and retrograde trafficking pathways determines steady-state cell surface expression of proteins. Endocytosis is the initial step in retrograde movement after which internalized proteins can follow multiple routes to different intracellular fates (13). One well-recognized outcome is the targeting of internalized proteins to lysosomes followed by degradation. Alternatively, trafficking through recycling endosomes allows proteins to return to the plasma membrane and protects them from degradation (14). Sorting at early endosomes to Rab-GTPase-specific compartments is now established as an important event determining the intracellular fate of internalized protein (15–17). Individual Rab proteins appear to have unique functional specializations (13). Rab5, for example, is involved in clathrin-mediated endocytosis (18, 19), Rab4 and Rab11 have been implicated in protein recycling (20–23), and Rab8 is proposed to regulate anterograde trafficking (13).

Although progress has been made with regard to a subset of ion channels in identifying mechanisms that regulate post-translational trafficking, surprisingly little is known about the mechanisms that regulate how Kv channels traffic into and out of the plasma membrane. Several studies have demonstrated that Kv channels undergo regulated endocytosis, however, the intracellular fate of these internalized channels is largely unknown (24–26). Recent results demonstrate that, in atrial...
myocytes, Kv1.5 internalizes via a microtubule-dependent pathway (25). However, the downstream mechanisms of Kv1.5 sorting and trafficking are unresolved. The present study was therefore designed to investigate the intracellular fate of Kv1.5 following internalization in HL-1 atrial myocytes and the mechanisms regulating this process. Specifically, we tested the hypothesis that internalized Kv1.5 recycles back to the plasma membrane in a Rab-GTPase-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—Kv1.5-GFP and Kv1.5-mCherry were generated by inserting a green fluorescent protein (GFP) or mCherry tag into the extracellular loop between the first and second transmembrane segments, respectively. All Rab constructs were a generous gift of Dr. José Esteban (University of Michigan, Ann Arbor, MI). Polyclonal anti-GFP, monoclonal anti-V5, and AlexaFluor secondary antibodies were purchased from Invitrogen. Polyclonal anti-EEA1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Kv1.5 antibody was purchased from Chemicon (Temecula, CA). Polyclonal anti-DsRed antibody was purchased from BD Biosciences Clontech. Horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Biotin-conjugated goat anti-rabbit secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cy5-Streptavidin secondary antibody was purchased from GE Healthcare. HL-1 cells were a generous gift from Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA).

**Western Blot**—HL-1 cells were harvested in 50 mM Tris-Cl, 10 mM EDTA, pH 8.0, containing complete protease inhibitors (Roche Applied Science). Membranes were isolated and separated by SDS-PAGE on a NuPAGE® Novex 4–12% bis-tris gel (Invitrogen). 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), and visualized using the Western lightning enhanced chemiluminescent reagent according to the manufacturer’s protocol (PerkinElmer Life Sciences). Images were captured using the EpiChemi3 darkroom (UVP, Inc., Upland, CA).

**Immunoprecipitation**—HL-1 cells were transiently transfected with Kv1.5 and one of GFP, Rab4-GFP, or Rab11-GFP. 48-h post-transfection, cells were harvested and solubilized in dilution buffer (50 mM Tris-Cl, 150 mM NaCl, 10 mM EDTA, pH 8.0, 1% Triton X-100) containing protease inhibitors. Rab proteins were activated using 100 μM GppNHP and 30 mM MgCl₂ and immunoprecipitated with anti-GFP antibodies conjugated to protein A-Sepharose beads overnight at 4°C. Following immunoprecipititation, the antibody-antigen complex was removed by centrifugation at 1000 × g for 60 s. The bead complex was washed three times with wash buffer (50 mM Tris-Cl, 150 mM NaCl, 10 mM EDTA, pH 8.0, 0.02% SDS) containing 0.1% Triton X-100 followed by a single wash with wash buffer containing no Triton X-100. Beads were then resuspended in 50-μl SDS sample buffer and separated on a NuPAGE® Novex 4–12% bis-tris gel (Invitrogen). Western blotting was performed as described above.

**Immunocytochemistry**—For surface labeling, live cell staining was performed on ice to decrease the rate of internalization. Cells were washed twice with ice-cold PBS, incubated with a polyclonal anti-GFP antibody (1:500) in 2% goat serum for 30 min; washed three times with PBS, incubated with goat anti-rabbit AlexaFluor 594 secondary antibody (1:500) in 2% goat serum for 30 min; and washed with PBS, fixed with 4% paraformaldehyde and mounted with ProLong Gold anti-fade reagent (Invitrogen). For permeabilized staining, cells were fixed, permeabilized with 0.1% Triton X-100 in 2% goat serum, and stained as described above. All images were collected on an Olympus Fluoview 500 confocal microscope. For studies with Rab proteins, Z-stacks were compressed, and total fluorescence was calculated for total Kv1.5 (GFP), surface Kv1.5 (Cy5), and Rab proteins (RF) using NIH ImageJ software. Background fluorescence was determined by measuring the fluorescent signal in neighboring untransfected HL-1 cells for all fluorescent channels tested (GFP, Cy5, and RF). To determine specific fluorescence, the background signal was subtracted from the total fluorescent signal. For quantitation, surface Kv1.5 fluorescent signal was normalized to total Kv1.5-GFP fluorescence in each cell.

**Internalization Assay**—48-h post-transfection, HL-1 cells transiently expressing Kv1.5-GFP were live cells stained with an anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, the cells were returned to 37°C for the indicated times. At this point, the cells were removed, and any remaining surface-labeled channels were saturated with goat anti-rabbit AlexaFluor 594 secondary antibody (1:250; Invitrogen). Cells were then fixed with 4% paraformaldehyde, permeabilized in 2% goat serum, 0.1% Triton X-100 in PBS, and internalized labeled channel was detected by first incubating with biotin-conjugated goat anti-rabbit secondary antibody (1:500) followed by incubation with Cy5-conjugated streptavidin antibody (1:500). For co-localization with EEA1, cells were incubated with EEA1 antiserum (1:500) followed by incubation with donkey anti-goat AlexaFluor 594 (1:500). Cover slips were mounted using ProLong Gold (Invitrogen). Images were collected on an Olympus Fluoview 500, and fluorescence was quantitated as described above.

**Recycling Assay**—48-h post-transfection, HL-1 cells transiently expressing Kv1.5-GFP were live cells stained with an anti-GFP antibody (1:500) for 30 min on ice. Following this incubation, the cells were returned to 37°C for 30 min, removed, and stained with goat anti-rabbit AlexaFluor 594 secondary antibody (1:250) for 30 min on ice. Cells were then returned to 37°C for the indicated times, removed, and incubated with biotin-conjugated goat anti-rabbit secondary antibody (1:500) for 30 min on ice followed by incubation with Cy5-conjugated streptavidin antibody (1:500) for 30 min on ice. Cells were then fixed, washed, and mounted with ProLong Gold. Images were collected on an Olympus FluoView 500 and fluorescence was quantitated as described above.

**Electrophysiology**—Whole-cell voltage clamp experiments were performed as described previously (27).
Results

Detection of Cell Surface Kv1.5 in HL-1 Atrial Myocytes—Cell surface labeling of Kv channels with antibodies has proven difficult. Kv channels are polytopic membrane proteins with short extracellular loops of limited antigenicity. To study Kv1.5 trafficking, we developed an extracellular-tagged channel in which GFP was inserted in the first extracellular loop between transmembrane segments S1 and S2 (Kv1.5-GFP). Importantly, the presence of the GFP tag did not affect the electrophysiologic properties of the channel (Fig. 1A and Table 1). Whole-cell patch clamp experiments revealed that Kv1.5-GFP was capable of generating nanoampere currents of which the steady-state biophysical properties were nearly identical to untagged channel. Furthermore, similar to untagged channel, Kv1.5-GFP was post-translationally modified by glycosylation (Fig. 1B). Therefore, Kv1.5-GFP is a valid and useful tool to study channel trafficking. For our studies, we used the immortalized HL-1 cardiomyocyte cell line, which maintains spontaneous contractility as well as a differentiated cardiac phenotype (28). We find that these cells express low levels of endogenous Kv1.5 channel protein (supplemental Fig. 1A). Heterologous expression of Kv1.5-GFP results in a small increase in channel expression as determined by Western blot. Fluorescence imaging of HL-1 cells transiently transfected with Kv1.5-GFP revealed a punctate distribution (Fig. 1C, left panel). Live cell staining with an anti-GFP antibody, however, detected only surface populations of Kv1.5 (Fig. 1C, middle panel). Antibody staining after fixation and permeabilization detected the total channel population, as indicated by complete co-localization.

Table 1

GFP tag does not modulate Kv1.5 currents in HL-1 cells

HL-1 cells expressing wild-type Kv1.5 or Kv1.5-GFP were examined by whole cell voltage clamp. The voltage dependence of activation was determined using 250-ms voltage-clamp pulses applied in 10-mV steps from −80 mV to +60 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 −60 mV to +40 mV followed by a 500-ms test pulse at +30 mV. Normalized activation and inactivation curves were fitted to the Boltzmann equation, and results are expressed as means ± S.E., where V_1/2 is the midpoint of the curve, and t is the time constant of activation or inactivation, respectively.

| Channel          | Voltage-dependence of activation | Steady-state inactivation |
|------------------|----------------------------------|--------------------------|
|                  | V_1/2 (mV) t_1 (ms) t_2 (ms)     | V_1/2 (mV) t_1 (ms) t_2 (ms) |
| Kv1.5            | −17.71 ± 2.01 5.00 ± 0.80 1.07 ± 0.19 | −20.50 ± 2.32 2391.4 ± 466 179.5 ± 91.1 |
| Kv1.5-GFP        | −23.27 ± 2.18 5.11 ± 2.03 1.24 ± 0.23 | −25.68 ± 2.76 2045.5 ± 153 77.8 ± 77.4 |

Figure 1. Detection of Kv1.5-GFP in HL-1 atrial myocytes. A, electrophysiological recordings from HL-1 cells transiently expressing either untagged Kv1.5 (top traces) or Kv1.5-GFP (bottom traces). Cells were maintained at −80 mV followed by 250 ms pulses applied in 10-mV steps from −80 mV to +60 mV. B, Western blot analysis of untagged Kv1.5 (lanes 1 and 2) or Kv1.5-GFP (lanes 3 and 4). Arrows indicate non-glycosylated Kv1.5, and arrowheads indicate glycosylated Kv1.5 or Kv1.5-GFP, respectively. Treatment with N-glycosidase F resulted in presence of only the non-glycosylated channel (lanes 2 and 4). C, HL-1 cells transiently transfected with Kv1.5-GFP (green, top left panel) were live cells stained with anti-GFP to detect surface channel populations (red, top middle panel). The merged image shows selective surface staining (top right panel). A single confocal image is shown. D, HL-1 cells transiently transfected with Kv1.2-GFP (green, left panel) were fixed and permeabilized prior to staining with anti-GFP antibodies (red, middle panel). The merged image shows complete co-localization of anti-GFP with Kv1.5-GFP (yellow, right panel). A single confocal image is shown. Scale bars, 10 μm.

Figure 2. Involvement of dynein motors in Kv1.5 internalization. HL-1 cells co-expressing Kv1.5 with p50-dynamitin (n = 36) resulted in a 2.5-fold increase in Kv1.5 surface levels compared with Kv1.5 alone (n = 31). Representative images are shown below each bar in the histogram. Data are expressed as mean ± S.E. from three separate experiments. Fluorescence was quantitated as described under “Experimental Procedures.” **, p < 0.002; scale bars, 10 μm.
Kv1.5 Internalization to Early Endosomes—A tight balance between retrograde and anterograde trafficking determines channel surface density. An important initial step in the retrograde movement of channel proteins involves regulated endocytosis, which helps limit surface protein levels. One documented strategy to interfere with endocytic trafficking is the targeted disruption of the dynein motor system (25, 29–31). Overexpression of p50/dynamitin, a component of the dynactin complex, uncouples the motor from its cargo and impedes retrograde trafficking. We used this approach to study the contribution of endocytosis and endosomal movement to steady-state Kv1.5 surface levels in HL-1 myocytes. As shown in Fig. 2, overexpression of p50/dynamitin in HL-1 cells increased cell surface expression levels of Kv1.5. These data are consistent with a recent report describing dynemin-dependent surface expression of Kv1.5 in HEK cells (25).

Although this approach reveals a role of internalization in setting the steady-state levels of Kv1.5, it does not provide information on the dynamics of channel internalization.

To directly measure Kv1.5 internalization, surface channels were initially tagged at low temperature with a primary antibody directed against the extracellular GFP epitope tag and returned to 37 °C (Fig. 3A). After allowing for internalization for the indicated times the channels remaining at the plasma membrane were labeled with first AlexaFluor secondary antibody, whereas the fraction that was internalized was detected with the second Cy5-labeled secondary antibody. Fig. 3B shows that

with GFP fluorescence (Fig. 1D, middle and right panels). Probing for the accessibility of the GFP epitope, therefore, permits the discrimination between surface and internal channels.

FIGURE 3. Time course of Kv1.5 internalization. A, schematic illustrating protocol used for measurement of Kv1.5 internalization. B, HL-1 cells expressing Kv1.5-GFP (green, top left panel) were stained with anti-GFP and were kept on ice instead of allowing for internalization (t = 0 min). The surface channel population was detected by AlexaFluor 594 (red, top middle panel), whereas internalized channel was detected by Cy5 (gray, top right panel). HL-1 cells expressing Kv1.5-GFP (green, lower left panel) were stained with anti-GFP prior to a 30-min incubation at 37 °C allowing for internalization. Cells were placed back on ice, and the remaining surface Kv1.5 population was detected using an AlexaFluor 594 secondary antibody (red, lower middle panel). Internalized Kv1.5 was detected by Cy5 staining (gray, lower right panel). C, HL-1 cells expressing Kv1.5-GFP were stained with anti-GFP detecting surface channel. Following incubation at 37 °C for the indicated time points, secondary antibody staining detected internalized protein. Fluorescence was quantitated as described under “Experimental Procedures.” Average data for internalized Kv1.5 were fit to a single exponential. The half-time of internalization is 11.2 min. Representative images are shown in the lower panels for the indicated times. D, internalized Kv1.5-GFP (green, left panel) co-localized with the early endosomal marker EEA1 (red, middle panel) as indicated in the merged image (yellow, right panel). Closer examination of the boxed region shows co-localization of these two proteins (inset, right panel). Scale bars, 10 μm.
at 0 min, all surface sites have been saturated and no internalized channel is detectable (Fig. 3B, lower left panel). Detection with the second secondary antibody indicates that this population originated on the cell surface and a significant fraction internalized to perinuclear regions (Fig. 3B, lower right panel). To measure the time course of internalization, Cy5-labeled internalized channel was measured at various times. At 5 min, only a minimal amount of internalized channel is detectable (Fig. 3C), whereas at 10 and 30 min, internalized channel is detected, and relocation of the channel from the cell surface to discrete intracellular puncta is clearly identifiable (Fig. 3C). Quantitative analysis of Cy5 fluorescence revealed that Kv1.5 internalized with a \( t_{1/2} \) of \( \sim 11 \) min (Fig. 3C). During Kv1.5 internalization, there is a concomitant decrease in channel surface population (supplemental Fig. 2). The puncta observed during the internalization assay are indicative of perinuclear endosomal compartments, including early endosomes (32). Interestingly, staining for EEA1 reveals that a significant portion of internalized Kv1.5 localizes to early endosomes (Fig. 3D, right panel).

Kv1.5 Recycles to the Plasma Membrane—Localization of internalized Kv1.5 channel to early endosomes suggests that the channel is either targeted for degradation or returned to the plasma membrane. To determine the intracellular fate of internalized channel, we investigated the possibility of Kv1.5 recycling. Surface populations were first tagged with an anti-GFP antibody at 4 °C (Fig. 4A). Cells were warmed to 37 °C for 30 min to allow for maximal internalization, as determined by the time course in Fig. 3B. Any remaining cell surface channels were then labeled at 4 °C with saturating concentrations of AlexaFluor 594-conjugated secondary antibody. At \( t = 0 \) min, cells were returned to 37 °C for increasing intervals to allow for recycling back to the plasma membrane. The previously tagged and internalized Kv1.5 channels, which returned to the surface, were then labeled with a biotin-conjugated anti-rabbit secondary antibody and detected with Cy5-conjugated streptavidin (Fig. 4A). The internalized channel population, originally labeled on the cell surface is therefore protected against detection with the AlexaFluor 594 secondary antibody, whereas Kv1.5 that has returned to the plasma membrane, but not newly synthesized channel, is detected with Cy5-conjugated streptavidin. As seen in Fig. 4B, at \( t = 0 \) min, no recycled channel is detectable (upper right panel). At 120 min, however, a significant amount of Cy5-streptavidin is observed, indicating recycled Kv1.5 channel (Fig. 4B, lower right panel). At 10, 30, and 60 min (Fig. 4C, lower panels), increasing levels of...
recycled channel are observed. An exponential fit of the time course data revealed that Kv1.5 returned to the plasma membrane with a half-time of ~29 min (Fig. 4C). These data show for the first time that a population of Kv1.5 originating on the cell surface recycled back to the plasma membrane.

Recycling of Kv1.5 Involves Rab4 and Rab11—Intracellular trafficking of membrane proteins is mediated by the Rab family of small GTPases (33, 34), which are thought to coordinate protein movement into and out of the plasma membrane. Rab-mediated retrograde trafficking involves orchestrated passage through specific compartments defined as early, late, and sorting or recycling endosomes (14). Previous studies have indicated a role for both Rab4 and Rab11 in protein recycling from early and recycling endosomes to the plasma membrane (18, 19, 23). Mutation of a single serine residue to asparagine in the nucleotide binding site of Rab proteins lowers their affinity for GTP and results in a GDP-locked, dominant negative protein, specific for each respective Rab isoform (35–38).

To test for a potential role of Rab proteins in the regulation of Kv1.5 surface levels, we co-expressed, in HL-1 atrial myocytes, dominant-negative forms of either Rab4 (Rab4DN) or Rab11 (Rab11DN) with Kv1.5-GFP. The RabDN proteins were tagged with red fluorescent protein (RFP) to ensure that each cell analyzed co-expressed both proteins of interest. Expression of each of these dominant-negative Rab constructs resulted in a significant decrease in Kv1.5 steady-state surface levels (Fig. 5A), with no significant change in total Kv1.5-GFP expression (supplemental Fig. 3A). Transfection of HL-1 cells with both Rab4DN and Rab11DN had no additional effect on Kv1.5 surface levels, indicating that these Rabs may act sequentially in the recycling pathway (Fig. 5A). Interestingly, neither Rab5 nor Rab8, which are expressed in cardiac myocytes (39–41), had any significant effect on Kv1.5 surface levels. No significant difference was detected in the expression of the individual RabDN isoforms (data not shown).

Given the effect of the Rab4DN and Rab11DN, we hypothesized that co-expression with constitutively active Rab4 and Rab11 GTPase-deficient mutants (Rab4CA and Rab11CA, respectively) would result in an increase in steady-state surface Kv1.5. Fig. 5B shows that co-expression with either Rab4CA or Rab11CA increased Kv1.5 surface levels without affecting total channel expression (supplemental Fig. 3B). Given the functional effects of the mutant Rab4 and Rab11 proteins on Kv1.5 surface levels, we predicted that the channel and Rab proteins might interact in complex at least transiently. In HL-1 cells co-expressing untagged Kv1.5 and GFP-tagged Rab proteins, anti-GFP antibodies immunoprecipitated Kv1.5 when co-expressed with either Rab4 or Rab11 (Fig. 6A, lanes 4 and 6) but not with Rab8 (lane 8) or soluble GFP alone (lane 2). Interestingly, the lower mobility Kv1.5 band was readily detected in the immunoprecipitation of Rab proteins. This was a consistent finding and may reflect relative differences in the expression levels between the two forms of the protein and perhaps, a more complex regulation of channel trafficking. To ensure that all proteins were expressed and immunoprecipitated, blots were stripped and reprobed with an anti-GFP antibody (Fig. 6B). Finally, to demonstrate the GTP-dependence of this interaction immunoprecipitation, experiments were performed in the presence of excess GDP, which locked Rab4 and Rab11 in their inactive conformation (Fig. 6, C and D) or in the presence of a non-hydrolyzable GTP analog (GppNHp). Activating either Rab4 or Rab11 by treatment with GppNHp enhanced the Rab interaction with Kv1.5 compared with the identical immunoprecipitation in the presence of excess GDP (Fig. 6C).

To further examine this interaction, we looked for colocalization of Kv1.5 and Rab proteins. We analyzed data from recycling experiments in which constitutively active Rab4-GFP or Rab11-GFP proteins were co-expressed with Kv1.5-mCherry. Under these stringent conditions, the interaction is predicted to be the most transient. As shown in Fig.
Rabs Modulate Kv1.5 Recycling in Atrial Myocytes

FIGURE 6. Kv1.5 is in complex with Rab4 and Rab11. A and B, co-immunoprecipitation of Kv1.5 with the indicated Rab proteins or soluble GFP. A, lysates from HL-1 cells expressing Kv1.5 with soluble GFP, Rab4-GFP, or Rab11-GFP were immunoprecipitated using anti-GFP antibodies. Blots were probed with an anti-V5 antibody to detect Kv1.5 channel. Arrow indicates Kv1.5 protein. Lane 1, Kv1.5 + GFP lysate; lane 2, Kv1.5 + GFP IP; lane 3, Kv1.5 + Rab4-GFP lysate; lane 4, Kv1.5 + Rab4-GFP IP; lane 5, Kv1.5 + Rab11-GFP lysate; lane 6, Kv1.5 + Rab11-GFP IP; lane 7, Kv1.5 + Rab8-GFP lysate; lane 8, Kv1.5 + Rab8-GFP IP. B, blots in A were stripped and re-probed using an anti-GFP antibody. Lanes are the same as in A. Arrowhead indicates soluble GFP immunoreactive band, whereas arrow indicates Rab immunoreactive band. (C and D, guanine nucleotide-dependence on the interaction of Kv1.5 with both Rab4 and Rab11). C, lysates from HL-1 cells expressing Kv1.5 with Rab4-GFP or Rab11-GFP were treated with either 100 μM GDP or 100 μM GppNHp and immunoprecipitated using anti-GFP antibodies. Blots were probed with an anti-V5 antibody to detect Kv1.5 channel. Arrow indicates Kv1.5 protein. Lane 1, Kv1.5 + Rab4-GFP + GDP lysate; lane 2, Kv1.5 + Rab4-GFP + GDP IP; lane 3, Kv1.5 + Rab4-GFP + GppNHp lysate; lane 4, Kv1.5 + Rab4-GFP + GppNHp IP; lane 5, Kv1.5 + Rab11-GFP + GDP lysate; lane 6, Kv1.5 + Rab11-GFP + GDP IP; lane 7, Kv1.5 + Rab11-GFP + GppNHp lysate; lane 8, Kv1.5 + Rab11-GFP + GppNHp IP. D, blots in C were stripped and re-probed using an anti-GFP antibody. Lanes are the same as in C. Arrow indicates Rab protein. The blots presented are representative of three different experiments. (E, HL-1 cells co-expressing Kv1.5 (red) with either Rab4CA or Rab11CA (green) were subject to a recycling assay as described under “Experimental Procedures.” Kv1.5 exhibits significant, but not complete, co-localization to intracellular compartments with both Rab4CA and Rab11CA. Arrows indicate regions of co-localization. Scale bars, 5 μm.

In this study, we show for the first time that a population of Kv1.5 originating on the cell surface recycles back to the plasma membrane, and that this process is mediated through interactions with the small GTPases Rab4 and Rab11. Sequential labeling of surface Kv1.5 channel permitted us to distinguish pre-existing and recycled channel pools at the plasma membrane. Using an externally epitope-tagged Kv1.5 channel, we found that Kv1.5 internalized via a dynein-dependent mechanism to early endosomes in HL-1 atrial myocytes. These results are consistent with a previous report showing dynein-dependent endocytosis of Kv1.5 in HEK cells and microtubule-regulated internalization in atrial myocytes (25). The mechanisms stimulating Kv1.5 internalization are unclear and likely multifactorial. Although antibody binding may, in part, trigger internalization in our system, we were most interested in the intracellular fate of internalized channel. Experiments in heterologous systems suggest that a portion of internalized Kv1.5 is susceptible to ubiquitin-mediated proteasomal degradation (42). Our results in atrial myocytes demonstrate that, once internalized, Kv1.5 is capable of returning to the plasma membrane, bypassing the protein degradative pathway. This process contributes to the regulation of steady-state surface levels by engaging components of the intracellular membrane sorting machinery controlled by specific Rab proteins. Together, these results identify an important

To further demonstrate that Rab4 and Rab11 are involved in the post-internalization trafficking of Kv1.5, we co-expressed each RabDN isoform with p50-dynamitin, which we had previously shown increases Kv1.5 surface levels. Interestingly, neither the Rab4DN nor the Rab11DN mutants altered the p50-dynamitin-mediated increase in Kv1.5 surface levels, indicating that both Rab4 and Rab11 were acting downstream of Kv1.5 internalization (Fig. 7A). Taken together, these data show that specific Rab proteins are involved in endocytic recycling of Kv1.5 and regulate the steady-state surface levels of channel in HL-1 atrial myocytes.

DISCUSSION

6, we observed significant, but not complete co-localization of Kv1.5 with both Rab4 and Rab11 in perinuclear endosomal compartments (Fig. 6E).

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To further demonstrate that Rab4 and Rab11 are involved in the post-internalization trafficking of Kv1.5, we co-expressed each RabDN isoform with p50-dynamitin, which we had previously shown increases Kv1.5 surface levels. Interestingly, neither the Rab4DN nor the Rab11DN mutants altered the p50-dynamitin-mediated increase in Kv1.5 surface levels, indicating that both Rab4 and Rab11 were acting downstream of Kv1.5 internalization (Fig. 7A). Taken together, these data show that specific Rab proteins are involved in endocytic recycling of Kv1.5 and regulate the steady-state surface levels of channel in HL-1 atrial myocytes.
new pathway regulating Kv channel density necessary for the maintenance of proper cell homeostasis.

Specific Rab GTPase isoforms, known to participate in endocytic recycling, govern cell surface expression of Kv1.5. Our results demonstrate a selective role for both Rab4 and Rab11, which localize to recycling endosomes and assist in the trafficking of internalized proteins back to the plasma membrane (13, 34). We show that Kv1.5 is in complex with both Rab4 and Rab11 in a guanine nucleotide-dependent manner, and that dominant-negative and constitutively active mutants of both proteins regulate steady-state cell surface levels of Kv1.5. Interestingly, co-expression of Rab4 and Rab11 dominant-negative constructs did not result in an additive effect on Kv1.5 suggesting that Rab4 and Rab11 may act in series within the same recycling pathway. Nevertheless, co-expression with p50-dynamitin indicated that both Rab4 and Rab11 were acting downstream of dynein-mediated internalization and did not participate in anterograde trafficking of the channel. In contrast, neither Rab8 nor Rab5, were found to regulate surface levels of Kv1.5. Rab8 has been implicated in Golgi-to-surface trafficking of select proteins in some cells, whereas Rab5 plays a well-documented role in clathrin-mediated internalization (13, 18). Therefore, in atrial myocytes, Kv1.5 may internalize by a clathrin-independent mechanism. This pathway may involve ADP-ribosylation factor 6, which has been recently implicated in the internalization of inwardly rectifying potassium channels (39). Alternatively, we have shown previously that Kv1.5 localizes to caveolin-containing lipid raft microdomains (2), which have been suggested to internalize via a Rab5-independent mechanism (19).

It is tempting to hypothesize that all Kv channels traffic via common mechanisms. Comparisons between the limited number of studies examining retrograde trafficking reveal that both similarities and differences exist among Kv channel family members. Comparable with the role of Rab11 in Kv1.5 recycling shown here, a recent study in oocytes demonstrated that KCNQ1/KCNE1 undergoes rapid exocytosis from an intracellular pool to the plasma membrane in a Rab11-dependent manner (23). Contrary to our results, KCNQ1/KCNE1 internalizes via a Rab5-dependent mechanism (23). This may reflect differences between the channel isoforms or the cell types studied. Recent evidence does demonstrate a role for clathrin- and Rab5-independent internalizations of K+ channels operating in HL-1 atrial myocytes (39). Nonetheless, it appears that multiple Kv channels use dynein motors during endocytosis. Our results (Fig. 2) together with others clearly show a role for the dynactin complex in the regulation of both Kv1.5 and Kv1.2 channel surface levels in multiple cell types. Collectively, these results suggest that cells utilize multiple mechanisms in an isoform-specific manner to regulate the intracellular fate of endocytosed channels.

An understanding of Kv1.5 channel trafficking will undoubtedly contribute to our understanding of the events underlying the pathophysiological conditions characterized by altered Kv1.5 surface expression. Alterations in Kv1.5 channel recycling may contribute to the reduction of channel protein observed in chronic pulmonary hypertension or in patients with persistent and paroxysmal atrial fibrillation (11, 12). Future studies are needed to determine whether channel activity or post-translational modifications, such as phosphorylation (26, 43), sumoylation (44), or thioacylation (45) contribute to endocytic recycling, or whether these and other pathophysiological states alter the intracellular fate of internalized channel. In addition to an important role in maintaining cell homeostasis, Kv1.5 recycling may also provide a rapid mechanism of adaptation allowing cells to dynamically tune their electrical excitability. Therefore, therapeutic strategies designed to manipulate specific Kv1.5 trafficking pathways may prove useful in the treatment of related cardiovascular channelopathies.

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