Kv1.5 Surface Expression Is Modulated by Retrograde Trafficking of Newly Endocytosed Channels by the Dynein Motor

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Abstract—In this article we have investigated the mechanisms by which retrograde trafficking regulates the surface expression of the voltage-gated potassium channel, Kv1.5. Overexpression of p50/dynamitin, known to disrupt the dynein–dynactin complex responsible for carrying vesicle cargo, substantially increased outward K⁺ currents in HEK293 cells stably expressing Kv1.5 (0.57±0.07 nA/pF, n=12; to 1.18±0.2 nA/pF, n=12, P<0.01), as did treatment of the cells with a dynamin inhibitory peptide, which blocks endocytosis. Nocodazole pretreatment, which depolymerizes the microtubule cytoskeleton along which dynein tracks, also doubled Kv1.5 currents in HEK cells and sustained K⁺ currents in isolated rat atrial myocytes. These increased currents were blocked by 1 mmol/L 4-aminopyridine, and the specific Kv1.5 antagonist, DMM (100 nM). Confocal imaging of both HEK cells and myocytes, as well as experiments testing the sensitivity of the channel in living cells to external Proteinase K, showed that this increase of K⁺ current density was caused by a redistribution of channels toward the plasma membrane. Coimmunoprecipitation experiments demonstrated a direct interaction between Kv1.5 and the dynein motor complex in both heterologous cells and rat cardiac myocytes, supporting the role of this complex in Kv1.5 trafficking, which required an intact SH3-binding domain in the Kv1.5 N terminus to occur. These experiments highlight a pathway for Kv1.5 internalization from the cell surface involving early endosomes, followed by later trafficking by the dynein motor along microtubules. This work has significant implications for understanding the way Kv channel surface expression is regulated. (Circ Res. 2005;97:363-371.)

Key Words: atrial myocyte ■ cardiomycocytes ■ intracellular protein transport ■ ion channels ■ potassium channels

Voltage-gated K⁺ channels (Kv channels) are intimately involved in the cellular regulation of excitation in all cardiovascular cells, and their activity depends on the presence of active channel subunits at the plasma membrane. Surface expression is regulated by changes in gene expression,1-3 interactions with accessory subunits, by phosphorylation, and by cellular components that regulate their trafficking to the cell surface. Trafficking can also provide an explanation for the mechanisms by which drugs may act to achieve their therapeutic actions.4

Although several groups have investigated motifs within Kv channels that affect trafficking,5-8 little is known about the molecules and machinery involved in these processes in the heart.

Surface expression requires movement from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane, and several studies have investigated channel determinants that affect this trafficking process. Motifs in the C termini and pore domains,6,7 of Kv channels have been implicated in their differential surface expression presumably through effects on forward trafficking.5 Functional expression of channels can also be regulated by the removal of extant channels from the cell surface, as recently shown for Kv1.2.

Surface expression is modulated by tyrosine phosphorylation which promotes the endocytosis of the channel.9 Dhani et al.,10 have implicated retrograde transport along the microtubule cytoskeleton in the regulation of CIC-2 chloride channel expression. CIC-2 interacts directly with the dynein motor and inhibition of this motor significantly increased the surface expression of the channel.

Here we have used a multi-pronged approach to show that dynein is also closely involved in the regulation of Kv1.5 surface expression, probably through its role in transporting newly formed endosomes from the cell surface to the cell interior. Direct interference with dynein function caused a significant increase in Kv1.5 surface expression as did disruption of the microtubule cytoskeleton or direct interference with endocytosis itself. We have identified specific residues in Kv1.5 that are essential to this process and shown the process to be relevant both in heterologous cell expression systems and in rat cardiac myocytes.

Materials and Methods
Many methods used in this study have been previously described.11 In electrophysiological experiments where controls were compared

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with cells overexpressing p50, dynamin inhibitory peptide, or compared with nocodazole pretreatment, control and experimental groups were prepared in parallel and the experimenter was blinded to their identity. Further methodological details can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Disruption of Dynein Function Increases Kv1.5 Surface Expression

Overexpression of p50/dynamitin is established as an effective and specific method for disrupting the dynein motor system. p50, an important component of the dynactin complex, is thought to link the cargo-interacting subunits to the dynein motor itself. Overexpression of this protein causes the dynactin complex to dissociate, decoupling the motor from its cargoes. As shown in Figure 1, overexpression of p50 in HEK cells significantly increased Kv1.5 currents on the day after transfection. Peak current densities at +80 mV were approximately doubled from 0.57±0.07 nA/pF to 1.18±0.2 nA/pF, P<0.01 (Figure 1A). This increase in response to p50 overexpression was not limited to HEK293 cells, and experiments in CHO cells yielded similar results (Figure 1B). Activation and inactivation kinetics were unchanged with increases in current across the whole activation range (Figure 1C), suggesting an increase in the number of functional channels at the cell surface.

To confirm that increased Kv1.5 currents were indeed caused by increased surface expression, Kv1.5 localization was examined by immunocytochemistry/confocal microscopy and channel Proteinase K sensitivity was assayed. In Figure 1D, p50 overexpression caused a substantial redistribution of Kv1.5 (digested form) and intracellular Kv1.5 (undigested form). The immunoblot at the bottom indicates that equal amounts of protein were loaded in each lane.

Figure 1. p50/dynamitin coexpression significantly increases Kv1.5 current levels and surface expression. A, Peak currents from HEK293 cells stably expressing Kv1.5 transfected with empty vector (pGFP) or p50-pGFP. From −80 mV, cells were depolarized to between −70 and +80 mV in 10-mV steps, followed by repolarization to −40 mV. B, CHO cells transiently expressing both Kv1.5 and p50-GFP (solid line) and control cells transfected with Kv1.5 pGFP alone (dashed line). Dotted line denotes the zero current level. C, Peak current amplitudes at +80 mV from controls (filled symbols) and p50-overexpressing cells (open symbols) were normalized to cell capacitance (n=12; **P<0.01). D, Confocal images of HEK293 cells stably expressing T7-tagged Kv1.5 (red) transfected with pGFP alone or p50-GFP (green). Scale bars=10 μm. E, HEK cells were cotransfected with T-7 tagged Kv1.5 and either p50-GFP or pGFP and treated with externally applied Proteinase K (lanes 3 and 4) or buffer alone (lanes 1 and 2). The arrows indicate the Western blot position of surface Kv1.5 (digested form) and intracellular Kv1.5 (undigested form). The immunoblot at the bottom indicates that equal amounts of protein were loaded in each lane.
sensitivity experiments yielded results consistent with this. When applied externally to living cells, Proteinase K cleaves exposed protein on the exterior of the cell although cytoplasmic proteins are unaffected. This protease is thus a sensitive test for cell surface expression and is useful also for determining the relative proportions of the channel at the cell surface and in the cytoplasm.6 Uncleaved T7-tagged Kv1.5 migrates at 83 kDa; the digested N-terminal fragment (roughly, the cytoplasmic N terminus plus S1), migrates at ≈47 kDa. Densitometric analysis of the results shown in Lanes 3 and 4 of Figure 1E confirmed that the intensity of the 47 kDa Kv1.5 band relative to the upper band intensity was significantly increased in cells overexpressing p50 (1.52±0.09, n=3, paired t test P<0.05) compared with control cells. That is, p50 overexpression increased Kv1.5 surface residency by ≈50%. This result is consistent with the 2-fold increase in Kv1.5 expression detected electrophysiologically and with the redistribution of the channel seen in the imaging experiments. Given that p50 is well established as a specific disruptor of dynein motor function,12–14 the dynein motor is strongly implicated in the regulation of Kv1.5 surface expression.

Kv1.5 Is Present in Early Endosomes

Dynein motor disruption by p50 overexpression might increase Kv1.5 surface expression by interfering with endocytosis, because dynein is required for transport of endosomes from the cell surface to the interior.14 To see whether Kv1.5 is trafficked via endosomes, we used confocal microscopy to look for colocalization of Kv1.5 with EEA1, an early endosomal marker. As shown in Figure 2A, Kv1.5 and EEA1 associated pixels in line scans across 9 groups of endosomes in this cell, as well as endosome-rich regions of several additional cells confirmed this. In all line scans the pixel correlation between Kv1.5 and EEA1 fluorescence was extremely high (P<0.0001, online Figure IA and IB). In contrast, there was no correlation of Kv1.5 and EEA1 associated pixels in line scans across regions where Kv1.5 was found but endosomal localization was not obviously present (see below).

If dynein disruption interferes with the removal of Kv1.5 from the plasma membrane, then direct inhibition of endocytosis should mimic this effect. Dynamin is responsible for pinching off the neck of the budding endocytotic vesicle and dynamin inhibitory peptide (DIP, Tocris, Bristol, UK) has been previously established to be an effective blocker of endocytosis.15 HEK293 cells stably expressing Kv1.5 were incubated overnight with 50 μmol/L DIP or a control scrambled peptide. As shown in Figure 2C, DIP incubation doubled Kv1.5 currents, an effect essentially identical to that elicited by p50 overexpression. Peak current densities at +80 mV were 0.36±0.05 nA/pF (n=15) in control cells and 0.76±0.05 nA/pF (P<0.01, n=11) in cells incubated overnight with DIP. Imaging analysis showed that colocalization of Kv1.5 and EEA1 in residual early endosomes was dramatically reduced in DIP-treated cells relative to controls. Line-scan analyses across these endosomes showed no significant pixel correlation between EEA1 expression and Kv1.5 (online Figure IC) with probability values between 0.065 and 0.420. These data suggest that dynin inhibition may increase Kv1.5 surface expression by interfering with the net removal of channels from the plasma membrane.

Nocodazole Treatment Mimics Dynein Inhibition in HEK Cells and Cardiomyocytes

If p50 overexpression exerts its actions through interference with dynein transport along microtubules, then disruption of the microtubule cytoskeleton should have similar effects on Kv1.5 currents. To test this prediction we used the microtubule depolymerizing agent, nocodazole, which strongly inhibits trafficking by dynein.16 Incubation of Kv1.5-expressing HEK cells with nocodazole substantially increased Kv1.5 currents (Figure 3A and 3B). Kv1.5 currents increased in a dose-dependent manner with a maximum effect at 70 μmol/L. p50 overexpression was additive to the nocodazole current increase at lower doses (open triangles, Figure 3B) where the drug had a submaximal action. However, current levels saturated with p50-overexpression and
overexpression is acting on a microtubule-dependent pathway, ie, very probably on dynein. Although the additivity of p50 overexpression and low-dose nocodazole may indicate that p50 acts also on a secondary pathway, it is more likely that p50 overexpression is not completely effective in preventing dynein motor complex assembly. Thus, only when microtubules are fully depolymerized at the higher nocodazole doses is the residual trafficking by dynein eliminated.

In parallel experiments, atrial myocytes were isolated from adult rats and treated for 6 hours with 35 \( \mu \text{mol/L} \) nocodazole. I-V relations in Figure 3C and 3D show that sustained currents were enhanced \( \approx 2 \times \) in the nocodazole-treated myocytes but the A-type currents, which underlie \( i_m \), in these cells were unaffected (Figure 3E). As shown in Figure 3D, the increase in sustained current was across the voltage range that Kv1.5 is expected to be open.

Imaging experiments confirmed an enhancement of Kv1.5 surface expression in these myocytes. Nocodazole-treated and control myocytes were stained with anti-Kv1.5 and anti-tubulin and visualized by confocal microscopy. This treatment resulted in a loss of microtubule integrity, as evidenced by a loss of reticulate structure and a general dispersal of tubulin staining (data not shown). Nocodazole treatment dramatically increased the intensity of Kv1.5 staining at the myocyte surface compared with controls (Figure 4A and 4B). Line scan analysis on 10 randomly chosen control and nocodazole-treated myocytes showed that 26.9 ± 2.1% (mean ± SEM) of Kv1.5-positive pixels were within the quartile of the cell nearest to (and including) the plasmalemma in control cells and 40.5 ± 2.6% (Figure 4C and 4D; unpaired \( t \) test, \( P < 0.001 \)) of positive pixels were within these boundaries in nocodazole-treated myocytes. The increased Kv1.5 staining in the outermost quartile supports the idea that enhancement of sustained currents in these cells by nocodazole was caused by a redistribution of Kv1.5 to the plasma membrane.

To determine the contribution of Kv1.5 to the increased sustained currents in nocodazole-treated myocytes, Kv1.5 blockers were used. 4-Aminopyridine is commonly used to block K’ channels by binding to the inner pore mouth. Susceptibility of the current to 4-AP eliminated the possibility that anion or other currents underlie the increase in myocyte current.

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**Figure 3.** Nocodazole pretreatment increases Kv1.5, but not transient currents in HEK cells and in rat atrial myocytes. A, Kv1.5 current density-voltage relationships at various doses of nocodazole in HEK cells overexpressing p50 or transfected with empty vector, as indicated (n=5). B, Peak current densities at +80 mV from A in nocodazole-treated cells transfected with p50 (open triangles) or empty vector. C through E, Currents from rat atrial myocytes treated with 35 \( \mu \text{mol/L} \) nocodazole or solvent (DMSO) alone. Cells were depolarized to +80 mV from −80 mV in 10-mV steps. D, Mean sustained current-voltage data: nocodazole. **\( P < 0.01 \), Student \( t \) test. E, Mean A-type peak current-voltage data: nocodazole. To avoid contamination by sustained currents, the Kv1.5-specific blocker DMM (100 nM) was included in the bath solution.
nocodazole-treated myocytes, 1 mmol/L 4-AP reduced sustained and A-type current (Figure 5A), confirming that nocodazole was indeed influencing potassium channel expression. However, whereas Kv1.5 reportedly underlies the bulk of the sustained current in rat atrial myocytes, other channels, like Kv2.1, may also be contributors to this current at the 1 mmol/L dose used here.\textsuperscript{19,20} To confirm that Kv1.5 underlies the enhanced sustained K$^+$ currents seen after nocodazole treatment, a Kv1.5-specific blocker, 2-(3,4-Dimethylphenyl)-3-(4-methoxyphenethyl) metathiazan-4-one (DMM), a gift from Cardiome Pharma (Vancouver, Canada), was used. The IC$\textsubscript{50}$ for Kv1.5 block (1.5 nM) with this drug was >2 orders of magnitude lower than for Kv2.1 or other K$^+$ channels and Na$^+$ channels present in heart (Figure 5B), and 100 nM DMM completely blocked Kv1.5 in HEK cells (Figure 5C). More importantly, DMM also blocked the nocodazole-increased sustained current in rat atrial myocytes (Figure 5D).

Figure 4. Nocodazole pretreatment increases Kv1.5 surface expression in rat atrial myocytes. A and B, Myocytes incubated for 6 hours without (control) or with 35 \mu mol/L nocardazole, fixed and immunostained for Kv1.5 (green). Scale bar=10 \mu m. C and D, Relative pixel intensity across cells divided into eighths and summed into quartile bins from edge to center, where 1 represents the total fluorescence intensity in the peripheral two-eighths of the cells and 4 represents the center-most quartile of the cells. **P<0.01, 1-way ANOVA.

Figure 5. Nocodazole-induced increase in atrial myocyte current is caused by $I_{KUR}$. A, Current traces from rat atrial myocytes (−80 to +80 mV) pretreated with 35 \mu mol/L nocodazole for 6 hours (above) and then exposed to 1 mmol/L 4-AP (below). B, Dose response curves for DMM on Kv1-Kv4 subfamily channels. Numbers indicate IC$\textsubscript{50}$ values, n=3 to 5 for each point. C, Kv1.5 HEK cell currents at +80 mV in control and 100 nM DMM. D, Sample current traces from rat atrial myocytes pretreated with 35 \mu mol/L nocodazole for 6 hours (above), and the same cell then exposed to 100 nM DMM.
Kv1.5 Coimmunoprecipitates With the Dynein Motor Complex

To test for an association between Kv1.5 and dynein, coimmunoprecipitations of Kv1.5 and the dynein intermediate chain (DIC), or p50 (an integral part of the dynein–dynactin complex) were performed on extracts of HEK cells heterologously expressing a T7-tagged Kv1.5 construct (Figure 6A), and rat ventricular myocyte lysates expressing the native channel (Figure 6B). A significant portion of Kv1.5 coimmunoprecipitated with the dynein intermediate chain in these experiments. Anti-T7 antibody brought down the DIC along with the targeted T7-tagged Kv1.5 in HEK cells and anti-Kv1.5 coimmunoprecipitated DIC in myocytes (Figure 6A, lower panel, and 6B). T7-tagged Kv1.5 was also efficiently coimmunoprecipitated with p50 (online Figure II). Given that dynein interacts with many cellular proteins and that plasmalemma-integrated Kv1.5 is unlikely to interact with dynein, this level of coimmunoprecipitation is highly significant. Thus, like CIC-2,10 Kv1.5 directly interacts with the motor complex that is likely trafficking it from near the cell surface in native cells to the interior.

A Proline Rich Region in Kv1.5 Is Essential to Regulation of Channel Expression by Dynein

Both the N and C termini of Kv channels have been implicated in regulating their surface expression.5-11 Mutants lacking the bulk of either the N terminus (ΔN209) or C terminus of Kv1.5 (ΔC51) were tested for responsiveness to p50-overexpression (Figure 7A). The ΔC51 mutant behaved like WT, as currents were roughly doubled, but Kv1.5ΔN209 was wholly unresponsive to disruption of dynein motor function. A more conservative deletion (ΔN91) also proved unresponsive to p50 overexpression, and included in the deletion is a proline rich region (aa 65 to 93) harboring two SH3 binding domains that are consensus src kinase binding sites.21 As tyrosine phosphorylation has been previously implicated in Kv1.5 surface expression22 and specifically in the regulation of Kv1.2 endocytosis,23 this region seemed a likely candidate for involvement in internalization of the channel. Deletion of aa65–93 completely eliminated the increased Kv1.5 currents seen with p50 overexpression (Figure 7B).

The 2 consensus SH3-binding domains in this region are separated by 4 residues and both have the sequence RPLP-PLP. We mutagenized the consensus sequences individually to RPLAALP (PI and PII, Figure 7C). Mutation of the second consensus domain (Kv1.5 P79/80A; P-II) had no effect on Kv1.5 responsiveness to p50 overexpression (Figure 8B), but
mutation of the first consensus SH3-binding domain (Kv1.5 P68/69A; P-I) eliminated the responsiveness of Kv1.5 to p50 overexpression (Figure 8A). Interestingly, the baseline current densities in PI mutant cells were unusually high, resembling levels seen with WT Kv1.5 cells overexpressing p50 or the dynamin inhibitory peptide (DIP, Figure 2). This suggested that Kv1.5 P68/69A had lost its ability to be upregulated by interference with the motor, or perhaps had been relieved of negative regulation by the dynein motor because the channel no longer interacted with it. However, given that block of endocytosis with a dynamin inhibitory peptide mimics the effect of p50 (Figure 2C) and that tyrosine phosphorylation has been implicated in the Kv1.5 downregulation22 and in endocytosis of the related Kv1.2 channel,9 we preferred the hypothesis that this loss of responsiveness might be attributable to a defect in endocytosis of the channel, which was thus no longer available for retrograde transport. We therefore repeated the PI mutant experiment with DIP to test whether that peptide could still affect surface expression of the PI-Kv1.5. As shown in Figure 8C, DIP had no significant effect on the expression of PI channel currents, although still affecting PI currents. Kv1.5 P68/69A expression was high whether or not the cells were pretreated with DIP. Currents were 2 to 3× those of Kv1.5 P79/80A which served as a control in these experiments. Neither DIP, nor p50 overexpression, nor a combination of the two (Figure 8D) had any effect on the expression of the PI mutant Kv1.5 channel. We conclude that the N-terminal most SH3-binding domain is essential for normal endocytosis of Kv1.5. Surface expression is regulated by endocytosis, followed by retrograde transport of the internalized channels in association with the dynein motor complex.

**Discussion**

**Dynein Motor Function Affects Kv1.5 Surface Expression**

This is the first report of an association of a K⁺ channel with the dynein motor complex. In the only other report of an ion channel-dynein interaction, Dhani et al10 demonstrated that a chloride channel, CIC-2, bound the heavy and intermediate dynein chains in vitro and that the channel could be coimmunoprecipitated with the dynein motor complex from murine hippocampal lysates. As with Kv1.5, interference with dynein function increased surface expression of CIC-2. Here, our data unequivocally demonstrate that inhibition of dynein motor function in HEK cells with p50 overexpression, or nocodazole in myocytes increases the presence of Kv1.5 at the cell surface by about 2-fold, by electrophysiological, biochemical and imaging data (Figures 1, 3, and 4). p50 overexpression is well established about 2-fold, by electrophysiological, biochemical and imaging data (Figures 1, 3, and 4). p50 overexpression is well established as a powerful and specific means to inhibit dynein motor function. However, it remains possible that p50 overexpression has additional, as yet undescribed effects on the cell that are independent of its role in the dynein–dynactin complex. To exclude this possibility, nocodazole was used as a second method to inhibit dynein function, and caused significant increases in Kv1.5 currents in HEK cells and in rat cardiac myocytes (Figure 3), that could be abolished by the K⁺ current-selective agent 4-AP, and the more specific Kv1.5 antagonist, DMM (Figure 5). Although there is no requirement for a direct interaction between the complex and channel for trafficking by the motor (Kv1.5 could be dragged along in endosomes bound to the dynein motor complex independently of a specific Kv1.5-dynein association), we were also able to find a direct interaction between Kv1.5 and components of the dynein complex, p50, and DIC in HEK cells and native myocytes (Figure 6). Also, it is interesting that, at low doses of nocodazole, the effects of the drug and p50 overexpression on Kv1.5 expression in HEK cells are additive (Figure 3). Either p50 overexpression is insufficient to fully inhibit dynein function or there is at least one additional pathway by which Kv1.5 is trafficked.

**Endocytosis and the Role of a Kv1.5 Consensus SH3 Binding Domain**

The most likely mechanism by which dynein block increases Kv1.5 surface expression is indirect, by affecting the balance
of channel delivery and removal from the plasma membrane. Valetti et al. have shown that endocytosis is normal in HeLa cells overexpressing p50, as is the recycling of endosomes to the cell surface. This does not mean, however, that Kv1.5 surface levels must remain in a steady state when retrograde trafficking is impaired. If even a fraction of the newly synthesized or internal pools of the channel continue to traffic to the cell surface, the failure of dynein to remove endocytosed channels would quickly result in an increased concentration of the channel in the endocytosing/recycling pool. Indeed, disruption of the dynein–dynactin complex by p50 overexpression has been shown to lead to a redistribution of endosomes toward the cell periphery in COS-7 cells, probably attributable to a failure of newly formed endosomes to be further internalized. Block of endocytosis using the dynamin-inhibitory peptide mimicked the effects of p50 overexpression and nocodazole treatment (Figure 2) and is consistent with this model. Mutation of 2 prolines to alanines in 1 Kv1.5 consensus SH3-binding domain also eliminated the Kv1.5 current response to p50 overexpression (Figures 7 and 8). This is almost certainly because the mutant channel is not efficiently endocytosed. Current levels are high (similar to those of the wild-type channel when dynein function or endocytosis are inhibited) and, more tellingly, the mutant is also insensitive to dynamin inhibition (Figure 8).

SH3 domains are important for the recruitment of tyrosine kinases, like src, to a protein. Tyrosine phosphorylation is well known to downregulate Kv1.5 currents, and src kinase has been shown to directly bind the proline-rich region in which this SH3-binding domain resides. There is a direct role for tyrosine phosphorylation in the regulation of endocytosis has recently been demonstrated for Kv1.2, a related potassium channel. Thus, the simplest explanation for the role of this consensus SH3-binding domain in Kv1.5 is that it is required to promote tyrosine phosphorylation of the channel and that this in turn regulates channel internalization. Lacking this domain, the channel remains in the plasma membrane, and is inaccessible to dynein so manipulations of the motor have no effect on channel surface expression (Figure 8).

There are 2 identical consensus SH3-binding domains in close juxtaposition in Kv1.5. However, mutation of the second SH3-binding domain does not affect the sensitivity of the channel to p50 overexpression. Perhaps the precise context of the domain is important. Also, although deletion of the entire proline rich domain (residues 65 to 93) eliminates the responsiveness of the channel to p50 overexpression, cells expressing this mutant do not display current densities above those of cells expressing the wild-type channel in the absence of p50 overexpression. Perhaps there are additional determinants in this region that affect delivery of the channel to the plasma membrane or otherwise affect its stability at that locale.

**Implications for Forward Trafficking**

Although pore and C-terminal motifs have been implicated in the trafficking of Kv to the cell surface, the mechanism(s) by which these motifs affect this movement remain unclear. They may relate to interactions with trafficking molecules or to channel assembly instead. However those signals operate, the fact that inhibition of dynein function and depolymerization of the microtubule cytoskeleton increases Kv1.5 surface expression has important implications for forward trafficking. Somehow, Kv1.5 is continuing to traffic to the membrane. Kinesins are unlikely to be involved in this process. Although they are generally responsible for anterograde transport, dynein and kinesin activities are coordinated. When retrograde transport is blocked, anterograde movement also ceases and vice versa. The dynactin complex, which connects the dynein motor to its cargo, may play an essential role in this regard.ＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡＡÅ

**Relevance to the Heart**

Nocodazole-treatment increased Kv1.5 surface expression as assayed by confocal imaging and measurement of sustained current densities in rat cardiac myocytes. The sensitivity of these increased currents to the specific Kv1.5 antagonist DMM demonstrated that they were specifically underlain by increased Kv1.5 expression in these cells. In addition, Kv1.5 was shown to directly interact with components of the dynein complex (Figure 6). It is highly likely, therefore, that the microtubule cytoskeleton is involved in the internal trafficking of Kv1.5 in both heterologous and physiological (cardiac) systems. It will be of great interest to determine how this system is used by the heart to modulate the expression of Kv1.5 and other ion channels as a mechanism to regulate cardiomyocyte excitability, and how other channels are affected by perturbations of retrograde trafficking. It is possible that in disease states associated with channel remodeling, such as atrial fibrillation and congestive cardiac failure, the trafficking or endocytosis mechanisms described above may be of key importance in determining the final population of surface-expressed channels. For example, it has been reported that microtubule abundance is increased in the myocytes of patients with congestive heart failure.
surface in HEK cells and myocytes, probably via a direct interaction between the channel and the dynein motor complex. This trafficking depends on efficient endocytosis of the channel, which is, in turn, dependent on an intact SH3-binding domain in the Kv1.5 N terminus. This is a previously unexplored avenue of Kv channel trafficking and regulation.

Acknowledgments
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References
Kv1.5 Surface Expression Is Modulated by Retrograde Trafficking of Newly Endocytosed Channels by the Dynein Motor

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Materials and Methods

Cell Preparation and Transfection

Unless otherwise indicated, hKv1.5 channels were studied in HEK293 cells as described previously. Transfections were by a liposome-mediated method. One day before transfection, cells were plated on a coverslip in 35-mm dishes at 40-50% confluence. After one day’s growth, transfections were performed using 1.5 µg of each relevant plasmid and LIPOFECTAMINE 2000™ transfection reagent (Invitrogen) according to the manufacturer's instructions. For nocodazole experiments, cells were incubated with or without 35 µM nocodazole for 6 hr, prior to electrophysiological analysis. In experiments with the dynamin inhibitory peptide, cells were incubated for 30 min with 50 µM mry-DIP (myristoylated dynamin inhibitory peptide; Tocris Cookson Ltd., Bristol, UK) prior to electrophysiological analysis.

Plasmid Constructs

The construction of hKv1.5 in pcDNA3, Kv1.5ΔN209, Kv1.5ΔN91 and Kv1.5ΔC51 were described previously. Human p50 in pEGFP was a gift of Richard Vallee (Columbia University, NY). Kv1.5Δ65-93 was constructed by a PCR-based method using appropriate primers. The PCR product was cloned into hKv1.5 in pcDNA3 as a HindIII-BspEI fragment, replacing the wild-type sequence in that region. All channel constructs used in this study were cloned into the pcDNA3 vector and various deletion mutants were prepared by PCR with HindIII and EcoRV insertions for directed cloning. Site-directed mutant constructs were prepared using the QuickChange Site-directed Mutagenesis Kit.
(Stratagene, La Jolla, CA). Following mutagenesis and sequencing, the mutant sequence was subcloned into the parent vector to ensure that no extraneous mutations were present in the constructs used. Plasmid DNA was prepared for transfection using the Qiagen Plasmid Midi Kit (Qiagen Inc, Valencia, CA).

**Electrophysiological Experiments and Solutions**

Solutions and methodology for the recording of ionic currents were as previously reported from our laboratory.\(^4,5\) The standard bath solution contained (in mM): NaCl, 135; KCl, 5; MgCl\(_2\), 1; sodium acetate, 2.8; HEPES, 10; CaCl\(_2\), 1; adjusted to pH 7.4 using NaOH. The standard pipette filling solution contained (in mM): KCl, 130; EGTA, 5; MgCl\(_2\), 1; HEPES, 10; Na\(_2\)ATP, 4; GTP, 0.1; adjusted to pH 7.2 with KOH. All chemicals were from Sigma (Mississauga, ON, Canada). Whole cell current recording and data analysis were done using an Axopatch 200B amplifier and pClamp 8 software (Axon Instruments, Foster City, CA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL) and polished by heating. We used pipettes have resistance of 1-3 M\(\Omega\). Compensation for capacitance and series resistance was performed manually in all whole cell recordings. HEK-293 cells were depolarized between –70 and +80 mV in 10 mV-step from a holding potential of –80 mV followed by –40 mV repolarization pulse to record tail currents. All whole cell recordings were performed at room temperature (20-23 °C), and control and experimental group identities were concealed from the experimenters.

**Myocyte Isolation and Electrophysiology**
Rat atrial myocytes were isolated from hearts of male Wistar rats weighing 250-300 g using a conventional method with normal Tyrode’s solution. In nocodazole experiments, after pretreatment with the drug, cells were depolarized between –80 and +80 in 10-mV steps for 800 ms from holding potential, –80 mV to record ionic current using the solution contained (in mM): NaCl, 135; KCl, 5; MgCl₂, 1; sodium acetate, 2.8; HEPES, 10; adjusted to pH 7.4 using NaOH. The pipette filling solution contained (in mM): KCl, 130; EGTA, 5; MgCl₂, 1; HEPES, 10; Na₂ATP, 4; GTP, 0.1; adjusted to pH 7.2 with KOH.

**Imaging**

Cells were prepared according to previously published methods. For studies on the effects of p50 overexpression, HEK293 cells stably expressing T7-tagged Kv1.5 were seeded onto coverslips and later transfected with p50-GFP and cultured for a further 12 hr prior to fixation. The cells were rinsed and fixed with 4% paraformaldehyde for 12 min at room temperature (RT). After three 5-min washes with 1×phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄), cells were incubated in a permeabilizing/blocking solution (PBS containing 2% BSA and 0.2% Triton X-100) for 30 min at RT. A mouse monoclonal antibody to the T7 Tag (1:1000; Novagen) was diluted in blocking solution and incubated with the cells for 2 h at RT. Cells were then washed three times for 5 min in PBS on a rotator before incubation with secondary antibodies, Alexa 594-conjugated goat anti-mouse IgG antibody (1:1000; Molecular Probes) for 1 h on the rotator at RT. Coverslips were then washed three times with PBS prior to mounting with 10 µl of a 90% glycerol, 2.5% w/v DABCO–PBS
solution. Images were collected on a Nikon C1 laser scanning confocal unit (Nikon D-Eclipse C1, Melville, NY) and processed using the operation software EZ-C1 for Nikon C1 confocal microscope (Nikon). Images were prepared using the Adobe PhotoShop software package.

For endosome imaging studies, HEK cells stably expressing Kv1.5 were labeled with antibodies against the C-terminus and against EEA1. For Kv1.5-EEA1 colocalization experiments, stable Kv1.5-T7 lines were plated onto coverslips and treated with cycloheximide supplemented media (Cycloheximide, Sigma, 200 µg/ml) for 4 hr to arrest protein synthesis. After the first 2 hr of treatment at 37°C, cells were placed into a 20°C incubator for the last 2 hr of cycloheximide treatment. This temperature allows normal internalization but is not compatible with trafficking out of early endosomes or recycling to the plasma membrane.10,12,13 The cells were rinsed and fixed with 4% paraformaldehyde for 12 min at room temperature (RT). After three 5-min washes with 1×phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4), cells were incubated in permeabilizing/blocking solution (PBS containing 2% BSA and 0.2% Triton X-100) for 30 min at RT. Rabbit Polyclonal C-terminal Kv1.5 antibody (1:500)1 and a mouse monoclonal EEA-1 antibody (1:250; BD Biosciences), were diluted in blocking solution and incubated with the cells at 4°C overnight. Cells were then washed three times for 5 min in PBS on a rotator. Secondary antibodies, Alexa 594-conjugated goat anti-mouse IgG antibody and Alexa 488-conjugated goat anti-rabbit IgG antibody (1:1000; Molecular Probes) were then incubated with the cells for 1 h on the rotator at RT. The coverslips were once again washed three times with PBS prior to mounting with 10 µl of a 90% glycerol, 2.5% w/v DABCO–PBS solution. Images were
collected using a Deltavision Deconvolution Microscope using a 60X lens using Softworks software. Images were later viewed and prepared using Adobe PhotoShop software.

For studies of the effects of nocodazole on rat atrial myocytes, the myocytes were treated with 35µM nocodazole (Sigma) for five hours then fixed and immunolabeled as previously described.¹ Myocytes were co-stained with anti-Kv1.5 and anti-β-tubulin (Sigma) to verify the depolymerizing actions of nocodazole.

**Image Analysis**

To quantitatively assess Kv1.5 distribution in myocytes, line scanning analysis was performed using Image J software (NIH imaging). For each cell, eight 0.25 micron confocal slices were taken along the long axis of the cell, at 45-55% of the cell depth, approximating the cell middle. 3 lines were drawn across the width of each slice chosen at positions approximately ¼, ½ and ¾ of the cell length from one end of the cell. The line was then divided into eight equal segments. Pairs of segments (i.e., the outermost eighths, the eighths immediately inside adjacent to those eighths, etc.) were combined to form 'quarters' across the width of the cell. These data from the lines at the four positions along the length of the cell were combined to get an average estimate of the fluorescence distribution in each cell. The fluorescence intensity for each quartile was expressed as a percentage of the total fluorescence intensity in the lines. Image J was used also for line scan analysis of Kv1.5 colocalization with EEA1. Lines were drawn through endosomes across several cells in confocal images and the intensities of Kv1.5 and EEA1 signals at each pixel were plotted on the same graph for comparison. Correlation coefficients, $r$,
between Kv1.5 and EEA1 signals in pixels across each line scan were calculated by the formula \( r = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}} \). To assess the significance of each \( r \) value, \( t \) was determined according to \( t = \frac{r}{\sqrt{1-r^2}/(N-2)} \) and compared to critical values of \( t \).

**Western Blotting and Densitometry**

Western blotting was performed using standard techniques. Following protein estimation by the Lowry method, cell extracts were subjected to PAGE and blotted to PVDF membranes. Antibodies were diluted to appropriate working concentrations in blocking buffer (5% skim milk powder, 0.1% Tween-20 in PBS) and incubated with the membranes. Following incubation with appropriate HRP-conjugated secondary antibody, protein bands were visualized by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and detected on X-ray film.

For densitometry, X-ray film images, were digitized using an Epson Perfection 1200U scanner and saved as .tif files. They were then analyzed using NIH Image J software according to the instructions provided on the NIH webpage. Optical Density calibration for the analysis was performed using a Kodak optical density step tablet.

**Co-immunoprecipitation Experiments**

HEK cells stably expressing T7 tagged Kv1.5 were lysed in lysis buffer (0.025M phosphate buffer, 150 mM NaCl, 2.5mM EDTA, 10% glycerol and 1% Triton). 2.5 µg of anti-T7 tag antibody (Novagen) with protein A Sepharose (Sigma) and 3 µg Dynein antibody (Anti-Dynein cytoplasmic; Chemicon International) with protein G Agarose
(Roche Diagnostics) were used for immunoprecipitation of T7 tagged Kv1.5 and Dynein intermediate chain respectively. Western blotting was performed using the T7 tag antibody (1:10000) and Dynein antibody (1:3000) and blots were developed using biotin-labeled goat anti-rabbit antibody (Amersham) and Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

**Proteinase K experiments**

Digestion of surfaced proteins in living HEK cells was conducted as described by Manganas, et al. For Proteinase K-sensitivity experiments, HEK cells expressing T7-tagged Kv1.5 alone or T7-tagged Kv1.5 plus p50 were washed, and then treated with 200 µg/mL Proteinase K for 30 min. Following extensive washing to remove residual Proteinase K, cells were lysed and processed for Western analysis. The N-terminally T7-tagged Kv1.5 was detected with anti-T7 monoclonal antibody (Novagen).

**References**


Figure Legends

Online Figure 1. Line scan analyses of Kv1.5-EEA1 colocalization. In several cell confocal images, lines were drawn in ImageJ through endosomes and the intensities of Kv1.5 and EEA1 signals at each pixel were plotted on the same graph for comparison. A and B are examples of plot profiles from cells that showed a high degree of colocalization between Kv1.5 and EEA1. C illustrates a plot profile across several endosomes in a cell pretreated with Dynamin inhibitory peptide (DIP) before staining and examining for EEA1-Kv1.5 colocalization. To ensure a stringent test for co-localization, pixel lengths ranged from 0.1064 microns to 0.134 microns in the scans across endosomes in which Kv1.5-EEA1 were expected to be present, and were 0.242 microns in the cells where Kv1.5 and EEA1 were not obviously present together in endosomes.

Online Figure 2. Co-immunoprecipitation of Kv1.5 with p50. HEK293 cells were co-transfected with T7-tagged Kv1.5 and either p50-GFP or empty vector (pGFP) as a control. The cells were harvested approximately 66 h post-transfection. Control and transfected cells were lysed and aliquots of the supernatants were employed for immunoprecipitation. Anti-GFP (Torrey Pines Biolabs) was added to aliquots containing equal amounts of protein from the control and the T7-Kv1.5-expressing cells and the immune-complexes were precipitated with Protein G agarose. The beads were pelleted, washed and boiled in SDS-sample buffer and subjected to Western analysis. Blots were probed with anti-T7 antibody (detecting Kv1.5) and the bands were visualized by chemiluminescence. Control (no GFP antibody and no Kv1.5) lanes are as indicated.
Specific co-immunoprecipitation of Kv1.5 was observed only when both p50-GFP and T7-Kv1.5 constructs are co-transfected and GFP antibody is included in the IP reaction (lane 4). IP : immunoprecipitation, WB : western blot.
Transfection:

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Online Supplement Figure 2