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# Intravascular pressure enhances the abundance of functional $K_v1.5$ channels at the surface of arterial smooth muscle cells

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## Abstract

Voltage-dependent potassium ($K_v$) channels are present in various cell types, including smooth muscle cells (myocytes) of resistance-sized arteries that control systemic blood pressure and regional organ blood flow. Intravascular pressure depolarizes arterial myocytes, stimulating calcium ($Ca^{2+}$) influx through voltage-dependent $Ca^{2+}$ ($Ca_v$) channels that results in vasoconstriction and also $K^+$ efflux through $K_v$ channels that oppose vasoconstriction. We hypothesized that pressure-induced depolarization may not only increase the open probability of plasma membrane–resident $K_v$ channels but also increase the abundance of these channels at the surface of arterial myocytes to limit vasoconstriction. We found that $K_v1.5$ and $K_v2.1$ proteins were abundant in the myocytes of resistance-sized mesenteric arteries. $K_v1.5$, but not $K_v2.1$, continuously recycled between the intracellular compartment and the plasma membrane in contractile arterial myocytes. Using ex vivo preparations of intact arteries, we showed that physiological intravascular pressure through membrane depolarization or membrane depolarization in the absence of pressure inhibited the degradation of internalized $K_v1.5$ and increased recycling of $K_v1.5$ to the plasma membrane. Accordingly, by stimulating the activity of $Ca_v1.2$, membrane depolarization increased whole-cell $K_v1.5$ current density in myocytes and $K_v1.5$ channel activity in pressurized arteries. In contrast, the total amount and cell surface abundance of $K_v2.1$ were independent of intravascular pressure or membrane potential. Thus, our data indicate that intra-vascular pressure–induced membrane depolarization selectively increased $K_v1.5$ surface abundance to increase $K_v$ currents in arterial myocytes, which would limit vasoconstriction.

## INTRODUCTION

Intravascular pressure stimulates membrane depolarization in small, resistance-sized arteries, leading to the activation of smooth muscle cell (myocyte) voltage-dependent calcium ($Ca^{2+}$) ($Ca_v$) channels, an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), and

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**Author contributions:**

M.W.K. designed and conducted all the experiments, performed all the data analysis, and wrote the manuscript. M.D.L. and J.P.B. taught and oversaw all the techniques and commented on the manuscript. J.H.J. designed the project, directed the experiments, and wrote the manuscript.

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The authors declare they have no competing interests.

vasoconstriction (1, 2). Myocyte membrane depolarization also activates voltage-dependent potassium ($K^+$) ($K_v$) channels, inducing a negative feedback mechanism that partially opposes depolarization and vasoconstriction (3–9). This “myogenic response” is an autoregulatory mechanism that modulates regional blood flow, systemic blood pressure, and sets a resting arterial diameter from which other stimuli can induce vasoconstriction or vasodilation.

$K_v$ channels comprise a large family of ~40 proteins that are subdivided into 12 classes ($K_v1$ to $K_v12$) (10). The genes encoding several $K_v$ channel family members are expressed, and the encoded proteins are functional in arterial myocytes, including $K_v1.2$, $K_v1.5$, $K_v2.1$, $K_v2.2$, $K_v7.1$, and $K_v7.4$ (5, 11–13). Functional channels may be composed of homomeric subunits or heteromultimeric $K_v$ subunits, both of which are also present in arterial myocytes (7, 14). Membrane potential directly controls the open probability ($P_O$) of surface $K_v$ channels to modulate arterial contractility. However, cellular $K_v$ current amplitude ($I$) is dependent not only on single-channel $P_O$ but also on the number of channels present in the plasma membrane ($N$) and single-channel amplitude ($i$), such that $I = NP_Oi$. Whether membrane potential regulates $K_v$ channel surface abundance is unclear, and the role of multiple $K_v$ channel subtypes in arterial myocytes is poorly understood. Conceivably, specific $K_v$ channel subtypes may respond to distinct stimuli or perform specific physiological functions in arterial myocytes.

Here, we investigated the hypothesis that intravascular pressure and membrane potential modulate the abundance of functional $K_v$ channels at the surface of myocytes of resistance-sized mesenteric arteries. Our data showed that pressure-induced membrane depolarization inhibited $K_v1.5$ channel degradation, stimulated $K_v1.5$ surface abundance, and enhanced $K_v1.5$ currents in arterial myocytes. In contrast, the amount of $K_v2.1$ at the myocyte surface was unaffected by intravascular pressure in these arteries. We also show that the pressure-induced increase of $K_v1.5$ channel surface abundance reduced arterial contractility. Thus, our data demonstrated that membrane depolarization activated $K_v1.5$ currents through two distinct pathways in arterial myocytes: (i) direct channel activation and (ii) subtype-specific enhancement of $K_v$ channel abundance at the cell surface.

## RESULTS

### The gene encoding $K_v1.5$ is highly expressed relative to other $K_v$ channel members in mesenteric artery myocytes

We performed reverse transcription polymerase chain reaction (PCR) on freshly isolated mesenteric artery myocytes and quantified the relative expression of six $K_v$ channel family members that are expressed in arterial myocytes (11–13). Analysis of transcripts encoding myosin heavy chain 11 (a myocyte marker), platelet endothelial cell adhesion molecule-1 (an endothelial cell marker), and fatty acid–binding protein 4 (adipocyte marker) served as controls for the purity of the myocyte preparations. We only detected the transcript encoding myosin heavy chain 11 in the preparations, indicating that complementary DNA (cDNA) was from pure myocytes (fig. S1A). Quantitative PCR analysis indicated that of the transcripts encoding the six $K_v$ channel subtypes measured (fig. S1B), mRNAs encoding $K_v1.5$ accounted for ~60%, $K_v2.1$ and $K_v2.2$ each accounted for ~15%, and $K_v1.2$, $K_v7.1$,

and $K_V$7.4 together represented ~10% of the $K_V$ transcripts (Fig. 1A). Because the genes encoding $K_V$1.5 and $K_V$2.1 were most highly expressed, we focused on the regulation of these channels to pressure and membrane potential in arterial myocytes.

## Arterial isolation leads to loss of surface $K_V$1.5, but not $K_V$2.1, channels

To measure the amount of $K_V$1.5 and $K_V$2.1 channels at the plasma membrane in arterial myocytes, we used surface biotinylation after confirming the specificity of the antibodies for these proteins when expressed in human embryonic kidney (HEK) 293 cells (fig. S2, A and B). The $K_V$1.5- and $K_V$2.1-specific antibodies did not detect any proteins in mock-transfected HEK293 cells (fig. S2, A and B). Each antibody detected proteins in arterial lysates, and the most intense bands were at similar molecular weights to their recombinant counterparts (fig. S2C). The lower molecular weight bands may be subunit cleavage products.

Freshly isolated (assayed immediately after isolation, 0 hour) mesenteric arteries had ~48% of total $K_V$1.5 and ~81% of total $K_V$2.1 channels at the cell surface (Fig. 1, B and C). We examined the time-dependent effects of the loss of intravascular pressure due to arterial isolation on the relative amounts of $K_V$1.5 and $K_V$2.1 at the myocyte surface. Three hours after arterial isolation and maintenance at 37°C, the amount of $K_V$1.5 at the cell surface was ~37% of that in freshly isolated (0 hour) arteries (Fig. 1, B and D). The total amount of $K_V$1.5 also decreased during 3 hours without pressure, but the relative amount of $K_V$1.5 at the cell surface compared to the total amount of $K_V$1.5 was unchanged (Fig. 1, B and C). In contrast, both the amount of $K_V$2.1 at the surface and the relative cellular distribution of $K_V$2.1 were unchanged after 3 hours without pressure (Fig. 1, B to D). These data indicated that arterial isolation reduced the amount of $K_V$1.5 at the cell surface but did not alter $K_V$2.1 surface protein or the relative cellular distribution of $K_V$1.5 or $K_V$2.1 channels in myocytes.

## Arterial isolation leads to time-dependent decrease in total $K_V$1.5 but not $K_V$2.1

After arterial isolation, we found that surface $K_V$1.5 decreased, but the abundance of the protein in the intracellular fraction did not increase, suggesting that the internalized $K_V$1.5 was degraded. We monitored the abundance of total $K_V$1.5 and $K_V$2.1 from isolation through 18 hours to investigate the differences in stability of the proteins in depressurized vessels. One hour after arterial isolation, total $K_V$1.5 was similar to that in freshly isolated arteries (Fig. 2, A and B). In contrast, 3 hours after arterial isolation, total $K_V$1.5 was ~33% of that in the freshly isolated arteries, and this reduction was maintained for 18 hours, the longest time point measured (Fig. 2, A and B). In contrast, total $K_V$2.1 did not change up to 18 hours after arterial isolation (Fig. 2, A and B). Quantitative analysis of transcript abundance showed that 3 hours after arterial isolation, $K_V$1.5- and $K_V$2.1-encoding mRNAs were similar to those in freshly isolated arteries, indicating that the reduction in $K_V$1.5 protein was not due to a decrease in mRNA for $K_V$1.5 (Fig. 2C). These data indicated that, in the absence of pressure, isolated artery $K_V$1.5, but not $K_V$2.1, is internalized and degraded within 3 hours.

Internalized proteins are either degraded in lysosomes, by the proteasome, or both degradation pathways may contribute. Arteries were placed in physiological saline solution (PSS) containing bafilomycin, an inhibitor of lysosomal degradation, or MG132, a blocker of proteasomal degradation, immediately after isolation to determine which pathways

contributed to $K_v1.5$ instability in the unpressurized arteries. Either inhibitor prevented the reduction in total $K_v1.5$, with bafilomycin more effective than MG132 (Fig. 2D). Neither inhibitor affected the amount of $K_v2.1$ (Fig. 2D) or the relative surface/intracellular distribution of either $K_v1.5$ or $K_v2.1$ (Fig. 2, E and F). Bafilomycin completely prevented the loss of $K_v1.5$ from the cell surface 3 hours after isolation and depressurized conditions (Fig. 2G). These data indicated that, after arterial isolation, myocyte $K_v1.5$ is degraded by both the lysosomal and proteasomal pathways. Blocking either of these intracellular degradation pathways maintained $K_v1.5$ at the myocyte surface.

## Intravascular pressure–induced membrane depolarization prevents arterial myocyte $K_v1.5$ loss and degradation

We hypothesized that intravascular pressure regulates $K_v1.5$ degradation and surface abundance. Pressurizing mesenteric arteries to 80 mmHg for 3 hours inhibited the reduction in total $K_v1.5$ (Fig. 3A) and limited the loss of $K_v1.5$ from the cell surface (Fig. 3, B and C), when compared to 0-hour controls. In contrast, the cell surface and total amounts of $K_v1.5$ were reduced in arteries maintained at 10 mmHg for 3 hours compared with that in 0-hour controls (Fig. 3, A to C). Intravascular pressure did not alter total or surface $K_v2.1$ (Fig. 3, A to C) or the relative surface/intracellular distribution of $K_v1.5$ or $K_v2.1$ (Fig. 3, B and D). These data indicated that intravascular pressure controls $K_v1.5$ degradation.

Intravascular pressure stimulates membrane depolarization (1). To investigate regulation of $K_v$ channel degradation by membrane potential, we compared the effects of an increase in $K^+$ in the PSS from 6 to 30 mM $K^+$ to depolarize arteries from ~−60 to ~−40 mV. This voltage shift is similar to that which occurs in arteries when increasing pressure from 0 mmHg to physiological intravascular pressure (2, 15). Whereas the 6 mM $K^+$ solution was ineffective at stabilizing $K_v1.5$, the 30 mM $K^+$ solution prevented the isolation-induced reduction in total and surface $K_v1.5$ (Fig. 4, A to C), as determined by Western blotting and cell surface biotinylation. In contrast, 30 mM $K^+$ did not alter total or surface amounts of $K_v2.1$ (Fig. 4, A to C) or the relative cellular distribution of $K_v1.5$ or $K_v2.1$ channels (Fig. 4, B and D). To test the hypothesis that $K_v1.5$ is trafficked between the surface and internal pools in arterial myocytes, we also analyzed the amount of $K_v1.5$ at the cell surface by immunofluorescence colocalization with wheat germ agglutinin, a plasma membrane marker (Fig. 4E). The 30 mM $K^+$ pre- vented the isolation-induced reduction of surface $K_v1.5$ in myocytes (Fig. 4E). These data indicated that membrane depolarization inhibits $K_v1.5$ channel internalization and degradation in mesenteric artery myocytes.

## Voltage-dependent $Ca^{2+}$ channel activity controls arterial myocyte $K_v1.5$ channel degradation

Membrane depolarization stimulates $K_v$ channels, which increases $K^+$ current mediated by the efflux of $K^+$ ions, and $Ca_v$ channels, which permits $Ca^{2+}$ influx, increasing $[Ca^{2+}]_i$ (1–9). To investigate contributions of intravascular pressure–induced depolarization and depolarization-induced $Ca^{2+}$ influx to $K_v1.5$ degradation and surface abundance, we applied nimodipine, a $Ca_v$ channel blocker to arteries maintained at 10 or 80 mmHg or in the presence of 6 or 30 mM $K^+$ PSS. Nimodipine blocked both pressure- and depolarization-induced stabilization of $K_v1.5$ (Fig. 5, A and B). In arterial myocytes, depolarization-

induced $Ca^{2+}$ influx stimulates ryanodine-sensitive $Ca^{2+}$-release (RyR) channels, leading to release of $Ca^{2+}$ from the sarcoplasmic reticulum (16). Ryanodine, an RyR channel inhibitor, did not alter depolarization-induced stabilization of $K_v1.5$ (Fig. 5B). Bay K8644, a $Ca_v$ channel activator, partially prevented isolation-induced $K_v1.5$ degradation that occurred after 3 hours in depressurized conditions (Fig. 5C). Nimodipine, ryanodine, and Bay K8644 did not change $K_v2.1$ abundance (Fig. 5, A to C). These data indicated that physiological intravascular pressure and depolarization-induced $Ca_v$ channel activation inhibits $K_v1.5$ degradation in arterial myocytes.

We tested the hypothesis that $K_v1.5$ continuously recycles and that voltage-dependent $Ca^{2+}$ influx inhibits both internalization and degradation, thereby enhancing surface abundance. Concanavalin A, an internalization inhibitor, prevented isolation-induced degradation of $K_v1.5$ at 3 hours, with-out affecting $K_v2.1$ (Fig. 6A). Concanavalin A also increased the proportion of total $K_v1.5$ that was located in the plasma membrane from ~45 to 72% in 6 mM $K^+$ PSS and from ~48 to 74% in 30 mM $K^+$ PSS (Fig. 6B). These data suggested that $K_v1.5$ channels continuously recycle to and from the plasma membrane and that membrane potential does not directly control this process. Instead, $Ca_v$-mediated $Ca^{2+}$ influx inhibits $K_v1.5$ degradation to enhance $K_v1.5$ surface abundance.

## $K_v1.5$ degradation reduces whole-cell $K_v1.5$ currents in arterial myocytes

We analyzed the effect of $K_v1.5$ abundance on myocytes isolated from mesenteric arteries by patch-clamp electrophysiology using conditions designed to isolate whole-cell $K_v$ currents and reduce contamination from other current types, including that from large-conductance $Ca^{2+}$-activated $K^+$ channels. Mean $K_v$ current density (at +50 mV) in myocytes from arteries isolated for 3 hours was significantly lower than the $K_v$ current density in myocytes from freshly isolated (0 hour) arteries (Fig. 7, A to C). To evaluate the portion of the total $K_v$ current that was mediated by $K_v1.5$-containing channels, we applied Psora-4, a $K_v1.5$ channel inhibitor (17), to myocytes and monitored the Psora-4–sensitive current (Fig. 7A). Psora-4 reduced the mean current density in freshly isolated myocytes from ~25.5 to ~8.8 pA/pF (Fig. 7, B and C), suggesting that $K_v1.5$-containing channels carried ~65% of the current at +50 mV. In contrast, the effect of Psora-4 on the current at +50 mV in myocytes 3 hours after isolation was less, reducing the current density from ~12.6 to ~8.9 pA/pF (~29%) (Fig. 7, A to C). Bafilomycin prevented the isolation-induced reduction in total $K_v$ current and the decrease in Psora-4–sensitive currents in myocytes (Fig. 7, A to C). We obtained similar results with diphenyl phosphine oxide-1 (DPO-1) (Fig. 7, B and C), a putative $K_v1.5$ channel–specific inhibitor (18, 19). These data indicated that $K_v1.5$-containing channels are a major contributor to whole-cell $K_v$ currents and that isolation-induced $K_v1.5$ degradation reduces $K_v1.5$-mediated current density in arterial myocytes.

## Intravascular pressure stimulates functional $K_v1.5$ channels in mesenteric arteries

To test the hypothesis that intravascular pressure controls the amount of functional $K_v1.5$ channels in arterial myocytes, we performed arterial myography on pressurized single third-order, abluminal mesenteric arteries in the presence of Psora-4 or the general $K_v$ inhibitor 4-aminopyridine (4-AP). The arteries were maintained in a temperature-controlled perfusion chamber at pre-experimental intravascular pressures of either 10 or 80 mmHg for 3 hours.

Arteries maintained at 10 mmHg pre-experimental pressure were then pressurized to 80 mmHg to stimulate the development of myogenic tone. Arteries in the 80 mmHg pre-experimental pressure group were continued at 80 mmHg, providing an equivalent time course for both experimental groups. Arteries from either the 10 or the 80 mmHg group responded to Psora-4 or 4-AP with a reduction in diameter, indicating vessel constriction (Fig. 8A). However, Psora-4 stimulated the contraction of arteries from the 80 mmHg pre-experimental pressure group by ~29 μm and from the 10 mmHg pre-experimental pressure group by ~10 μm, which was ~65% less (Fig. 8, A and B). As expected, 4-AP caused a greater reduction in diameter, consistent with a stronger constriction response when more classes of $K_v$ channel were inhibited, than that observed for Psora-4 (Fig. 8B). However, both Psora-4 and 4-AP produced significantly greater constriction responses in the 80 mmHg pre-experimental pressuregroup, consistent with pressure maintaining $K_v$ channels and $K_v1.5$-containing channels at the cell surface to counter vasoconstriction.

## DISCUSSION

Myogenic responsiveness requires a balance of ion channels that mediate the vasoconstriction response and those that mediate vasodilation. As critical mediators of membrane hyperpolarization, $K_v$ channels are components that counter vasoconstriction in response to depolarization. Here, we found that membrane potential selectively controls the amount of functional $K_v1.5$-containing channels at the myocyte surface (*N*, the number of channels) to modulate $K_v$ currents and, hence, vasoconstrictive tone in resistance-sized arteries. Quantitative PCR experiments revealed that $K_v1.5$-encoding mRNA accounts for most $K_v$ channel transcripts in mesenteric artery myocytes. Our data indicated that $K_v1.5$ continuously recycled between the intracellular compartment and the plasma membrane and that membrane potential through $Ca^{2+}$ signaling modulated $K_v1.5$ surface abundance in arterial myocytes. Loss of intravascular pressure and the associated membrane hyperpolarization stimulated degradation of internalized $K_v1.5$ through a mechanism dependent on both proteasomal and lysosomal pathways, resulting in a reduction in both total and plasma membrane–localized $K_v1.5$. We propose that physiological membrane depolarization activates $Ca_v$ channels, which inhibited $K_v1.5$ degradation, enabling the internalized $K_v1.5$-containing channels to return to the plasma membrane. Our data indicated that the regulation of $K_v1.5$ degradation by intravascular pressure and membrane potential determined $K_v1.5$ current density and functionality. In contrast, $K_v2.1$, another $K_v$ channel subtype detected in arterial myocytes, did not appear to internalize over the same time course, and total and surface proteins were not modulated by intravascular pressure, membrane potential, or $Ca_v$ channel activity. Mechanisms that underlie the $K_v1.5$ specificity of this regulatory pathway may involve amino acid sequence dissimilarity of the channel proteins, differences in regulation by auxiliary subunits, or modulation by signal messengers. In summary, our data indicated that intravascular pressure selectively controls functional $K_v1.5$ surface abundance to regulate myocyte $K^+$ currents and arterial contractility.

Membrane depolarization activates $Ca_v$ channels in arterial myocytes, leading to an increase in $[Ca^{2+}]_i$ and vasoconstriction (1, 2). Concomitant $K_v$ channel activation limits depolarization and, thus, $Ca_v$ channel activity and the associated vasoconstriction (1, 2).

Here, our data showed that physiological intravascular pressure and membrane depolarization inhibit $K_v1.5$ degradation by activating $Ca_v$ channels. In contrast, ryanodine did not inhibit depolarization-induced $K_v1.5$ protection. In arterial myocytes, $Ca_v$ channel activation leads to global, nanomolar increases in $[Ca^{2+}]_i$, whereas ryanodine receptors generatelocalized, micromolarintracellular $Ca^{2+}$transients, termed $Ca^{2+}$ sparks, and propagating cytosolic $Ca^{2+}$ waves. Thus, our data indicated that the global nanomolar increase in $[Ca^{2+}]_i$ inhibited $K_v1.5$ degradation, which may involve $Ca^{2+}$-sensing proteins, such as $Ca^{2+}$/calmodulin (20,21), that have affinities for $Ca^{2+}$ in the nanomolar range. Evidence exists linking $K_v$ channels and calmodulin because apocalmodulin associateswith $K_v7$ channels in neurons to promote trafficking to the axonal surface (22). Cells degrade proteins through lysosomal and proteasomal pathways (23, 24). Here, inhibitors of either lysosomal or proteasomal degradation stabilized $K_v1.5$, suggesting that lysosomal and proteasomal pathways act in series, rather than in parallel, to degrade $K_v1.5$ in arterial myocytes. In summary, our data indicated that membrane depolarization stimulates $K_v$ channels both directly through voltage-dependent activation and indirectly by inhibiting $K_v1.5$ channel degradation.

Although the detailed mechanisms that control $K_v$ channel trafficking in arterial myocytes are unclear, experiments with concanavalin A, an internalization inhibitor, suggested that $K_v1.5$ constantly recycles to the plasma membrane through a membrane potential–independent mechanism. Concanavalin A prevented the loss of 65% of total $K_v1.5$, suggesting that this amount of the protein may be available for internalization and either recycling to the plasma membrane or degradation. Degradation inhibitors prevented loss of both surface and total $K_v1.5$ proteins. Because the cell surface/intracellular ratio of $K_v1.5$ was similar in pressurized and nonpressurized arteries, our data suggested that membrane potential controls $K_v1.5$ degradation but does not appear to regulate surface trafficking or internalization in arterial myocytes. In HL-1 mouse atrial myocytes, H9c2 rat cardiac myoblasts, and HEK293 cells stably expressing $K_v1.5$, constitutively internalized $K_v1.5$ is recycled to the plasma membrane through a Rab4- and Rab11-dependent pathway, implicating involvement of both recycling and early endosomes (25, 26). In HL-1 cells exposed to oxidative stress, internalized $K_v1.5$ colocalized with heat shock protein 70, a chaperone that targets misfolded proteins for ubiquitylation and degradation (27, 28). Oxyhemoglobin also reduced the abundance of $K_v1.5$ at the cell surface through a protein tyrosine kinase–dependent endocytosis mechanism in rabbit cerebral artery myocytes (29). Future experiments should be designed to elucidate trafficking mechanisms by which membrane potential regulates $K_v1.5$ degradation and surface abundance in arterial myocytes.

Several studies have provided insights into trafficking as a mechanism to regulate ion channel function in arterial myocytes (30–33). Trafficking of $Ca_v1.2$ to the surface in arterial myocytes requires the $\alpha_2\delta$-1 auxiliary subunit (30). The abundance of both subunits at the cell surface is increased in rats with genetic hypertension, which increases $Ca_v1.2$ current density and produces a non-inactivating current that stimulates vasoconstriction (30). Pharmacological and molecular targeting of $\alpha_2\delta$-1 reduces $Ca_v1.2$ surface abundance in arterial myocytes, leading to vasodilation (30, 34). Protein kinase Cδ increases the surface abundance of TRPM4, a melastatin transient receptor potential channel, in cerebral artery myocytes (32). Rapid delivery of large-conductance $Ca^{2+}$-activated $K^+$ (BK) channel β1

subunits to the cell surface activates BK channels, leading to vasodilation (33). This pathway is activated by nitric oxide and is a mechanism by which this endothelium-derived factor activates BK channels to dilate cerebral arteries (33). Here, we showed that intravascular pressure, by controlling membrane potential, selectively enhanced the abundance of functional $K_v1.5$-containing channels at the myocyte surface. Myocyte $K_v1$ versus $K_v2$ function may exhibit some vascular bed specificity, suggesting that the regulated trafficking pathway described here may be more prominent in some vessels than others (3, 4, 14). Similarly, $K_v$ channels in arterial myocytes can comprise heteromultimers containing $K_v1.5$ or other subunits (7, 14). Regulated $K_v1.5$ trafficking may not only alter the surface expression of Kv1.5 homomultimeric channels but also modulate $K_v$ channel heteromultimer formation. Future studies should be designed to investigate these hypotheses and mechanisms of trafficking of other $K_v$ subunits that are present in arterial myocytes (13). In atrial myocytes, increased mechanical stress activates an integrin signaling pathway, leading to an increase in functional surface $K_v1.5$ channels (35). Whether there is overlap in mechanisms by which pressure and shear stress increase the abundance of functional $K_v1.5$ at the surface of arterial and atrial myocytes is unclear. Together, these studies indicate that multiple, unique trafficking pathways, which differ with respect to time course and mechanisms involved, exist to modulate the activity of different ion channels in myocytes, thereby controlling vascular contractility.

Patch-clamp electrophysiology and myography in the presence of $K_v1.5$ channel blockers supported the biochemical evidence that changes in $K_v1.5$ surface abundance control myocyte $K_v$ currents and pressurized artery contractility. Psora-4, a Psoralen compound, blocks $K_v1.3$ and $K_v1.5$ channels with similar low nanomolar potency but is a less effective blocker of other $K_v1$ family members (17). There is little to no evidence for expression of the $K_v1.3$-encoding gene or $K_v1.3$ function in contractile arterial myocytes, with studies supporting the presence of $K_v1.2$, $K_v1.5$, and $K_v1.6$ members of the $K_v1$ family in this cell type (3, 7, 17, 36, 37). Psoralens are also weak blockers of $K_v2$, Kir2.1, $Ca_v1.2$, and BK channels, which are present in arterial myocytes but at concentrations 25 to 150 times higher than those used here (8, 17, 33, 34, 38). DPO-1 binds to the open state of $K_v1.5$ channels, and high selectivity for $K_v1.5$ over $I_{to}$, $IK_1$, $I_{Kr}$, $I_{Ks}$, and $K_v3.1$ has been reported in cardiac myocytes (18, 19). Thus, the data with Psora-4 and DPO-1 indicated that changes in intravascular pressure affect the abundance of functional $K_v1.5$ channels at the cell surface of arterial myocytes to control $K_v$ currents and vascular tone.

## MATERIALS AND METHODS

### Tissue preparation and cell isolation

Animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center (UTHSC). Male Sprague-Dawley rats (200 to 250 g body mass) were euthanized with sodium pentobarbital (150 mg/kg). The mesenteric artery bed was removed and placed into ice-cold PSS that contained 112 mM NaCl, 6 mM KCl, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, and 10 mM glucose, gassed with 21% $O_2$, 5% $CO_2$, and 74% $N_2$ (pH 7.4).

Individual third- and fourth-order mesenteric arteries (~100 to 200 μm in diameter) were cleaned of adventitial tissue in ice-cold PSS and were dissected for experiments. Arteries were maintained at 37°C for 3 hours in 6 or 30 mM $K^+$ PSS in the absence or presence of bafilomycin, MG132, nimodipine, ryanodine, Bay K8644, or concanavalin A as required.

Myocytes were dissociated from mesenteric arteries in isolation solution containing 55 mM NaCl, 80 mM sodium glutamate, 5.6 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, with pH adjusted to 7.4 with NaOH, with papain (0.7 mg/ml), dithioerythreitol (1 mg/ml), and bovine serum albumin (BSA) (1 mg/ml) for 15 to 20 min at 37°C. Arteries were then immediately transferred to isolation solution containing collagenase F (0.66 mg/ml), collagenase H (0.33 mg/ml), BSA (1 mg/ml), and 100 μM $CaCl_2$ at 37°C for 5 to 10 min. Arteries were washed in ice-cold isolation solution and triturated with a fire-polished glass Pasteur pipette to yield single myocytes.

## Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard tissue culture conditions (37°C, 21% $O_2$/5% $CO_2$). HEK293 cells were transiently transfected using Effectene (Qiagen) with pRBG4 encoding full-length rat $K_v1.5$ [a gift from J. Trimmer, University of California (UC) Davis], pEGFP-N1 encoding full-length mouse $K_v1.5$ tagged with green fluorescent protein (a gift from J. Nerbonne, Washington University, St. Louis), or pCMV6 encoding full-length mouse $K_v2.1$ (a gift from K. O'Connell, UTHSC, Memphis). Cells were used 72 hours after transfection.

## Quantitative real-time PCR

Total RNAwas prepared from ~500 isolated myocytes, individually selected using an enlarged patch pipette and a microscope as we have described previously (39), or from whole mesenteric arteries. First-strand cDNA was synthesized through reverse transcription PCR with AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene). Quantitative PCR was then performed with TaqMan hydrolysis probes (Roche) on a Roche Light-Cycler 480 thermal cycler using the following protocol: 95°C for 5 min, then 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 10 s. Each PCR contained 5 μl of TaqMan LightCycler 480 Master Mix, 0.1 μl of forward primer, 0.1 μl of reverse primer, 0.1 μl of the hydrolysis probe, 2 μl of cDNA template, and 2.7 μl of ribonuclease-free water. A negative control lacking cDNA template was performed in each experiment. Relative mRNA abundance was calculated as the difference in threshold ($C_t$) values ($\Delta C_t$) of each $K_v$ isoform using the $\Delta C_t$ method. Fold changes in *$K_v1.5$* and *$K_v2.1$* mRNA were calculated as $100 \times (2^{-\Delta\Delta C_t})$, where $\Delta\Delta C_t$ is the difference between the $\Delta C_t$ values. *Myh11* was used as the reference gene. Primer sequences and probes were as follows: $K_v1.2$: GGAAGAGAACCTCAGCTCCTG (forward), AGACCCAGAGCCTTCTGTGA (reverse), probe #75; $K_v1.5$: AGGCTCCTCAGGATGCAG (forward), GCAACCCGGAGATGTTTATG (reverse), probe #9; $K_v2.1$: TGCTGTGCAGAGAAGAGGAA (forward), GGACGATGAACATGATCGAG (reverse), probe #120; $K_v2.2$: TGCCCAGAGAAAAGGAAGAA (forward), TAGACACGATGGCCAGGAT (reverse), probe #67; $K_v7.1$:

GCGAGATGTCATCGAGCAG (forward), TTCCCGATGGACTGATCC (reverse), probe #81; $K_v$ 7.4: TGTTGGGATCGGTGGTCTA (forward), GAAGATGAGCACCAG-GAACC (reverse), probe #17; and Myh11: CCTGCTAGTCCACCCCAGTA (forward), ACTGAGCTGCCCTTTCTGTG (reverse), probe #119. PCR products were separated on 1.5% agarose gels.

### Surface biotinylation

Mesenteric arteries were incubated for 1 hour at 4°C with EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin (1 mg/ml each) (Thermo Scientific) in phosphate-buffered saline (PBS) (Gibco). Unbound biotin was quenched with glycine, and arteries were washed with PBS. Arteries were then homogenized in radioimmunoprecipitation assay (RIPA) buffer with 1% Triton X, and biotinylated surface proteins were separated using avidin beads in spin columns. Biotinylated proteins were eluted from the avidin beads by boiling in SDS buffer with 5% 2-mercaptoethanol. The entire biotinylated and nonbiotinylated samples from each preparation were loaded onto separate lanes in the same gel and probed using Western blotting.

### Western blotting

Protein lysates were prepared in RIPA buffer with 1× SDS and 5% 2-mercaptoethanol, separated on 7.5% SDS–polyacrylamide gels, and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk, then incubated with primary antibodies for $K_v$1.5, $K_v$2.2 (1:1000; NeuroMab, UC Davis), or actin (1:10000; Millipore) overnight at 4°C in tris-buffered solution with 0.1% Tween (TBS-T) and 5% nonfat dry milk. After washing with TBS-T, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour and washed again. Membranes were then developed using enhanced chemiluminescence (Thermo Scientific) with a Kodak In Vivo F Pro Imaging System. Band intensity was analyzed using ImageJ software.

### Mesenteric artery bed pressurization

To investigate the regulation of $K_v$ channel total and surface protein by intravascular pressure, a section of intact mesenteric vasculature was used. A freshly isolated, single superior mesenteric artery was cannulated in a temperature-controlled perfusion chamber (Living Systems Instrumentation), and downstream fourth-order arteries were occluded using cotton ties to create a closed, no-flow vascular network. Intra-vascular pressure was controlled using an attached reservoir and monitored using a pressure transducer. Arterial pressure was maintained at either 10 or 80 mmHg for 3 hours, after which, third- and fourth-order arteries were dissected and harvested from the preparation to evaluate total and surface protein expression.

### Immunofluorescence

Isolated myocytes were plated on poly-L-lysine–coated coverslips and fixed immediately (0 hour) or after 3 hours in either 6 or 30 mM $K^+$ PSS at 37°C. Myocytes were labeled with Alexa 488–conjugated wheat germ agglutinin (Invitrogen), a plasma membrane stain, before permeabilization with 0.1% Triton X-100. Myocytes were blocked with 5% BSA in PBS and

incubated with the same $K_v$1.5-specific antibody used for Western blotting experiments at 1:100 in 0.5% BSA in PBS overnight at 4°C. Myocytes were washed with PBS and incubated with Alexa 546–conjugated secondary antibody. After washing, coverslips were secured to slides using mounting media (1:1 PBS/glycerol), and images were acquired using a laser scanning confocal microscope (LSM Pascal, Carl Zeiss). Alexa 488 and 546 were excited at 488 and 543 nm with emission detected at 505 to 530 and ≥560 nm, respectively. Pixel colocalization was measured using weighted colocalization coefficient values with Zeiss Pascal system embedded software. The RG2B Colocalization plugin for ImageJ was used to isolate colocalized pixel data with automatic selection threshold values and express the data as the average of the corresponding red and green channels.

### Patch-clamp electrophysiology

Patch-clamp electrophysiology was performed on isolated myocytes that were allowed to adhere to a glass coverslip for 15 min before experimentation. Whole-cell currents were measured using an Axopatch 200B amplifier and Clampex 9.2 (Axon Instruments). $K_v$ currents were activated from a holding potential of −70 mV by applying stepwise depolarizations to between −60 and +50 mV. Bath solution contained 120 mM NaCl, 3 mM $NaHCO_3$, 4.2 mM KCl, 1.2 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 1 mM tetraethylammonium, and 10 mM Hepes, with pH adjusted to 7.4 with NaOH. Pipette solution contained 110 mM potassium gluconate, 30 mM KCl, 0.5 mM $MgCl_2$, 5 mM $Na_2ATP$, 1 mM guanosine triphosphate, 10 mM EGTA, and 5 mM Hepes, with pH adjusted to 7.2 with KOH. $K_v$ currents were digitized at 5 kHz and filtered at 1 kHz. Offline analysis was performed using Clampfit 9.2 (MDS Analytical Technologies).

### Pressurized artery segment myography

Experiments were performed using individual endothelium-denuded, third-order mesenteric artery segments (0.5 to1 mm in length and 150 to 200 μm in diameter) cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation). Intravascular pressure was modulated using a reservoir and monitored by a pressure transducer. Pressure was maintained at either 10 or 80 mmHg for 3 hours. Arteries maintained at 10 mmHg pre-experimental pressure were then pressurized to 80 mmHg to stimulate the development of myogenic tone. Arteries in the 80 mmHg pre-experimental pressure group were continued at 80 mmHg, providing an equivalent time course for both experimental groups. Arterial diameter was measured using calibrated IonWizard edge detection software (IonOptix).

### Statistical analysis

All datawere analyzed using paired or unpaired Student's *t* test or analysis of variance (ANOVA) with Newman-Keuls post hoc test using GraphPad Prism 5. All data are expressed as means ± SEM. $P < 0.05$ was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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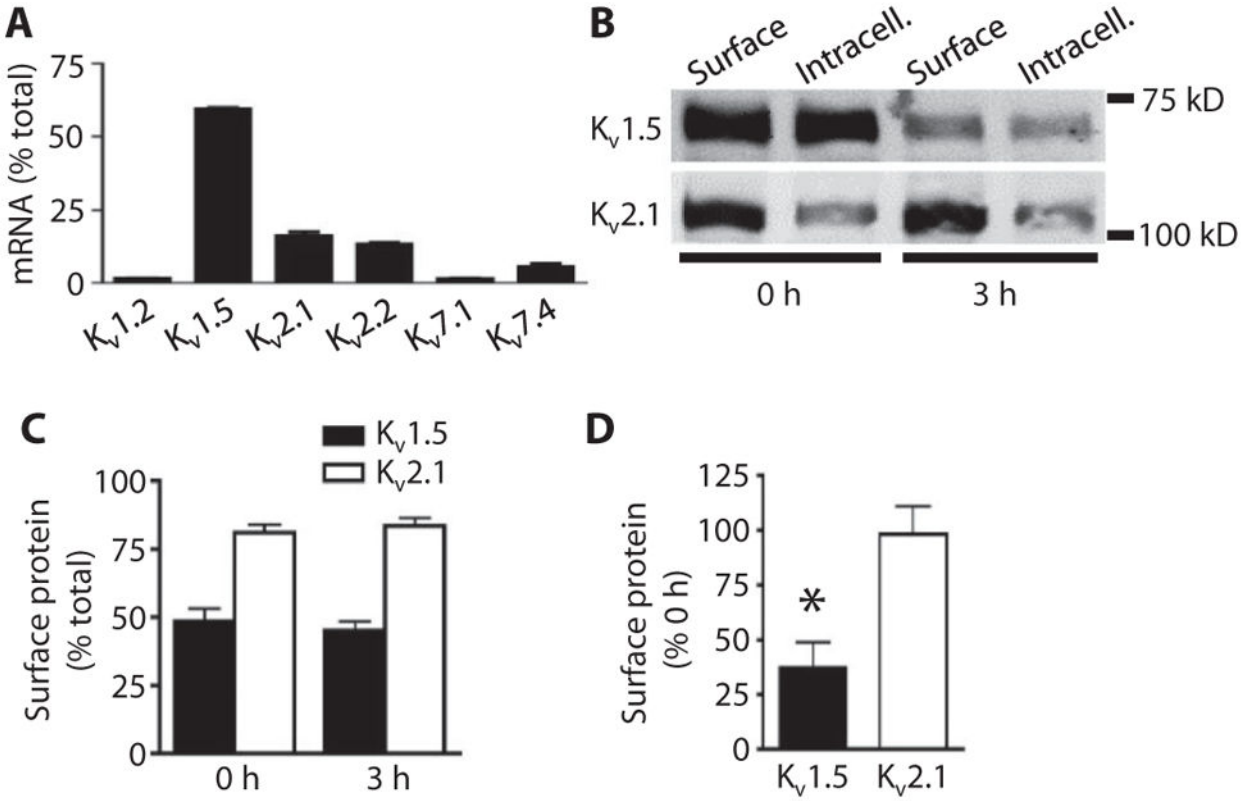



**Fig. 1. Arterial isolation leads to a decrease in total and surface $K_v1.5$**

(**A**) Quantitative real-time PCR mean data for transcripts of six $K_v$ family members in pure arterial myocytes. $n = 3$ for each. Data are expressed as the percent of total $K_v$ transcripts. (**B**) Representative Western blot images of arterial biotinylation samples showing the nonbiotinylated [intracellular (Intracell.)] and biotinylated (Surface) abundance of $K_v1.5$ and $K_v2.1$ in mesenteric arteries immediately after (0 hour) and 3 hours after arterial isolation. (**C**) Mean data showing the percent of total $K_v1.5$ and $K_v2.1$ at the cell surface for arteries immediately and 3 hours after isolation. $n = 6$ for each. (**D**) Mean data showing percent of $K_v1.5$ and $K_v2.1$ remaining at the cell surface after 3 hours relative to the 0-hour samples. $n = 6$ for each. $^*P < 0.05$ versus 0 hour.

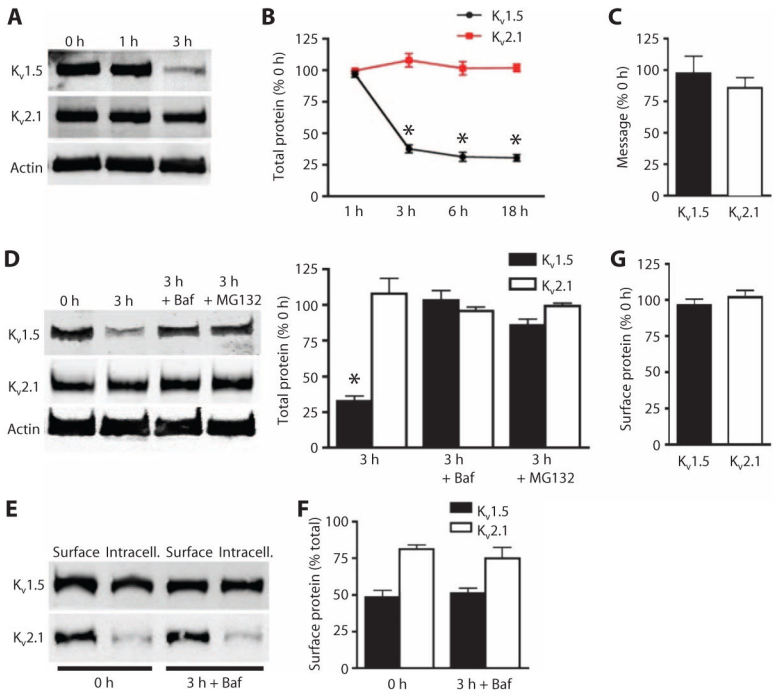



**Fig. 2. Bafilomycin and MG132 prevent $K_V$1.5 loss in mesenteric arteries**

(**A**) Representative Western blot images of total $K_V$1.5 and $K_V$2.1at 0, 1, and 3 hours of isolation. (**B**) Quantification of total $K_V$1.5 and $K_V$2.1 at the indicated times after isolation. *n* = 5 for the 1-hour group, *n* = 6 for the remaining groups. **P* < 0.05 versus 0 hour. (**C**) Quantitative PCR data expressed as the percent of transcripts for each channel remaining after 3 hours with the amount of transcript at 0 hour set at 100%. *n* = 3 for each. (**D**) Representative Western blots and quantification of total $K_V$1.5 and $K_V$2.1 0 and 3 hours after arterial isolation, and the effect of bafilomycin (Baf; 50 nM) or MG132 (10 μM). *n* = 6 for each. **P* < 0.05 versus 0 hour. (**E**) Representative Western blot images of arterial biotinylation samples showing the abundance of surface and intracellular $K_V$1.5 and $K_V$2.1 at 0 and after 3 hours of arterial isolation in the presence of bafilomycin. (**F**) Mean data showing the percent of total $K_V$1.5 and $K_V$2.1 at the cell surface for arteries immediately and 3 hours after isolation. *n* = 5 for each. (**G**) Mean data showing percent of $K_V$1.5 and $K_V$2.1 remaining at the cell surface after 3 hours in the presence of bafilomycin relative to the 0-hour samples. *n* = 5 for each.

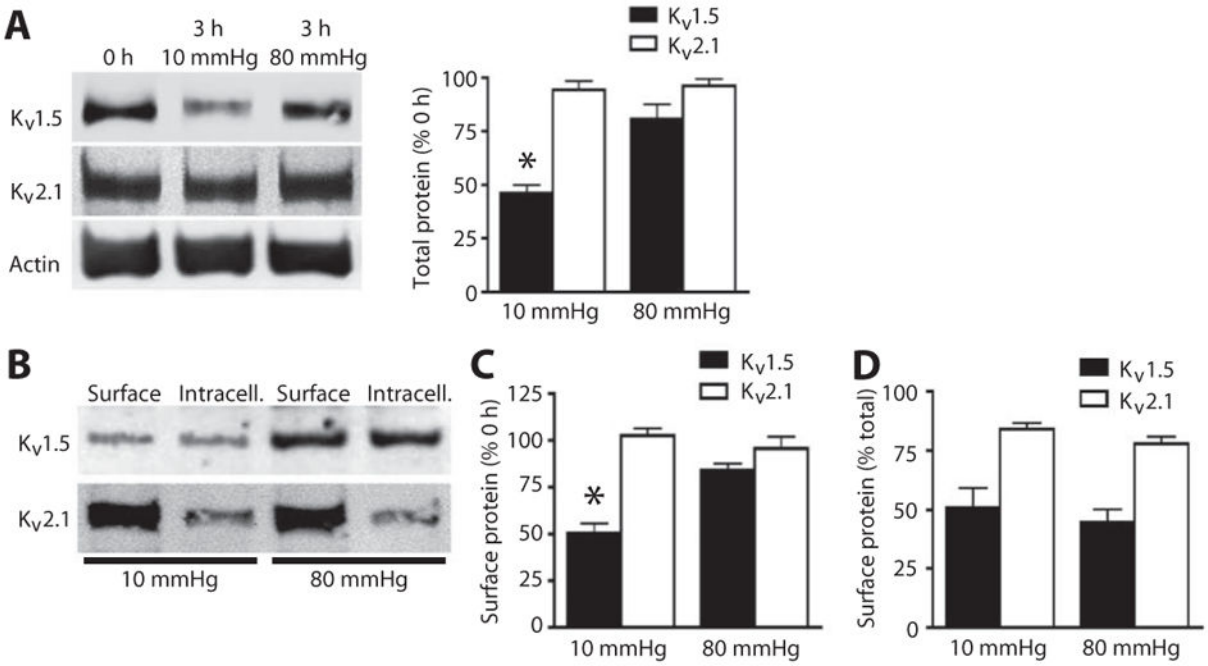



**Fig. 3. Intravascular pressure inhibits $K_v1.5$ degradation and maintains surface expression**

(**A**) Representative Western blots and quantitative data of total $K_v1.5$ and $K_v2.1$ in control arteries (0 hour) or arteries exposed to 10 or 80 mmHg intravascular pressure for 3 hours. (**B**) Representative Western blot of arterial biotinylation samples showing the abundance of surface and intracellular $K_v1.5$ and $K_v2.1$. (**C**) Mean data showing the percent of $K_v1.5$ and $K_v2.1$ at the cell surface compared to the amount at isolation (0 hour) after 3 hours at 10 and 80 mmHg intravascular pressure. (**D**) Mean data showing the percent of $K_v1.5$ and $K_v1.2$ at the cell surface as a percent of the total of each channel protein in arteries exposed to the indicated pressure for 3 hours. For all quantitative data, $n = 5$ for each group and $^*P < 0.05$ versus 0 hour.

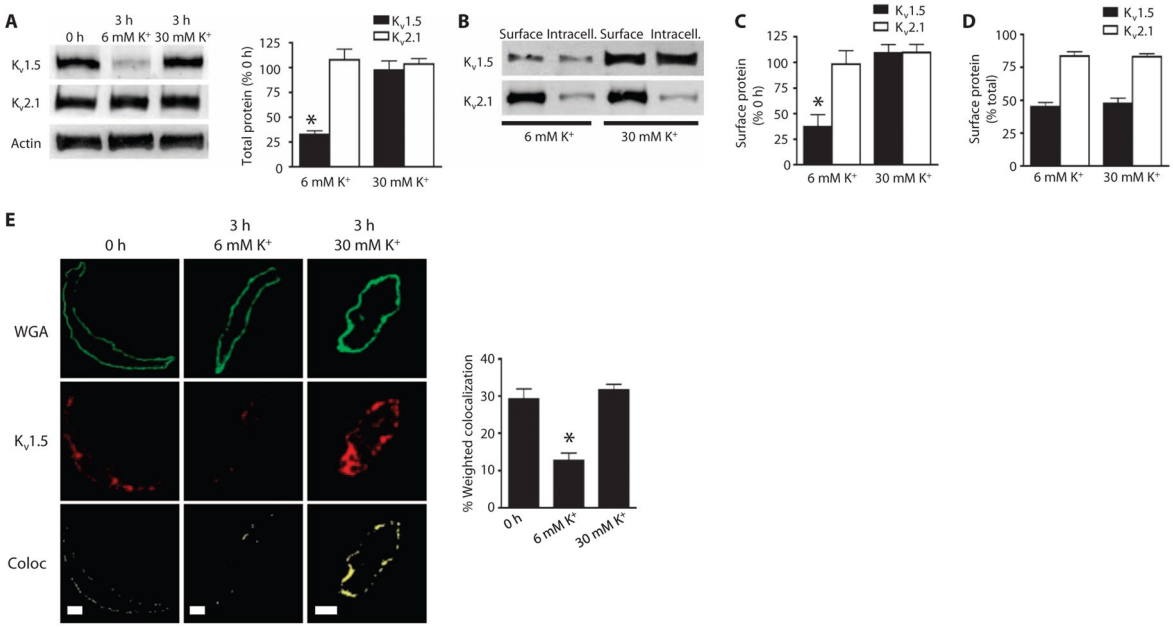



**Fig. 4. Membrane depolarization maintains $K_V1.5$ at the cell surface**

(**A**) Representative Western blots and quantitative data of total $K_V1.5$ and $K_V2.1$ in arteries exposed to 6 or 30 mM $K^+$ PSS for 3 hours. $n = 6$ for each. $^*P < 0.05$ versus 0 hour. (**B**) Western blot of arterial biotinylation samples showing the abundance of surface andintracellular $K_V1.5$ and $K_V2.1$ from arteries exposed to 6 or 30 mM $K^+$ PSS for 3 hours. (**C**) Mean data showing the percent of $K_V1.5$ and $K_V2.1$ at the cell surface compared to the amount at isolation (0 hour) after 3 hours in 6 or 30 mM $K^+$ PSS. $n = 6$ for each. $^*P < 0.05$ versus 0 hour. (**D**) Mean data showing $K_V1.5$ and $K_V2.1$ at the cell surface as a percent of the total of each channel protein in arteries after 3 hours in 6 or 30 mM $K^+$ PSS. $n = 5$ for each. (**E**) Immunofluorescence images and quantitative analysis of colocalization of wheat germ agglutinin (WGA) (green) and $K_V1.5$ (red) in myocytes isolated from arteries at 0 or 3 hours after maintenance in either 6 or 30 mM $K^+$ PSS. Yellow is pixel colocalization analyzed using the RG2B plug-in for ImageJ. Scale bars, 5 μm. $n = 7$ for each. $^*P < 0.05$ versus 0 hour.

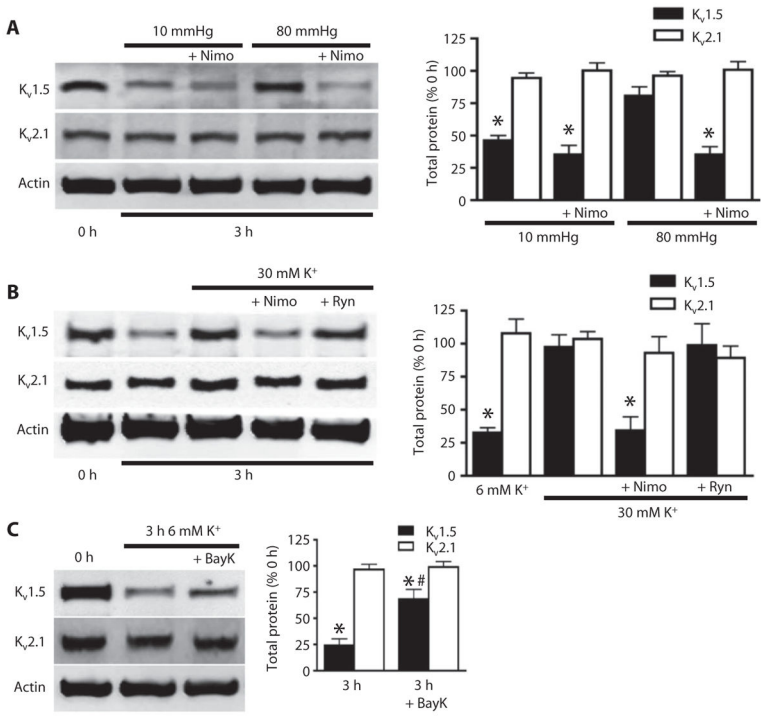



**Fig. 5. Voltage-dependent $Ca^{2+}$ channel activity controls $K_V$1.5 channel degradation**

(**A**) Representative Western blots and quantitative data showing the amount of total $K_V$1.5 and $K_V$2.1 in arteries exposed to 10 and 80 mmHg intravascular pressure for 3 hours in the presence or absence of nimodipine (Nimo; 1 μM). $n = 5$ for each. $^*P < 0.05$ versus 0 hour. (**B**) Western blot and quantitative data showing the amount of total $K_V$1.5 or $K_V$2.1 in arteries maintained for 3 hours in 6 or 30 mM $K^+$ PSS in the presence or absence of nimodipine (1 μM) or ryanodine (Ryn; 10 μM). $n = 6$ for each. $^*P < 0.05$ versus 0 hour. (**C**) Western blot and quantitative data showing the amount of total $K_V$1.5 and $K_V$2.1 from arteries exposed to 6 mM $K^+$ PSS for the indicated times with or without Bay K8644 (BayK; 100 nM). $n = 5$ for each. $^*P < 0.05$ versus 0 hour, $^{\#}P < 0.05$ versus 3 hour.

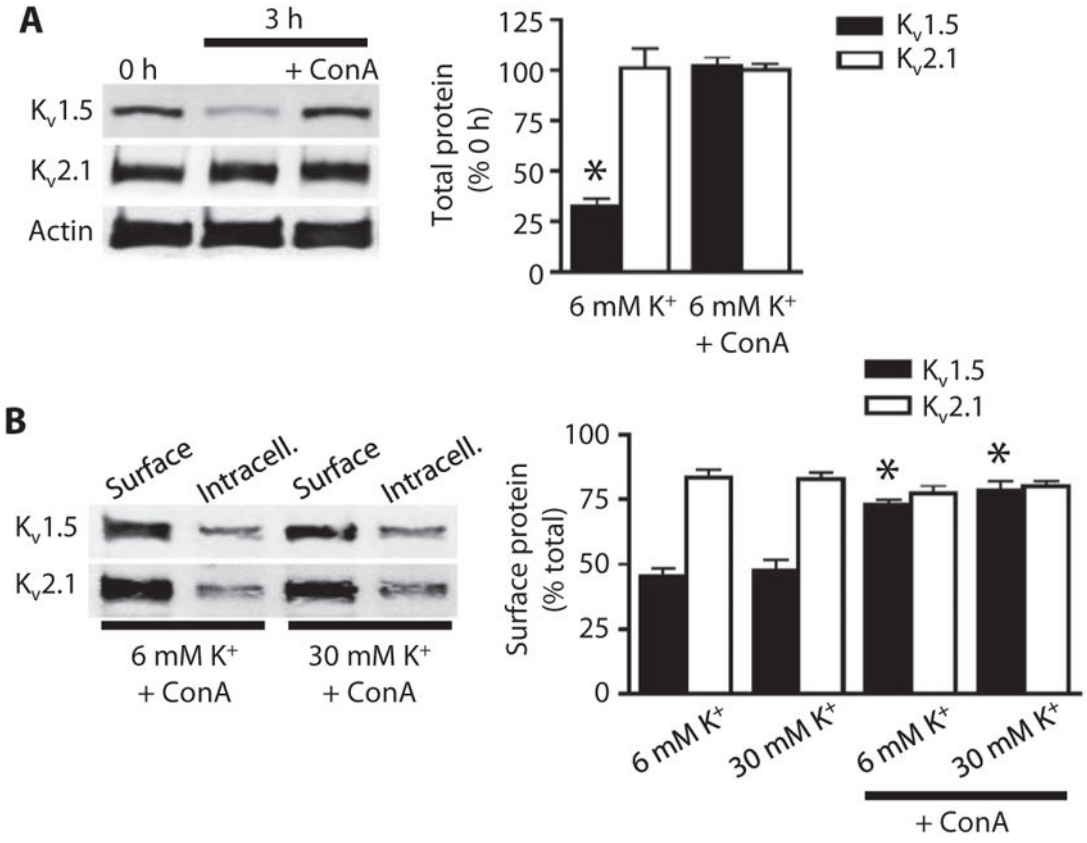
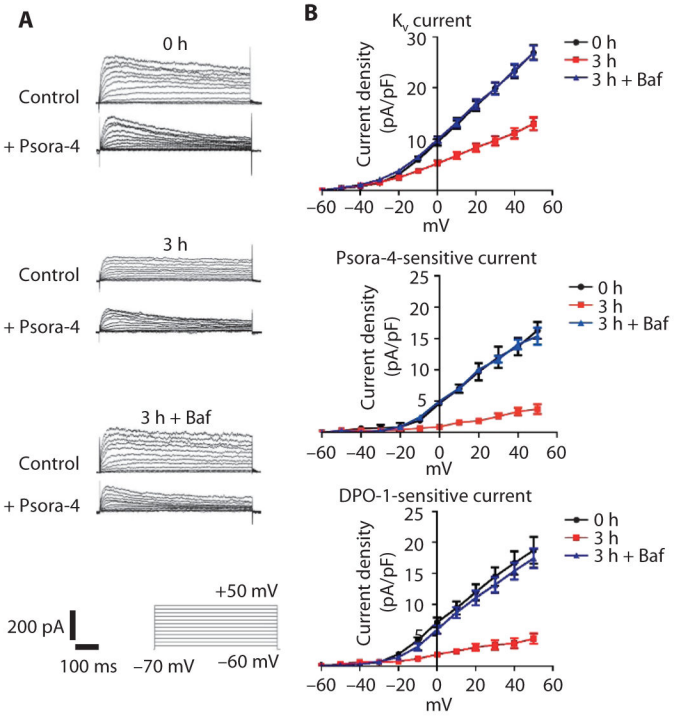



**Fig. 6. Concanavalin A prevents $K_v$1.5 degradation and increases surface abundance**

(**A**) Representative Western blot and quantitative data showing the amount of total $K_v$1.5 and $K_v$2.1 in arteries at 0 hour, 3 hours after isolation, and 3 hours after isolation with concanavalin A (ConA; 10 μM). $n = 4$ for each. $*P < 0.05$ versus 0 hour. (**B**) Western blot of arterial biotinylation samples showing the abundance of surface and intracellular $K_v$1.5 and $K_v$2.1 from arteries 3 hours after isolation in 6 or 30 mM $K^+$ PSS in the presence or absence of ConA. Mean data show the percentage of total $K_v$1.5 or $K_v$2.1 located at the cell surface. $n = 5$ for each. $*P < 0.05$ versus respective samples in the absence of ConA.




C

| | $K_v$ | Psora-4-sensitive | DPO-1-sensitive |
|---|---|---|---|
| 0 h | 25.5 ± 1.4 pA/pF | 16.7 ± 1.3 pA/pF | 18.7 ± 2.1 pA/pF |
| 3 h | 13.0 ± 1.2 pA/pF* | 3.7 ± 0.8 pA/pF* | 4.5 ± 0.8 pA/pF* |
| 3 h + Baf | 25.1 ± 1.5 pA/pF | 15.4 ± 1.3 pA/pF | 17.4 ± 1.6 pA/pF |

**Fig. 7. Arterial isolation reduces Psora-4– and DPO-1–sensitive whole-cell $K_V$ currents in arterial smooth muscle cells**

(**A**) Representative $K_v$ current recordings and the effect of Psora-4 (100 nM) in myocytes either isolated immediately from arteries (0 hour) or 3 hours after isolation and maintenance at 37°C without (3 hour) or with 50 nM bafilomycin (3 hours + Baf). Bottom panel shows voltage step protocol and scale. (**B**) Mean current-voltage relationships for whole-cell (top, $n$ = 12 for each), Psora-4 (100 nM)–sensitive ($n$ = 5 to 6), and DPO-1 (1 μM)–sensitive ($n$ = 5 to 6) currents at time points indicated with and without bafilomycin. (**C**) Mean current density values at +50 mV. *$P$ < 0.05 versus 0 hour.

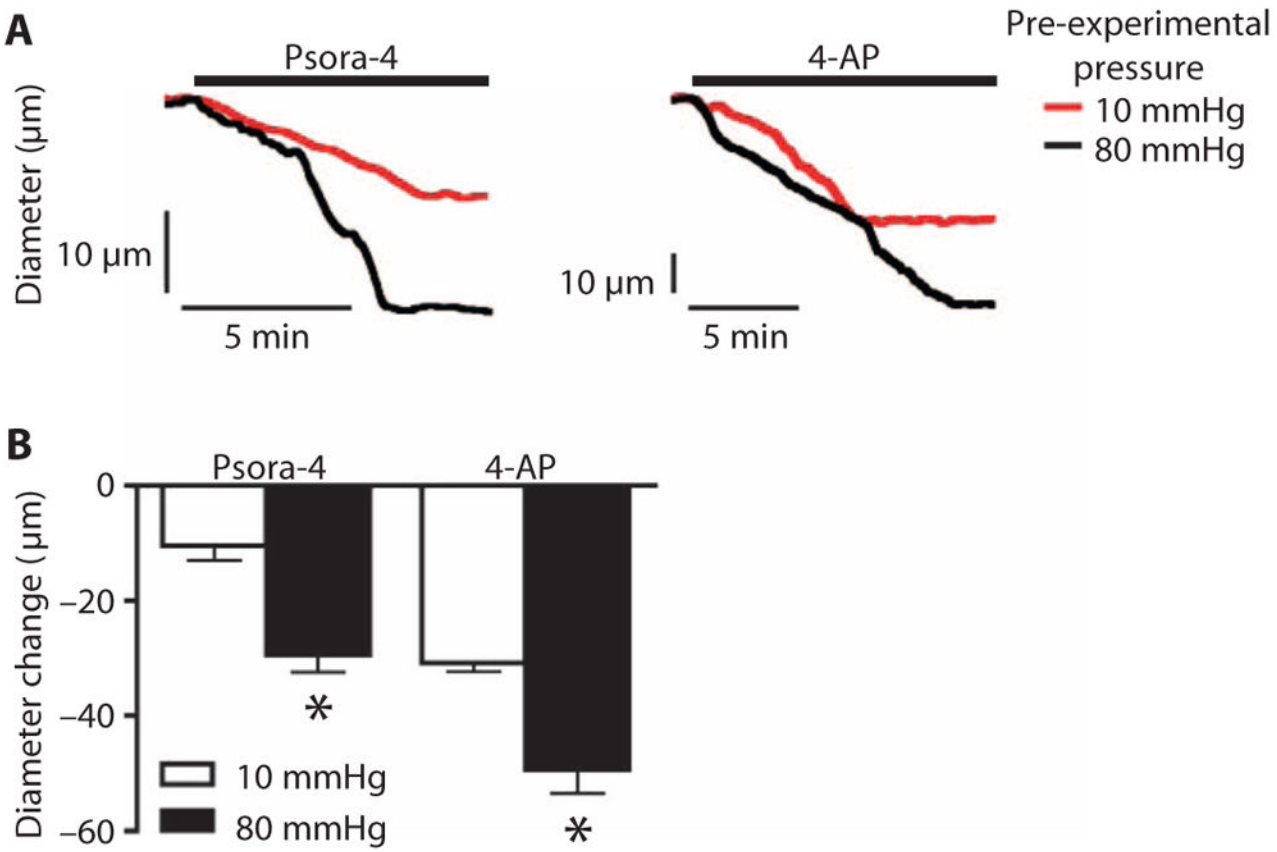



**Fig. 8. Intravascular pressure maintains functional $K_V1.5$ channels in arterial myocytes**

(**A**) Representative diameter traces showing vasoconstriction induced by Psora-4 (100 nM) or 4-AP (1 mM) at 80 mmHg for arteries maintained for 3 hours at pre-experimental pressures indicated. (**B**) Mean data showing the reduction in diameter in arteries exposed to the indicated drugs and held at the indicated pre-experimental pressures. $n = 5$ for each. $^*P <$ 0.05 versus 10 mmHg pre-experimental pressure group.