Angiotensin II reduces the surface abundance of $K_V1.5$ channels in arterial myocytes to stimulate vasoconstriction

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Key points

- Several different voltage-dependent K^+ (K_V) channel isoforms are expressed in arterial smooth muscle cells (myocytes).
- Vasoconstrictors inhibit K_V currents, but the isoform selectivity and mechanisms involved are unclear.
- We show that angiotensin II (Ang II), a vasoconstrictor, stimulates degradation of $K_V1.5$, but not $K_V2.1$, channels through a protein kinase C- and lysosome-dependent mechanism, reducing abundance at the surface of mesenteric artery myocytes.
- The Ang II-induced decrease in cell surface $K_V1.5$ channels reduces whole-cell $K_V1.5$ currents and attenuates $K_V1.5$ function in pressurized arteries.
- We describe a mechanism by which Ang II stimulates protein kinase C-dependent $K_V1.5$ channel degradation, reducing the abundance of functional channels at the myocyte surface.

Abstract Smooth muscle cells (myocytes) of resistance-size arteries express several different voltage-dependent K^+ (K_V) channels, including $K_V1.5$ and $K_V2.1$, which regulate contractility. Myocyte K_V currents are inhibited by vasoconstrictors, including angiotensin II (Ang II), but the mechanisms involved are unclear. Here, we tested the hypothesis that Ang II inhibits K_V currents by reducing the plasma membrane abundance of K_V channels in myocytes. Angiotensin II (applied for 2 h) reduced surface and total $K_V1.5$ protein in rat mesenteric arteries. In contrast, Ang II did not alter total or surface $K_V2.1$, or $K_V1.5$ or $K_V2.1$ cellular distribution, measured as the percentage of total protein at the surface. Bisindolylmaleimide (BIM; a protein kinase C blocker), a protein kinase C inhibitory peptide or bafilomycin A (a lysosomal degradation inhibitor) each blocked the Ang II-induced decrease in total and surface $K_V1.5$. Immunofluorescence also suggested that Ang II reduced surface $K_V1.5$ protein in isolated myocytes; an effect inhibited by BIM. Arteries were exposed to Ang II or Ang II plus BIM (for 2 h), after which these agents were removed and contractility measurements performed or myocytes isolated for patch-clamp electrophysiology. Angiotensin II reduced both whole-cell K_V currents and currents inhibited by Psora-4, a $K_V1.5$ channel blocker. Angiotensin II also reduced vasoconstriction stimulated by Psora-4 or 4-aminopyridine, another K_V channel inhibitor. These data indicate that Ang II activates protein kinase C, which stimulates $K_V1.5$ channel degradation, leading to a decrease in surface $K_V1.5$, a reduction in whole-cell $K_V1.5$ currents and a loss of functional $K_V1.5$ channels in myocytes of pressurized arteries.

(Resubmitted 19 August 2016; accepted after revision 30 November 2016; first published online 13 December 2016)
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Abbreviations 4-AP, 4-aminopyridine; Ang II, angiotensin II; AT_1, angiotensin II type 1 receptor; Baf, bafilomycin A; BIM, bisindolylmaleimide; BSA, bovine serum albumin; $Ca_V1.2$, voltage-dependent calcium channel; DAG, diacylglycerol; DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; K^+, potassium; K_V, voltage-dependent potassium channel; PKC, protein kinase C; PSS, physiological saline solution; WGA, wheat germ agglutinin.

DOI: 10.1113/JP272893

Introduction

In small, resistance-size arteries, intravascular pressure stimulates smooth muscle cell (myocyte) membrane depolarization, which activates voltage-dependent calcium ($Ca_V1.2$) channels, leading to an increase in $[Ca^{2+}]_i$ and vasoconstriction (Knot & Nelson, 1998; Davis & Hill, 1999). Arterial myocyte voltage-dependent potassium (K_V) channels are also activated by membrane depolarization, stimulating a negative feedback mechanism that opposes pressure-induced depolarization and vasoconstriction (Nelson & Quayle, 1995; Yuan, 1995; Albarwani *et al.* 2003; Plane *et al.* 2005; Amberg & Santana, 2006). This mechanism sets an equilibrium of arterial contractility that can be further modulated by vasoconstrictors and vasodilators to regulate regional organ blood flow and systemic blood pressure.

The K_V channels are a family of ∼40 proteins divided into 12 subclasses (K_V1–K_V12; Gutman *et al.* 2005). Several K_V family members are expressed in myocytes, including $K_V1.2$, $K_V1.5$, $K_V2.1$, $K_V2.2$, $K_V7.1$ and $K_V7.4$ (Xu *et al.* 1999; Albarwani *et al.* 2003; Fountain *et al.* 2004; Yeung *et al.* 2007). The whole-cell current amplitude (I) generated by a population of K_V channels is equally dependent on single-channel current (i), open probability (P_O) and the number of channels at the cell surface (N) such that $I = NP_Oi$. P_O is directly controlled by membrane potential, which modifies the conformation of the voltage-sensing, arginine-rich S4 segment in K_V channel subunits (Nelson & Quayle, 1995; Jiang *et al.* 2003). Membrane potential also regulates the number (N) of plasma membrane-resident $K_V1.5$ channels in arterial myocytes (Kidd *et al.* 2015). These mechanisms control functional K_V channel activity (NP_O) in arterial myocytes (Nelson & Quayle, 1995; Kidd *et al.* 2015). Whether vasoregulatory stimuli other than membrane potential, including vasoconstrictors, also modulate arterial contractility by controlling the surface abundance of K_V channels is unclear.

Angiotensin II (Ang II) is a vasoconstrictor produced by the renin–angiotensin system (Chappell, 2016). Angiotensin II binds angiotensin II type 1 (AT_1) receptors in arterial myocytes, stimulating $G_{q/11}$, leading to phospholipase C activation (Berk & Corson, 1997; Higuchi *et al.* 2007; Wynne *et al.* 2009). Phospholipase C cleaves phosphoinositide 4,5-bisphosphate into inositol triphosphate and diacylglycerol (DAG; Higuchi *et al.* 2007; Wynne *et al.* 2009). Diacylglycerol stimulates protein kinase C (PKC), which modulates a wide variety of ion channels in arterial myocytes, including $Ca_V1.2$, ATP-sensitive K^+ and Ca^{2+}-activated K^+ channels (Hughes, 1995; Nelson & Quayle, 1995; Taguchi *et al.* 2000; Higuchi *et al.* 2007; Wynne *et al.* 2009; Leo *et al.* 2015). Angiotensin II also inhibits K_V currents in arterial myocytes through a PKC-dependent mechanism, leading to vasoconstriction (Rainbow *et al.* 2009). Whether Ang II reduces surface K_V channel protein to inhibit K_V currents in arterial myocytes is unclear.

Here, we tested the hypothesis that Ang II controls surface levels of $K_V1.5$ and $K_V2.1$ channels, the predominant K_V isoforms expressed in rat mesenteric artery myocytes (Kidd *et al.* 2015). Data suggest that Ang II decreases total and surface $K_V1.5$ protein in arterial myocytes through a PKC- and lysosome-dependent mechanism, which reduces whole-cell $K_V1.5$ currents and $K_V1.5$ function in pressurized arteries. In contrast, $K_V2.1$ total and surface protein were unaltered by Ang II. These data suggest that Ang II controls surface $K_V1.5$ protein abundance to modulate arterial contractility.

Methods

Ethical approval

All animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, in accordance with the standards set by the National Institutes of Health. All experiments adhere to the ethical principles of *The Journal of Physiology* and comply with the journal's animal ethics checklist.

Preparation of tissues and isolated cells

Adult male Sprague–Dawley rats (200–250 g body mass) were purchased from Harlan Labs (Indianapolis, IN, USA) and fed *ad libitum*. Rats were administered CO_2 in a specially designed housing chamber until immobile, to reduce any discomfort, before being killed by I.P. injection of sodium pentobarbitone (150 mg kg^{-1}). The mesenteric vasculature was removed into ice-cold physiological saline solution (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 to pH 7.4. Third- and fourth-order mesenteric arteries (∼100–200 μm in diameter) were cleaned of adventitial tissue and dissected for experiments.

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

Surface biotinylation

Arteries were incubated with 1 mg ml^{-1} each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin (Thermo Scientific, Waltham, MA, USA) in ice-cold PSS for 1 h at 4°C. Unbound biotin was quenched with ice-cold 100 mM glycine in PBS, then arteries were washed with ice-cold PBS. Arteries were homogenized in ice-cold radioimmunoprecipitation assay buffer containing (mM): 50 Tris, 150 NaCl and 5 EDTA, with 0.1% SDS and 1% Triton-X. Avidin beads in spin columns were used to separate the biotinylated surface protein. Biotinylated proteins were freed from the avidin beads by boiling in SDS buffer containing 5% 2-mercaptoethanol. The whole volumes of either biotinylated or non-biotinylated protein samples from each preparation were loaded into separate lanes of the same polyacrylamide gel for electrophoresis.

Western blotting

Arteries were homogenized in radioimmunoprecipitation assay buffer with 1× SDS and 5% 2-mercaptoethanol. Arterial protein lysates were separated on 7.5% SDS–polyacrylamide gels, then transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T), then incubated with mouse monoclonal primary antibodies for K_V1.5, K_V2.1 (1:1000; NeuroMab, UC Davis, CA, USA) or actin (1:10000; Millipore, Billerica, MA, USA) in blocking solution overnight at 4°C. Membranes were washed with TBS-T before being incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, followed by a second wash with TBS-T. Protein bands were developed using Super-Signal enhanced chemiluminescence substrate (Thermo Scientific) with a Kodak In Vivo Pro Imaging System. Band intensity was analysed using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence

Isolated myocytes were plated on poly-L-lysine-coated coverslips and maintained for 2 h in 30 mM K^+ PSS alone, with Ang II or with Ang II in the presence of bisindolylmaleimide (BIM) before being fixed with formalin and incubated with Alexa 488-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA, USA), a plasma membrane stain. Myocytes were permeabilized with 0.1% Triton-X 100 and then blocked in 5% BSA before incubation with a mouse monoclonal K_V1.5 primary antibody (1:100 in 0.5% BSA; NeuroMab, UC Davis) overnight at 4°C. Myocytes were washed and incubated with an Alexa 546-conjugated donkey polyclonal secondary antibody to mouse IgG (1:100; Life Technologies, Carlsbad, CA, USA). Coverslips were washed, then secured to microscope slides using 1:1 PBS:glycerol. Images were acquired using a laser scanning confocal microscope (LSM Pascal; Carl Zeiss, Thornwood, NY, USA). Alexa 488 and 546 were excited at 488 and 543 nm, respectively, with emission collected at 505–530 and ≥560 nm, respectively. Zeiss Pascal system software was used to calculate weighted pixel colocalization coefficient values. The RG2B plugin for ImageJ was used to isolate colocalization pixel data, with automatic selection intensity threshold values for the red and green channels. Colocalization data were expressed as the average of the corresponding red and green channels, producing an image displaying only colocalized pixels.

Arteries were incubated with 1 mg ml^{-1} each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin (Thermo Scientific) in PSS for 1 h at 4°C. Unbound biotin was quenched with ice-cold 100 mM glycine in PBS, and arteries were washed with ice-cold PBS. Arteries were then postfixed with 4% paraformaldehyde in PBS overnight. After dehydration, vessels were embedded in paraffin and 5-μm-thick sections cut using a microtome and mounted on slides. Sections were deparaffinized and blocked in 5% BSA before incubation with Alexa Fluor 546 streptavidin (Molecular Probes, Eugene, OR, USA) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Thermo Scientific) for 1 h at 37°C. Slides were then washed in PBS. Images were acquired using a laser-scanning confocal microscope and a ×63 objective (Zeiss 710; Carl Zeiss). The DAPI and Alexa 546 were excited at 405 and 561 nm, respectively, with emission collected at ≤437 and ≥552 nm, respectively.

Patch-clamp electrophysiology

Experiments were performed on isolated myocytes that were allowed to adhere to a glass coverslip for ∼15 min before experimentation. Whole-cell currents were recorded using an Axopatch 200B amplifier and Clampex 10.4 (Molecular Devices, Sunnyvale, CA, USA). The 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 (mM): 120 NaCl, 3 $NaHCO_3$, 4.2 KCl, 1.2 KH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 1 TEA and 10 Hepes, and pH was adjusted to 7.4 with NaOH. Pipette solution contained (mM): 110 potassium gluconate, 30 KCl, 0.5$MgCl_2$, 5 Na_2ATP, 1 GTP, 10 EGTA and 5 Hepes, and pH was adjusted to 7.2 with KOH. The K_V currents were digitized at 5 kHz and filtered at 1 kHz. Offline analysis was performed using Clampfit 10.4 (Molecular Devices).

Pressurized artery myography

Third-order mesenteric artery segments (0.5–1 mm in length, 150–200 μm diameter) were denuded of

endothelium by introducing air into the lumen for ∼1 min. Arteries were then cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation, St Albans City, VT, USA) and continuously perfused with PSS. Intravascular pressure was modulated using an attached reservoir and monitored with a pressure transducer. Arterial diameter was measured using a closed-circuit camera and IonWizard edge-detection software (IonOptix, Westwood, MA, USA). Myogenic tone was calculated as: $100 \times (1 - D_{Active}/D_{Passive})$, where D_{Active} is active arterial diameter and $D_{Passive}$ is arterial diameter in Ca^{2+}-free PSS supplemented with 5 mM EGTA.

Statistical analysis

Data were analysed using ANOVA with Newman–Keuls *post hoc* test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). All data are expressed as mean values ± SEM. A value of $P < 0.05$ was considered significant.

Results

Angiotensin II reduces plasma membrane $K_V1.5$ channels via protein kinase C activation in arterial myocytes

Membrane potential controls both the surface abundance and total protein of $K_V1.5$ channels in arterial myocytes (Kidd *et al.* 2015). Arterial isolation (2 h) in PSS containing 6 mM K^+ at 37°C led to a decrease in $K_V1.5$ total protein to ∼31% of that in fresh isolated (0 h) control arteries (Fig. 1*A* and *B*). $K_V1.5$ protein loss was prevented by maintenance in a PSS containing 30 mM K^+, which depolarizes arterial myocytes to ∼−40 mV, a membrane potential similar to arteries at physiological intravascular pressure (Fig. 1*A* and *B*; Knot & Nelson, 1998). In contrast, $K_V2.1$ total protein was unaltered by arterial isolation (2 h) in PSS containing either 6 or 30 mM K^+ (Fig. 1*A* and *B*). These data are consistent with previous findings (Kidd *et al.* 2015).

We have previously demonstrated that biotin labels plasma membrane proteins, but not intracellular proteins, in rat resistance-size cerebral arteries (Bannister *et al.* 2009). Arterial biotinylation has been used to measure the abundance and cellular distribution of surface and intracellular ion channels and auxiliary subunits in arterial myocytes (Adebiyi *et al.* 2010; Bannister *et al.* 2009, 2016; Crnich *et al.* 2010; Thomas-Gatewood *et al.* 2011; Narayanan *et al.* 2013; Evanson *et al.* 2014; Nourian *et al.* 2014; Leo *et al.* 2015). To determine whether biotin permeates and labels proteins in the wall of rat mesenteric arteries, immunofluorescence was performed. Mesenteric arteries were exposed to biotin reagents, after which sections were cut and incubated with Alexa Fluor 546-labelled streptavidin. Confocal imaging demonstrated that biotin labelled proteins throughout the wall of mesenteric arteries, indicating efficient labelling consistent with data in cerebral arteries (Fig. 1*C*; Bannister *et al.* 2009).

The regulation of surface $K_V1.5$ and $K_V2.1$ protein by Ang II, a vasoconstrictor, was studied using arterial biotinylation in depolarized (30 mM K^+) arteries. Angiotensin II (applied for 2 h) reduced both total and surface $K_V1.5$ to ∼38 and ∼42% of control values, respectively (Fig. 1*A*, *B*, *D* and *E*). In contrast, Ang II did not alter $K_V1.5$ cellular distribution, which was calculated as the percentage of total $K_V1.5$ protein present at the cell surface (Fig. 1*D* and *F*). Angiotensin II also did not alter total or surface $K_V2.1$ protein or $K_V2.1$ cellular distribution (Fig. 1*A*, *B*, *D*, *E* and *F*). To ensure that the avidin beads used to pull down biotinylated proteins were 100% efficient, Western blots were probed with horseradish peroxidase-conjugated avidin. Horseradish peroxidase labelling was observed only in the surface lane and not in the intracellular lane, indicating that biotinylated proteins did not escape pull-down and contaminate the non-biotinylated protein fraction (Fig. 1*G*). These data indicate that Ang II reduces both total and surface $K_V1.5$ to a similar extent, but does not alter $K_V2.1$ protein, in mesenteric arteries.

The binding of many vasoconstrictors, including Ang II, to their plasma membrane receptors stimulates $G_{q/11}$ in arterial myocytes (Berk & Corson, 1997). $G_{q/11}$ activates phospholipase C, leading to an elevation in DAG, which stimulates PKC (Berk & Corson, 1997). We tested the hypothesis that Ang II controls $K_V1.5$ protein through a PKC-mediated mechanism. Bisindolylmaleimide, a PKC-specific inhibitor, blocked the Ang II-induced decrease in both total and surface $K_V1.5$ protein (Fig. 1*B*–*F*). In contrast, BIM, when applied alone, did not alter total or surface $K_V1.5$ protein or $K_V1.5$ cellular distribution (Fig. 1*B*–*F*). Bisindolylmaleimide also did not change total or surface $K_V2.1$ when applied either alone or together with Ang II (Fig. 1*B*–*F*). A membrane-permeant, myristoylated PKC inhibitory peptide (Myr-RFARKGALRQKNV) also blocked the Ang II-induced reduction in both total and surface $K_V1.5$ protein, but did not alter $K_V2.1$ protein (Fig. 1*B*–*F*). These data indicate that Ang II reduces total and surface $K_V1.5$ protein through a PKC-dependent mechanism in mesenteric arteries.

Immunofluorescence was performed to measure the regulation of $K_V1.5$ localization by Ang II in isolated mesenteric artery myocytes. Angiotensin II reduced $K_V1.5$ channel immunofluorescence colocalization with wheat germ agglutinin, a plasma membrane stain, from ∼29 to ∼9%, and this effect was prevented by BIM (Fig. 2*A* and *B*). Taken together, these data suggest that Ang II-induced PKC activation reduces surface and total $K_V1.5$ channel protein in arterial myocytes.

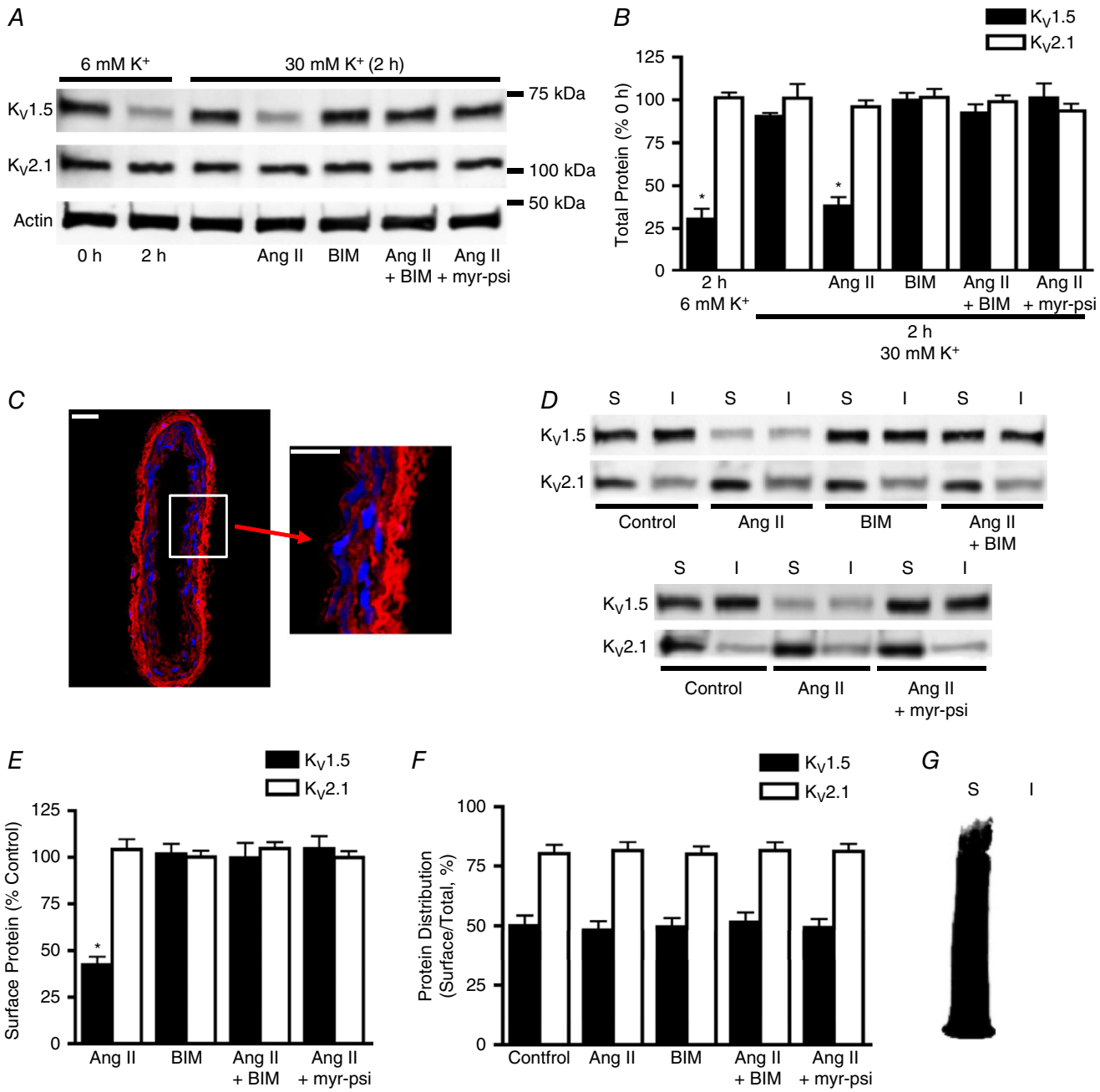

Figure 1. Angiotensin II reduces total and surface $K_V1.5$ channel protein through protein kinase C (PKC) activation in mesenteric arteries

A, representative Western blot images of total arterial $K_V1.5$, $K_V2.1$ and actin protein at 0 or 2 h in 6 mM K^+ physiological saline solution (PSS), 30 mM K^+ PSS, or 30 mM K^+ PSS with angiotensin II (Ang II; 100 nM), bisindolylmaleimide (BIM; 10 μM), Ang II plus BIM, or a myristoylated PKC inhibitory peptide (myr-psi; 100 μM). *B*, mean data. $n = 6$ for each. $^*P < 0.05$ *vs.* 0 h. *C*, immunofluorescence of a section cut from a biotinylated mesenteric artery labelled with Alexa Fluor 546 streptavidin (red) and DAPI (blue), a nuclear stain. Scale bars represent 20 μm. *D*, representative Western blot images of arterial biotinylation samples illustrating surface (S) and intracellular (I) $K_V1.5$ and $K_V2.1$ following 2 h in 30 mM K^+ alone (control) or in the presence of Ang II (100 nM), BIM (10 μM), Ang II plus BIM, or Ang II plus myr-psi (100 μM). *E*, mean data for surface protein in conditions from experiments shown in *C* calculated as a percentage of control surface protein. $n = 6$ for each. $^*P < 0.05$ *vs.* control. *F*, quantitative analysis of the relative cellular distribution of total $K_V1.5$ and $K_V2.1$ protein in conditions from *C* calculated as the percentage of total protein located at the cell surface. $n = 6$ for each. *G*, representative image of a Western blot probed with horseradish peroxidase-conjugated avidin, illustrating that the avidin bead pull-down procedure is 100% efficient and that no biotinylated proteins are present in the non-biotinylated protein fraction.

Angiotensin II stimulates $K_V1.5$ channel degradation in arterial myocytes

$K_V1.5$ continuously recycles between the plasma membrane and the intracellular compartment in mesenteric artery myocytes (Kidd *et al.* 2015). Conceivably, Ang II might stimulate $K_V1.5$ channel internalization, degradation or both in arterial myocytes. To test these possibilities, we studied regulation by bafilomycin A, a lysosomal v-ATPase and, thus, lysosomal degradation inhibitor. Bafilomycin A prevented the Ang II-mediated decrease in surface $K_V1.5$ protein (Fig 3*A* and *B*). In contrast, bafilomycin alone did not alter surface $K_V1.5$ protein (Fig. 3*A* and *B*). To determine whether Ang II altered channel internalization, effects on $K_V1.5$ cellular distribution were calculated. Angiotensin II, bafilomycin, or Ang II applied in the presence of bafilomycin did not alter the relative cellular distribution of $K_V1.5$ (Fig. 3*A* and *C*). Total and surface $K_V2.1$ were unaltered by Ang II, bafilomycin, or Ang II applied with bafilomycin (Fig. 3*A*–*C*). These data suggest that Ang II stimulates lysosomal degradation of internalized $K_V1.5$ protein, but indicate that Ang II does not stimulate $K_V1.5$ internalization in arterial myocytes.

Angiotensin II-induced PKC activation reduces $K_V1.5$ current density in arterial myocytes

To determine the functional effect of an Ang II-mediated reduction in surface $K_V1.5$ channels, patch-clamp electrophysiology was performed to measure $K_V1.5$ current density in isolated arterial myocytes. Arteries were exposed to 30 mM K^+ PSS alone (control), together with Ang II, or with both Ang II and BIM for 2 h, after which these agents were removed and myocytes isolated and used for electrophysiology. Patch-clamp solutions were designed to isolate whole-cell K_V currents and reduce contamination from other current types, including large-conductance Ca^{2+}-activated K^+ channels. Angiotensin II treatment reduced mean K_V current density from ~31 to ~14 pA pF^{-1}, or to ~45% (at +50 mV) of that in control myocytes (Fig. 4*A* and *B*). Involvement of $K_V1.5$ channels was investigated using Psora-4, a $K_V1.5$ open channel blocker that inhibits currents activated during voltage pulses (Fig. 4*A*; Marzian *et al.* 2013). Psora-4 reduced mean K_V current density (measured at the end of voltage pulses) in control myocytes by ~18 pA pF^{-1} (at +50 mV; Fig. 4*A* and *B*). In contrast, in Ang II-treated myocytes, Psora-4 reduced mean K_V current density by ~5 pA pF^{-1} (at +50 mV; Fig. 4*A* and *B*). The Psora-4-induced reduction

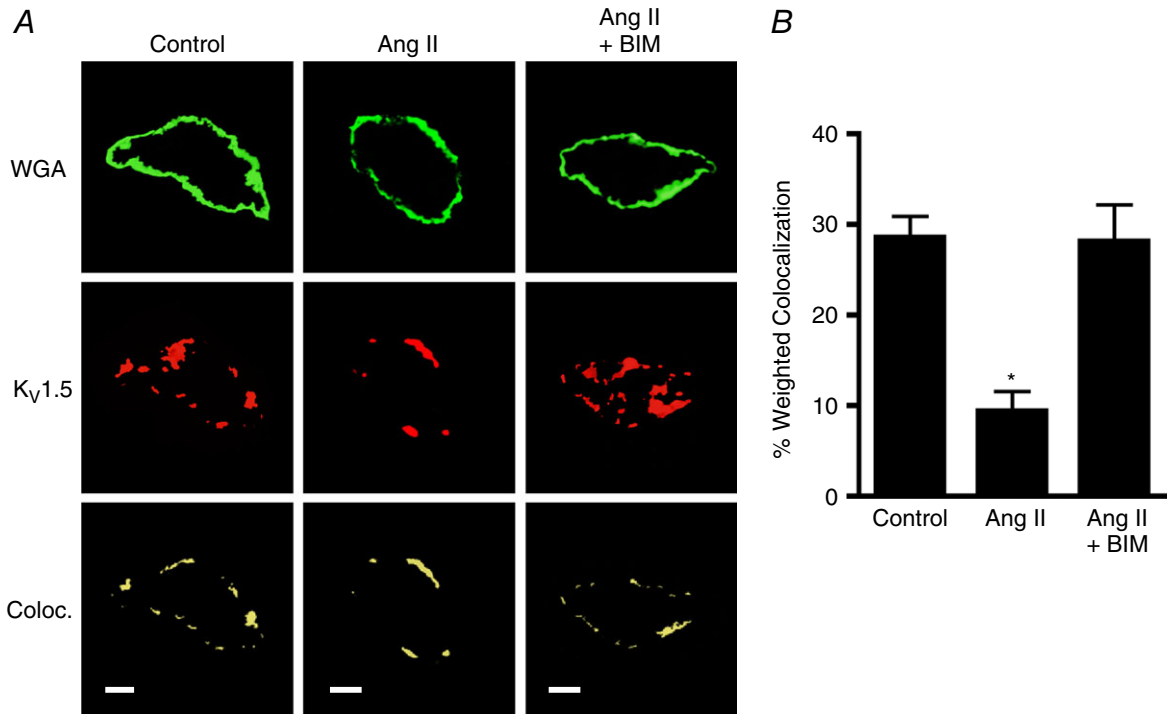

Figure 2. Angiotensin II reduces surface $K_V1.5$ channels in isolated arterial myocytes
A, immunofluorescence images illustrating colocalization of $K_V1.5$ (red) and wheat germ agglutinin (WGA, green) in isolated myocytes maintained for 2 h in 30 mM K^+ alone (control), with Ang II (100 nM) or with Ang II plus BIM (10 μM). Scale bars represent 5 μm. *B*, quantitative analysis for the percentage of WGA pixels that colocalize with $K_V1.5$. $n = 8$ for each. $^*P < 0.05$ *vs.* control.

in K_V current in Ang II-treated myocytes was ∼28% of that in control myocytes (at +50 mV; Fig. 4*A* and *B*). Bisindolylmaleimide prevented the Ang II-induced reduction in both whole-cell K_V and Psora-4-sensitive K_V currents (Fig. 4*A* and *B*). These data illustrate that Ang II-induced PKC activation reduces surface $K_V1.5$ channel protein, which decreases both whole-cell and $K_V1.5$ current density in arterial myocytes.

Angiotensin II inhibits functional $K_V1.5$ channel activity in pressurized arteries

To investigate the significance of an Ang II-induced reduction in surface $K_V1.5$ channels in arterial myocytes, myography was performed on pressurized, endothelium-denuded myogenic third-order mesenteric arteries. Arteries were exposed to 30 mM K^+ PSS alone, with Ang II, or with both Ang II and BIM for 2 h. These conditions were then removed, arteries were pressurized to 80 mmHg in PSS containing 6 mM K^+, and myogenic tone and acute vasoconstriction in response to Psora-4 or 4-aminopyridine (4-AP), a broad K_V channel inhibitor, were measured.

In myogenic arteries that were maintained in 30 mM K^+ PSS (control), Psora-4 or 4-AP stimulated acute mean vasoconstrictions of ∼20.6 and ∼40.4 μm, respectively (Fig. 5*A* and *B*). In contrast, Psora-4 or 4-AP contracted arteries that had been exposed to Ang II by ∼8.8 and ∼24.2 μm, respectively, or ∼42.7 and ∼59.9% of that in control arteries (Fig. 5*A* and *B*). Bisindolylmaleimide blocked the Ang II-induced reduction in Psora-4- and 4-AP-mediated vasoconstriction (Fig. 5*A* and *B*). In contrast, vasoconstriction in response to 60 mM K^+ was not altered by Ang II (Fig. 5*C*). Myogenic tone was also similar in all three conditions: control, 16.2 ± 0.86% (n = 6); Ang II, 15.0 ± 0.83% (n = 6); and Ang II plus BIM, 15.8 ± 0.73% (n = 6). These data suggest that Ang II-induced PKC activation reduces the abundance of functional surface $K_V1.5$ channels in arterial myocytes.

Discussion

Angiotensin II inhibits K_V currents in arterial myocytes, but the mechanisms involved are unclear. Here, we show that Ang II reduces the surface abundance of $K_V1.5$ channels in mesenteric artery myocytes. Angiotensin II-induced PKC activation stimulates $K_V1.5$ channel degradation, which reduces whole-cell K_V currents in myocytes and attenuates K_V function in myogenic arteries. In contrast, Ang II does not modulate surface $K_V2.1$ channel abundance in arterial myocytes. Thus, our data suggest that Ang II selectively decreases

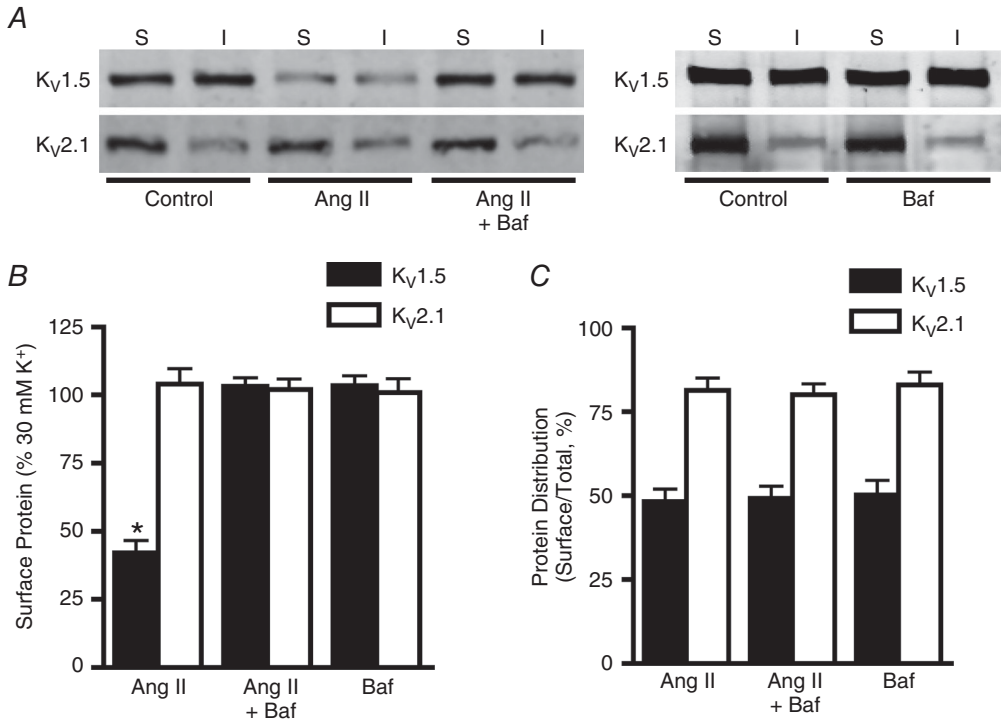

Figure 3. Angiotensin II stimulates $K_V1.5$ lysosomal degradation in mesenteric arteries
A, representative Western blot images illustrating surface and intracellular $K_V1.5$ and $K_V2.1$ protein after 2 h in 30 mM K^+ (control), 30 mM K^+ plus Ang II (100 nM) or 30 mM K^+ plus Ang II and bafilomycin (Baf, 50 nM). *B*, mean data illustrating surface $K_V1.5$ and $K_V2.1$ protein normalized to control. n = 6 for each. *P < 0.05 *vs.* control. *C*, quantitative data showing relative cellular distribution of total $K_V1.5$ and $K_V2.1$ protein. n = 6 for each.

surface $K_V1.5$ protein, thereby reducing K_V current density in arterial myocytes and inhibiting vasodilatation by these channels, leading to vasoconstriction.

Our data here and a previous study show that membrane potential and Ang II both regulate the surface abundance of $K_V1.5$ channel protein in arterial myocytes (Kidd *et al.* 2015). Angiotensin II binds to AT_1 receptors, which are coupled to $G_{q/11}$ proteins that stimulate phospholipase C in vascular myocytes (Berk & Corson, 1997). Phospholipase C activation elevates DAG, a PKC activator (Berk & Corson, 1997). Protien kinase C directly phosphorylates myosin light chain kinase, which then phosphorylates myosin light chain, promoting an interaction between actin and myosin, resulting in vasoconstriction (Gallagher *et al.* 1997). Data suggest that Ang II stimulates degradation of internalized $K_V1.5$ channels through a PKC-dependent mechanism. Bisindolylmaleimide (a selective PKC inhibitor) or a myristoylated, cell-permeant PKC inhibitory peptide prevented the Ang II-mediated reduction in surface and total $K_V1.5$ channels. Three isoforms of PKC are present in vascular myocytes; PKCα and PKCβ are DAG and Ca^{2+} dependent, and PKCε is stimulated by DAG (Newton, 1995). Angiotensin II-mediated PKCε activation reduces whole-cell K_V currents in arterial myocytes, and PKCε inhibition reduces Ang II-induced vasoconstriction in mesenteric artery rings (Rainbow *et al.* 2009). In contrast to Ang II, membrane depolarization inhibits $K_V1.5$ channel degradation via $Ca_V1.2$ channel activation (Kidd *et al.* 2015). Here, to study Ang II regulation of $K_V1.5$ channels in myocytes, experiments were performed in arteries depolarized with PSS containing 30 mM K^+, which depolarizes myocytes to ~−40 mV, a membrane potential similar to arteries at physiological intravascular pressure (Knot & Nelson, 1998). Although the kinases involved have not been identified, membrane potential and Ang II are likely to control $K_V1.5$ degradation through different mechanisms, as PKC stimulates $K_V1.5$ degradation and $Ca_V1.2$ channel activation inhibits $K_V1.5$ degradation. Angiotensin II-induced $K_V1.5$ degradation described here may be attributable to PKCε, whereas depolarization-induced $K_V1.5$ protection may occur as a

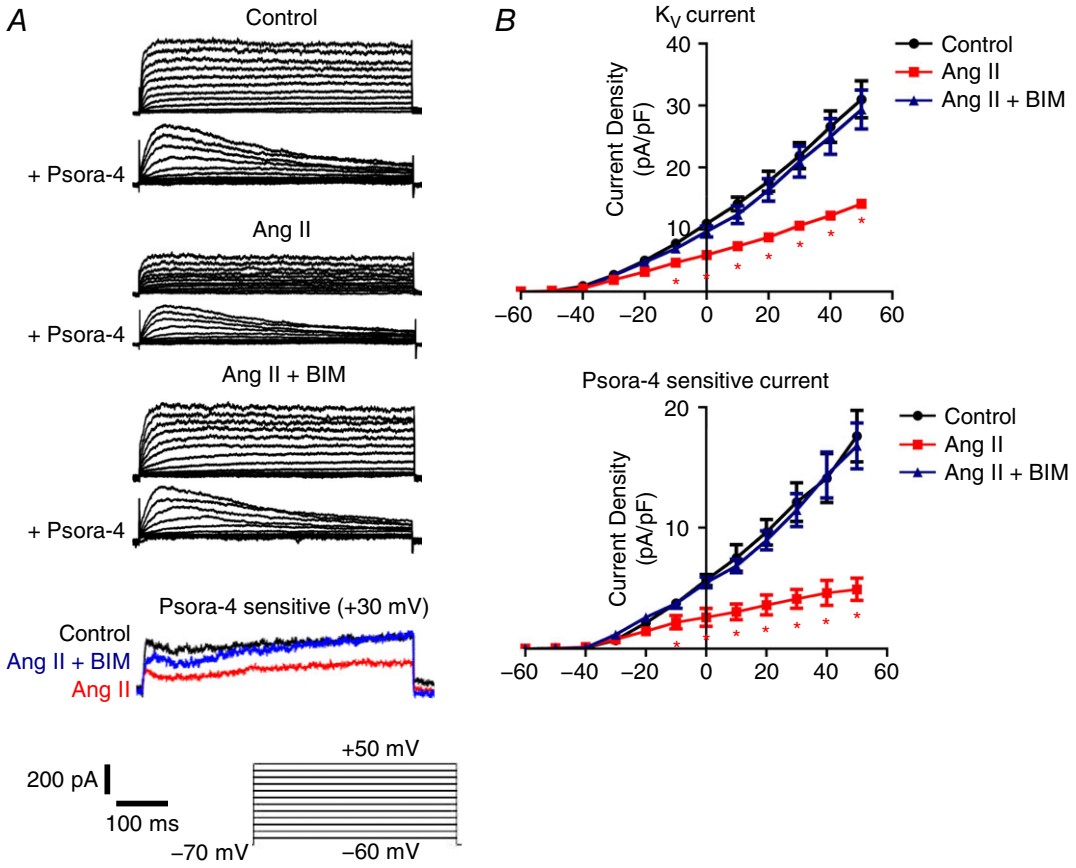

Figure 4. Angiotensin II reduces whole-cell K_V and $K_V1.5$ currents in arterial myocytes
A, representative whole-cell K_V current recordings in myocytes isolated from arteries that had been maintained for 2 h in 30 mM K^+ (control), 30 mM K^+ plus Ang II (100 nM) or 30 mM K^+ plus Ang II and BIM (10 μM). Currents were measured in myocytes immediately following isolation. *B*, mean current–voltage relationships for whole-cell (top, $n = 7$ for each) and Psora-4-sensitive currents (bottom, $n = 7$ for each). $^*P < 0.05$ *vs.* control.

result of Ca^{2+}-dependent PKCs or other Ca^{2+}-activated kinases. Future studies should be designed to test these hypotheses.

Bafilomycin, an inhibitor of lysosomal degradation, prevented the Ang II-induced reduction in surface and total $K_V1.5$ protein, but did not change $K_V1.5$ relative cellular distribution in arterial myocytes. These data suggest that Ang II stimulates $K_V1.5$ channel degradation, but does not activate $K_V1.5$ internalization or recycling in arterial myocytes. $K_V1.5$ channels continuously recycle in mesenteric artery myocytes, suggesting that Ang II-induced PKC activation shuttles internalized channels into the lysosomal degradation pathway (Kidd *et al.* 2015). In contrast, $K_V2.1$ channels do not recycle over the same time course, which may be one reason why Ang II does not stimulate degradation of these proteins. $K_V1.5$ channels recycle through Rab4- and Rab11-dependent pathways in HL-1 mouse atrial myocytes, H9c2 rat cardiomyoblasts and HEK293 cells expressing recombinant $K_V1.5$, suggesting involvement of recycling and early endosomes (McEwen *et al.* 2007; Zadeh *et al.* 2008). Oxidative stress increased colocalization of internalized $K_V1.5$ channels with the chaperone heat shock protein 70, which targets proteins for ubiquitylation and degradation, in HL-1 cells (Pratt *et al.* 2010; Svoboda *et al.* 2012). Oxyhaemoglobin stimulates $K_V1.5$ channel internalization through a tyrosine kinase-dependent mechanism in rabbit cerebral artery myocytes (Ishiguro *et al.* 2006). Angiotensin II-induced regulation of $K_V1.5$ transcription is highly unlikely to explain the mechanism we describe here. Arteries were exposed to Ang II for 2 h, which is a period of time unlikely to alter $K_V1.5$ protein through transcription. Supporting this conclusion are our data showing that bafilomycin blocked the Ang II-mediated reduction in $K_V1.5$ protein. We have also previously shown that arterial depressurization for 3 h reduces $K_V1.5$ protein by >60% without any change in $K_V1.5$ mRNA, indicating that protein processing and transcription can occur independently in arterial myocytes (Kidd *et al.* 2015).

Several studies have investigated trafficking pathways that modulate ion channel abundance at the cell surface in arterial myocytes. $\alpha_2\delta$-1, an auxiliary subunit of voltage-dependent Ca^{2+} channels, is required for trafficking of $Ca_V1.2$ to the cell surface in cerebral artery myocytes (Bannister *et al.* 2009). Myocyte surface expression of both $Ca_V1.2$ and $\alpha_2\delta$-1 subunits is increased in rats with genetic hypertension, elevating $Ca_V1.2$ current density and producing a non-inactivating current that stimulates vasoconstriction (Bannister *et al.* 2012). The surface abundance of TRPM4, a melastatin transient receptor potential channel, is

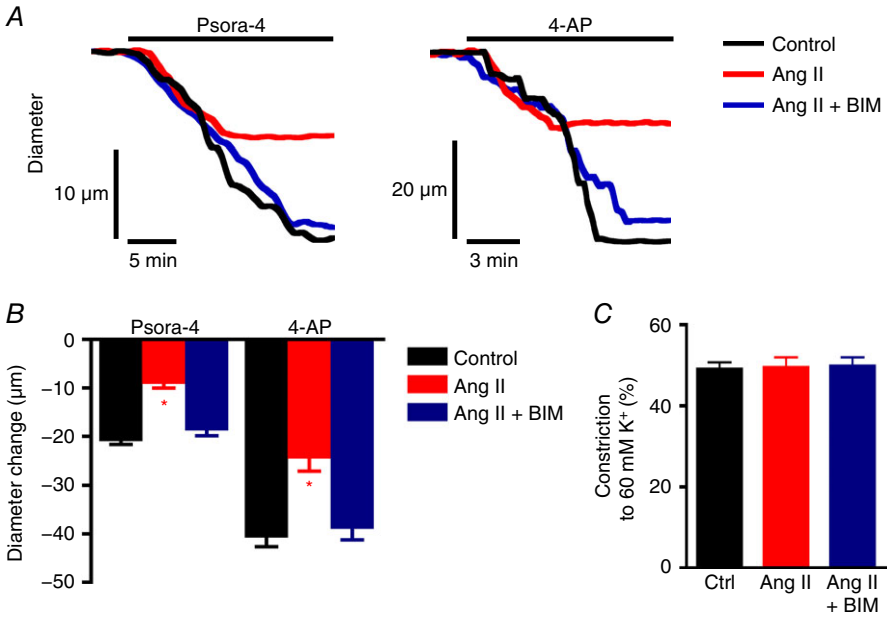

Figure 5. Angiotensin II reduces functional $K_V1.5$ channel activity in pressurized arteries
A, representative diameter traces for vasoconstriction stimulated by Psora-4 (100 nM) or 4-aminopyridine (4-AP, 1 mM) at an intravascular pressure of 80 mmHg for arteries maintained for 2 h in 30 mM K^+ alone (control) or plus Ang II (100 nM) or plus Ang II with BIM (10 μM). Initial arterial diameters before application of Psora-4 or 4-AP were normalized. *B*, mean data illustrating vasoconstriction stimulated by Psora-4 or 4-AP. $n = 6$ for each. Mean passive diameters were similar for arteries used in each data set ($P > 0.05$ for each, e.g. mean passive diameter for control arteries was 231.6 ± 4.7 μm, for Ang II-treated arteries 228.0 ± 4.1 μm, and for arteries treated with Ang II and BIM 232.8 ± 4.8 μm). *$P < 0.05$ *vs.* control. *C*, mean data for vasoconstrictive response to 60 mM K^+ PSS at 80 mmHg intravascular pressure. $n = 6$ for each.

increased by PKCδ in cerebral artery myocytes (Crnich *et al.* 2010). Nitric oxide stimulates rapid trafficking of large-conductance Ca^{2+}-activated K^+ (BK) channel β1 subunits to the myocyte surface to activate BK channels and produce vasodilatation in cerebral arteries (Leo *et al.* 2014). Serotonin, a neurotransmitter and vasoconstrictor, reduced surface K_V1.5 channels, as determined using immunofluorescence, in pulmonary artery myocytes (Cogolludo *et al.* 2006). Physiological intravascular pressure, via depolarization-induced voltage-dependent Ca^{2+} channel activity, stimulates K_V1.5 channel surface expression in mesenteric artery myocytes (Kidd *et al.* 2015). Here, we show that Ang II reduces surface K_V1.5, the predominant K_V channel subtype, in mesenteric artery myocytes (Kidd *et al.* 2015). Myocytes of other vascular beds may express different populations of K_V channel subtypes (Kerr *et al.* 2001; Albarwani *et al.* 2003; Amberg & Santana, 2006; Zhong *et al.* 2010; Jepps *et al.* 2011). As such, the regulatory pathway described here for K_V1.5 may be more relevant in some vessels than others. K_V1.5 and K_V1.2 form functional heteromultimeric channels in rat mesenteric artery myocytes and rabbit portal vein myocytes (Kerr *et al.* 2001; Plane *et al.* 2005). Angiotensin II-mediated modulation of surface K_V1.5 may alter K_V channel heteromultimer formation and abundance in arterial myocytes. Collectively, these studies indicate that multiple, distinct trafficking pathways, with differing time courses and mechanisms, modulate the surface abundance and activity of a variety of ion channels in arterial myocytes to regulate contractility.

The use of Psora-4, a K_V1.5 channel inhibitor, in patch-clamp electrophysiology and myography experiments supports our biochemical data showing that Ang II regulates K_V currents and vasocontractility by modulating K_V1.5 surface expression. Psora-4 is a psoralen compound that blocks both K_V1.3 and K_V1.5 channels at low nanomolar concentrations, but is a less effective blocker of other K_V1 family members (Vennekamp *et al.* 2004). There are few to no data suggesting the expression or function of K_V1.3 in contractile arterial myocytes (Cidad *et al.* 2010; Cheong *et al.* 2011). Psoralens also weakly inhibit K_V2, K_{ir}2.1, BK and Ca_V1.2 channels in arterial myocytes, but at concentrations 25–150 times higher than used here (Nelson & Quayle, 1995; Vennekamp *et al.* 2004; Schmitz *et al.* 2005). Arteries treated with Ang II developed the same amount of myogenic tone as control arteries despite the reduction in K_V1.5 function measured with Psora-4 or 4-AP. The reasons for this are unclear, but one explanation is that Ang II modulates the surface abundance of other ion channels that control arterial contractility, leading to no net effect on myogenic tone. Vasoconstriction stimulated by 60 mM K^+ was unaltered, indicating that Ang II does not cause non-specific alteration of contractility and does not appear to modify voltage-dependent Ca^{2+} channel protein or activity. Data indicate that the Ang II-induced reduction in K_V1.5 surface protein inhibits K_V currents and arterial contractility regulation by K_V1.5 channels.

In summary, we show that Ang II selectively stimulates degradation of K_V1.5 channels to reduce functional K_V1.5 channel surface abundance in mesenteric artery myocytes. Angiotensin II-mediated PKC activation stimulates lysosomal degradation of internalized K_V1.5 channels, reducing protein available for recycling. Through this mechanism, Ang II inhibits whole-cell K_V1.5 currents, thereby reducing their functional modulation of arterial contractility.

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Additional information

Competing interests

The authors declare they have no conflict of interest.

Author contributions

M.W.K. designed and performed the experiments, conducted the data analysis, prepared the figures and wrote the manuscript. S.B. performed the experiments, prepared the figures and wrote the manuscript. J.H.J. conceived the project, designed and directed the experiments and wrote the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work was supported by National Institutes of Health grants HL67061, 094378 and HL110347 to J.H.J.

Acknowledgements

We thank Drs M. Dennis Leo and Padmapriya Muralidharan for comments on the manuscript.