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Membrane depolarization activates BK channels through ROCK-mediated β1 subunit surface trafficking to limit vasoconstriction

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Abstract

Membrane depolarization of smooth muscle cells (myocytes) in the small arteries that regulate regional organ blood flow leads to vasoconstriction. Membrane depolarization also activates large-conductance calcium (Ca^{2+})–activated potassium (BK) channels, which limits Ca^{2+} channel activity that promotes vasoconstriction, thus leading to vasodilation. We showed that in human and rat arterial myocytes, membrane depolarization rapidly increased the cell surface abundance of auxiliary BK β1 subunits but not that of the pore-forming BKα channels. Membrane depolarization stimulated voltage-dependent Ca^{2+} channels, leading to Ca^{2+} influx and the activation of Rho kinase (ROCK) 1 and 2. ROCK1/2-mediated activation of Rab11A promoted the delivery of β1 subunits to the plasma membrane by Rab11A-positive recycling endosomes. These additional β1 subunits associated with BKα channels already at the plasma membrane, leading to an increase in apparent Ca^{2+} sensitivity and activation of the channels in pressurized arterial myocytes and vasodilation. Thus, membrane depolarization activates BK channels through stimulation of ROCK- and Rab11A-dependent trafficking of β1 subunits to the surface of arterial myocytes.

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SUPPLEMENTARY MATERIALS

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Fig. S1. Effect of different antagonists on depolarization- and SNP-induced changes in the surface abundance of BKα and β1 protein.
Fig. S2. Depolarization-induced surface trafficking of β1 subunits in human cerebral arteries.
Fig. S3. Overexpression of β1 subunits in HEK293 cells and colocalization analysis of BKα-β1 FRET with WGA.
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Fig. S6. Effect of ROCK 1 or ROCK2 knockdown and HA1100 on respective protein abundance.
Fig. S7. Rab11A knockdown does not alter vasoconstriction by membrane depolarization or myogenic tone.

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INTRODUCTION

Membrane potential regulates physiological functions in virtually all cell types, including neurons, endocrine cells, and smooth muscle cells (myocytes) of resistance-size arteries that control regional organ blood flow and systemic blood pressure (1). Membrane depolarization in small arteries leads to the activation of myocyte voltage-dependent calcium (Ca^{2+}) ($Ca_V1.2$) channels, leading to an increase in intracellular Ca^{2+} concentration and vasoconstriction. In contrast, membrane hyperpolarization reduces voltage-dependent Ca^{2+} channel activity in arterial myocytes, leading to vasodilation. Investigating the physiological processes that control membrane potential is essential to better understand the mechanisms that regulate vascular contractility.

Large-conductance Ca^{2+}-activated potassium (BK) channels are found in many different cell types, including arterial myocytes (2). Membrane potential is a key regulator of functional BK channel activity, although mechanisms involved in native cell types are poorly understood (3). Depolarization-induced BK channel activation attenuates voltage-dependent Ca^{2+} channel activity in arterial myocytes, limiting vasoconstriction (4, 5). BK channels are tetramers of pore-forming α (BKα) subunits that can couple to auxiliary β subunits, which comprise a family of four isoforms (β1-β4) (2, 6). β-subunit isoforms are distributed in a tissue-specific manner and can regulate the apparent Ca^{2+} sensitivity and gating properties of BK channels to customize cellular functionality (7). Both BKα and β1 subunits are present in arterial myocytes, and β1 subunits increase the apparent Ca^{2+} sensitivity of BK channels (8). β1 subunits are essential for BK channels to control arterial myocyte contractility and systemic blood pressure (7, 8). Cardiovascular diseases, including atherosclerosis, stroke, and hypertension, are associated with pathological alterations in BK channel β1 subunit abundance and function (9). Whether membrane potential controls BK channel activity by modulating interactions between BKα and β1 subunits is poorly understood, particularly in native cell types.

Membrane current (I) generated by a population of ion channels, including BK channels, is the product of the number of channels (N), open probability (P_o), and single-channel current (i). Membrane de-polarization and a rise in intracellular Ca^{2+} concentration increase the open probability of BK channels, but whether these activators control the number of plasma membrane BK channel subunits in arterial myocytes and other cell types to modulate cellular functions is unclear (3). In resting arterial myocytes, BKα subunits are essentially plasma membrane–localized proteins that are surface-trafficked by Rab4A-positive early endosomes (10). In contrast, only a small proportion of total β1 subunit protein is located at the plasma membrane, with much of the intracellular β1 protein stored within mobile Rab11A-positive recycling endosomes (11). Here, we tested the hypothesis that membrane potential and the intracellular Ca^{2+} concentration control the surface abundance of BKα and β1 subunits to regulate channel activity and contractility in arterial myocytes.

We showed that membrane depolarization, through the stimulation of voltage-dependent Ca^{2+} channels and Ca^{2+} influx, activated Rho kinase (ROCK) 1 and 2, which phosphorylated Rab11A, leading to the rapid delivery of intracellular β1 subunits to the plasma membrane in arterial myocytes. These β1 subunits increased the apparent Ca^{2+}

sensitivity of plasma membrane–resident BK channels, resulting in an increase in BK channel open probability and vasodilation. Thus, we describe a mechanism by which membrane potential regulates functional BK channel activity through the modulation of ROCK-dependent trafficking of β1 subunits to the surface.

RESULTS

Membrane depolarization increases the surface abundance of BK channel β1 subunits in arterial myocytes

Cellular distribution of BKα and β1 subunits was measured in myocytes of intact resistance-size (diameter of ~200 μm) cerebral arteries using surface biotinylation. Regulation by membrane potential was studied by increasing the extracellular K^+ concentration in the bath solution from 6 to either 30 or 60 mM, which depolarizes arteries from ~−60 to −40 or −20 mV, respectively (12). In rat cerebral arteries maintained in physiological saline solution (PSS) containing 6 mM extracellular K^+, ~95.5% of total BKα and ~7.7% of total β1 subunits were plasma membrane–localized (Fig. 1, A and B, and fig. S1, A to D). Increasing bath extracellular K^+ to 30 or 60 mM stimulated a ~2.7- or ~3.2-fold increase in surface β1 protein, respectively (Fig. 1, A and B). Depolarization increased surface β1 protein within 1 min, the shortest time point measured (Fig. 1, A and B). In contrast, membrane depolarization did not alter surface BKα protein (Fig. 1A and fig. S1, C and D). Arterial re-polarization, produced by returning depolarized arteries to a bath solution containing 6 mM extracellular K^+, reversed the depolarization-induced increase in surface β1 protein by ~75% in 1 min and completely in 10 min (fig. S1, E and F). Similar data were obtained in human cerebral arteries, where depolarization (30 mM K^+) increased surface β1 ~2.4-fold but did not alter surface BKα (fig. S2, A and B). Brefeldin A, an endoplasmic reticulum–to–Golgi trafficking inhibitor, blocked depolarization-induced β1 subunit surface trafficking but did not alter surface BKα (Fig. 1, A and B, and fig. S1, C and D). β1 subunit antibody specificity was confirmed with lysates from recombinant β1-transfected human embryonic kidney (HEK) 293 cells. The β1 antibody did not identify a protein in lysate from mock-transfected HEK293 cells but detected a band of ~34 kDa in cells expressing recombinant β1, which was similar in molecular mass to that of arterial β1 protein (fig. S3A). These data indicate that membrane depolarization stimulates intracellular β1 subunits to traffic to the plasma membrane in rat and human cerebral artery myocytes.

Immunofluorescence Förster resonance energy transfer (FRET) imaging was used to examine the regulation of BKα and β1 subunit spatial proximity by membrane depolarization in isolated arterial myocytes. The Förster coefficient for the Alexa 488–Alexa 546 FRET pair used for these experiments is ~6.3 nm. Membrane depolarization, produced by increasing extracellular K^+ concentration in the bath solution from 6 to 60 mM, contracted arterial myocytes and increased mean normalized FRET between BKα- and β1-bound secondary antibodies ~3.4-fold (Fig. 1, C and D). Returning depolarized (60 mM K^+) myocytes to a bath solution containing 6 mM K^+ (10 min) reversed the increase in normalized FRET (Fig. 1, C and D). Brefeldin A abolished the depolarization-induced increase in BKα-β1 normalized FRET (Fig. 1, C and D). In depolarized cells (30 mM K^+), colocalization of FRET with wheat germ agglutinin (WGA), a plasma membrane marker,

produced a coefficient of ~93.3 ± 1.3%, indicating that most of the BKα-β1 signal occurs at the surface (fig. S3B). These data suggest that membrane depolarization stimulates surface trafficking of β1 subunits, which then associate with plasma membrane–resident BKα channels in arterial myocytes.

By acting through soluble guanylyl cyclase (sGC) and guanosine 3′,5′-monophosphate–dependent protein kinase (PKG), nitric oxide (NO) stimulates the surface trafficking of β1 subunits that reside within Rab11A-positive recycling endosomes in arterial myocytes (11). Here, we investigated whether membrane depolarization and NO mobilize the same, or different, β1 protein pools. The NO donor sodium nitroprusside (SNP) increased surface β1 protein ~2.5-fold in arteries in a 6 mM K^+–containing PSS and ~3.6-fold in arteries in 30 mM K^+ and did not further increase surface β1 protein in arteries in 60 mM K^+ when compared to 30 mM K^+ (Fig. 1B and fig. S1B). The sGC inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the protein kinase G inhibitor DT-2 blocked the surface trafficking of β1 subunits induced by SNP but not by depolarization (fig. S1, A and B). SNP, ODQ, and DT-2 did not alter surface BKα protein (fig. S1, C and D). These data indicate that membrane depolarization and NO activate different signaling pathways to stimulate an increase in the surface abundance of β1 subunits.

To investigate the source of β1 subunits mobilized by membrane depolarization, we used RNA interference. Short hairpin RNAs (shRNAs) targeting Rab11A reduced Rab11A protein to ~48% of scrambled shRNA controls in arteries (fig. S4, A and C). Rab11A knockdown abolished the depolarization-induced increase in β1 surface abundance in arteries and the increase in normalized FRET between BKα and β1 subunits in isolated myocytes (Fig. 2, A to D). A Rab11A dominant-negative (Rab11AS25N) mutant also blocked the depolarization-induced increase in the surface abundance of β1 subunits (Fig. 2, E and F, and fig. S4, B and C). Rab11A knockdown or the Rab11A dominant-negative mutant did not alter surface BKα protein (fig. S4D). These data indicate that membrane depolarization and NO activate distinct intracellular signaling pathways to mobilize the same intracellular pool of β1 subunits that reside within Rab11A-positive recycling endosomes.

Voltage-dependent Ca^{2+} channel activity controls β1 subunit surface abundance

Next, we investigated signaling pathways by which membrane potential controls the surface abundance of β1 subunits. Membrane depolarization stimulates voltage-dependent Ca^{2+} channels, leading to an increase in the intracellular Ca^{2+} concentration in arterial myocytes (1, 13). Ca^{2+}-free PSS or the $Ca_V1.2$ channel blocker nimodipine inhibited depolarization-induced β1 subunit trafficking (Fig. 3, A and B). $Ca_V1.2$ small interfering RNA (siRNA) decreased $Ca_V1.2$ protein by ~60% and reduced depolarization-induced β1 subunit trafficking by ~75% of scrambled siRNA controls (fig. S5, B and C, and Fig. 3, C and D). These findings confirmed the role of Ca^{2+} influx in mediating the β1 surface trafficking through $Ca_V1.2$. Membrane depolarization stimulates local intracellular Ca^{2+} transients termed Ca^{2+} sparks, which activate BK channels in arterial myocytes (4). Ryanodine, a ryanodine-sensitiveCa^{2+}-releasechannel(RyR)blocker that inhibits Ca^{2+} sparks, did not alter depolarization-induced β1 trafficking (Fig. 3B). $Ca_V1.2$ knockdown, nimodipine, or ryanodine did not alter BKα surface protein (Fig. 3A and fig. S5, A and D). These data

indicate that membrane potential control of voltage-dependent Ca^{2+} channel activity and Ca^{2+} influx regulates β1 subunit surface abundance.

Membrane depolarization stimulates ROCK-dependent phosphorylation of Rab11A to activate β1 subunit trafficking

An increase in intracellular Ca^{2+} concentration can activate several kinases, including ROCK, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), phosphoinositide 3-kinase (PI3K), and protein kinase C (PKC) in arterial myocytes (14–17). The ROCK inhibitor HA1100 attenuated depolarization-induced β1 surface trafficking (Fig. 3, A and B). In contrast, the CaMKII inhibitor KN93, the PI3K inhibitor LY294002, or the PKC inhibitor bisindolylmaleimide (BIM II) did not alter depolarization-induced β1 trafficking (Fig. 3B).

Both ROCK1 and ROCK2 are present in arterial myocytes (18). To examine ROCK isoforms involved, we transfected arteries with siRNA targeting either ROCK1 or ROCK2. ROCK1 siRNA reduced ROCK1 protein to ~50% of scrambled siRNA controls, whereas ROCK2 siRNA reduced ROCK2 protein to ~47% of controls (fig. S6, A and B). Each siRNA did not alter the amount of the other ROCK isoform, indicating selectivity (fig. S6, A and B). ROCK1- and ROCK2-specific knockdown each reduced depolarization-induced β1 trafficking by ~91 and ~70%, respectively, of scrambled siRNA controls (Fig. 3, E and F). In contrast, ROCK1 or ROCK2 knockdown did not alter surface BKα (Fig. 3, E and F, and fig. S6C). These experiments suggest that depolarization activates ROCK1 and ROCK2, which stimulate β1 subunit anterograde trafficking, leading to an increase in β1 surface abundance in arterial myocytes.

Next, we tested the hypothesis that activated ROCK phosphorylated Rab11A. Membrane depolarization increased guanosine 5′-triphosphate–bound active Rab11 in arteries, an effect that was inhibited by HA1100 (Fig. 4, A and B). In contrast, depolarization alone or in the presence of HA1100 did not alter Rab11A, β1, or BKα total protein (Fig. 4A and fig. S6D). Together, these data indicate that membrane depolarization stimulates voltage-dependent Ca^{2+} channels, leading to Ca^{2+} influx and the activation of ROCK1/2, which phosphorylates Rab11A, thereby promoting the surface trafficking of Rab11A-positive recycling endosomes that deliver β1 subunits to the plasma membrane.

Depolarization-induced β1 subunit trafficking increases the apparent Ca^{2+} sensitivity of BK channels in arterial myocytes

β1 subunits increase the apparent Ca^{2+} sensitivity of BK channels, leading to channel activation (7, 19). To study the regulation of BK channel activity by depolarization-induced β1 subunit trafficking, we performed patch-clamp electrophysiology. Inside-out patches were pulled from myocytes that had been exposed to a bath solution containing either 6 mM K^+ (control), 30 mM K^+, or 30 mM K^+ plus HA1100. After patch excision, bath solutions were then changed so that the apparent Ca^{2+} sensitivity of BK channels was measured in membrane patches under identical conditions, in symmetrical K^+ at −40 mV, a physiological membrane voltage. In control myocytes, the mean apparent dissociation constant (K_d) for Ca^{2+} of BK channels was ~34 μM, with a maximum open probability of ~0.80 ± 0.02 (Fig. 4, C and D). In patches pulled from myocytes placed in 30 mM K^+ bath solution, the mean

K_d for Ca^{2+} of BK channels was ~9 μM or 3.8-fold higher, without an alteration in maximal open probability (0.80 ± 0.03; Fig. 4, C and D). HA1100 blocked the 30 mM K^+ bath solution from increasing the Ca^{2+} sensitivity of BK channels but did not change maximal open probability (0.08 ± 0.03; Fig. 4, C and D). These data indicate that membrane depolarization activates ROCK to increase BK channel apparent Ca^{2+} sensitivity in arterial myocytes.

Lithocholate, a specific activator of β1 subunit–containing BK channels, was used to study regulation of BK channels. We examined whether lithocholate would be a more effective activator of BK channels that contain additional depolarization-trafficked β1 subunits. Lithocholate increased BK channel mean open probability in inside-out patches pulled from both control (6 mM K^+) and 30 mM K^+–treated myocytes (Fig. 4, C and E). Moreover, lithocholate increased mean open probability 2.74 ± 0.02–fold more in 30 mM K^+–treated myocytes than in 6 mM K^+–treated myocytes, consistent with the hypothesis being tested (Fig. 4, C and E). HA1100 attenuated the depolarization-induced increase in BK channel open probability (Fig. 4, C and E). These data indicate that depolarization-stimulated β1 subunit surface trafficking increases BK channel Ca^{2+} sensitivity and activation by a β1 ligand and that, overall, depolarization increases the amount of β1 subunits associated with BK channels.

Intravascular pressure stimulates BK channels through Rab11A activation in arterial myocytes

Intravascular pressure stimulates BK channels in arterial myocytes (20). We tested whether Rab11A-mediated β1 subunit trafficking contributed to intravascular pressure–induced BK channel activation in arterial myocytes. ROCK could not be manipulated for these contractile measurements because this kinase contributes to myocyte contraction through multiple mechanisms, including phosphorylation of myosin phosphatase, myosin light chain (MLC) kinase, and CPI-17 (21). In pressurized, myogenic (60 mmHg) control arteries transfected with scrambled siRNA, the BK channel activator NS1619 stimulated a mean vasodilation of ~34.7 μm, and the specific BK channel inhibitor, iberiotoxin, produced a mean constriction of ~25.5 μm (Fig. 5, A and B). Lithocholate dilated control arteries by ~9.7 μm (Fig. 5, A and C). Rab11A knockdown reduced mean NS1619- and lithocholate-induced vaso-dilation and iberiotoxin-induced vasoconstriction to ~17.6, 4.6, and 13 μm, respectively, which are ~49.3, ~47.0, and ~51.0% of those in scrambled controls (Fig. 5, A to C). Rab11A knockdown did not alter vasoconstriction stimulated by membrane depolarization or myogenic tone (fig. S7, A and B). These data demonstrate that intravascular pressure stimulates BK channels through Rab11A-induced β1 subunit trafficking in arterial myocytes.

DISCUSSION

Intravascular pressure–induced membrane depolarization activates BK channels in arterial myocytes (4, 5). Here, we described a mechanism by which intravascular pressure and membrane potential controlled BK channel activity by modulating the plasma membrane abundance of auxiliary β1 subunits in arterial myocytes. Our data indicated that membrane

depolarization stimulated voltage-dependent Ca^{2+} channels, leading to Ca^{2+} influx and ROCK1 and ROCK2 activation. We showed that ROCK phosphorylated Rab11A, stimulating anterograde trafficking of Rab11A-positive recycling endosomes, which delivered intracellular β1 subunits to the plasma membrane. These β1 subunits associated with plasma membrane–resident BK channels, increasing their apparent Ca^{2+} sensitivity and leading to channel activation and vasodilation. Thus, we showed that membrane depolarization, through ROCK-dependent stimulation of Rab11A-dependent β1 surface trafficking, activated BK channels in arterial myocytes to modulate arterial contractility.

Physiological intravascular pressures stimulate arterial depolarization between ~−60 and −35 mV, with the entire contractile range of cerebral arteries occurring between membrane potentials of ~−60 and −25 mV (4). This depolarization range stimulates voltage-dependent Ca^{2+} channels, leading to an increase in “global” intracellular Ca^{2+} concentration from ~100 to 350 nM in arterial myocytes (4). BK channels are sensitive to micromolar intracellular Ca^{2+} concentrations, as shown here and elsewhere (11, 22–24). Thus, global intracellular Ca^{2+} concentration is inadequate to substantially alter BK channel open probability (4). BK channels in arterial myocytes are activated by Ca^{2+} sparks, which are local micromolar Ca^{2+} transients that occur due to the release of sarcoplasmic reticulum Ca^{2+} through RyR channels (4). A single Ca^{2+} spark activates several nearby BK channels, leading to a transient BK current (4). Here, data indicated that membrane depolarization rapidly stimulated anterograde trafficking and plasma membrane insertion of intracellular β1 subunits, which associated with surface BKα and increased apparent Ca^{2+} sensitivity. We showed that a small proportion of total β1 subunits are present in the plasma membrane of resting (~−60 mV) arterial myocytes and that depolarization within a physiological membrane potential range rapidly increased surface β1 protein abundance. Several lines of evidence support a requirement for β1 subunits in depolarization-induced BK channel activation. Ca^{2+} spark to BK channel coupling and contractility regulation by BK channels are abolished in β1 subunit knockout mice (8). Analysis of recombinant proteins demonstrates that a BK channel tetramer can contain between one and four β1 subunits and that the α/β1-tetramer ratio can shift BK channel voltage and Ca^{2+} dependence (25–27). Consistent with these findings, BK channel Ca^{2+} sensitivity and transient BK current amplitude were both low at resting potentials and increased with physiological membrane depolarization [here and in (24)]. β1 subunits increase BK channel apparent Ca^{2+} sensitivity in arterial myocytes (6). Our data indicated that membrane depolarization stimulated Rab11A-positive recycling endosomes to deliver β1 subunits to the plasma membrane. The β1 subunit–mediated increase in Ca^{2+} sensitivity should enhance BK channel coupling to Ca^{2+} sparks, leading to an increase in transient BK currents and vasodilation.

Previous studies of recombinant BK channel proteins have shown that the association of β1 with BKα either does not alter the slope of the P_o-[Ca^{2+}] curve or can increase the slope (28–30). Whether β1 subunits regulate the slope of the P_o-[Ca^{2+}] curve in arterial myocytes was unclear. Data here and in a previous study (11) both demonstrated that depolarization-induced and NO-induced surface trafficking of β1 subunits increased the apparent Ca^{2+} sensitivity of BK channels but did not alter the slope of the P_o-[Ca^{2+}] curve in arterial myocytes. Several factors may explain different results obtained from investigations of both recombinant and native arterial myocyte BK channels, including splice variants and mutants

studied and the presence or absence of other proteins. Regulatory proteins may also be involved, including auxiliary γ1 subunits, which modify both BK channel modulation by β1 subunits and BK channel properties in arterial myocytes (31, 32).

We showed that depolarization, through a $Ca_V1.2$- and Ca^{2+} influx–dependent mechanism, activated ROCK, leading to Rab11A-mediated β1 subunit trafficking in arteries. In contrast, PKC, PI3K, or CaMKII were not involved in depolarization-induced β1 subunit trafficking. A depolarization-induced increase in intracellular Ca^{2+} concentration stimulates Rho, leading to ROCK activation in rabbit aortic myocytes (17), providing further support for a Ca^{2+}-dependent mechanism. The ROCK pathway regulates multiple cellular functions, including contraction and proliferation, and is associated with cardiovascular diseases (18). Substrates of ROCK include MLC and myosin phosphatase target subunit 1, modulation of which regulates myosin Ca^{2+} sensitivity and vascular myocyte contractility (18). Here, knockdown of either ROCK1 or ROCK2 inhibited depolarization-induced β1 trafficking, indicating a requirement for both isoforms. Several reasons may explain why both isoforms are necessary, including that they function in a series signaling cascade. ROCKs homodimerize, suggesting that they may also form a heterodimeric protein that controls β1 trafficking in arterial myocytes (33). ROCK may stimulate recycling endosome trafficking by directly phosphorylating Rab11A or may act upstream by phosphorylating one or more kinases that control Rab11A activity. The Rab11A dominant-negative mutant used here (S25N), which blocked depolarization-induced β1 trafficking, is guanosine diphosphate–bound and inactive. Analysis of the Rab11A amino acid sequence [using an open-source online software, Prediction of PK-Specific Phosphorylation (PPSP) (34)] revealed five potential phosphorylation sites for ROCK, at Thr^{32}, Thr^{43}, Thr^{77}, Thr^{98}, and Ser^{149}. It was beyond the scope of this study to determine whether ROCK indirectly activated Rab11A or directly phosphorylated Rab11A and which, if any, of these potential phosphorylation sites are involved in β1 trafficking. Future studies should test these hypotheses. In summary, data indicated that membrane depolarization activated $Ca_V1.2$ channels, leading to the stimulation of ROCK1 and ROCK2, which activated Rab11A to deliver β1 subunits to the plasma membrane in arterial myocytes.

NO stimulates a rapid increase in the surface abundance of β1 subunits in arterial myocytes, leading to BK channel activation and vasodilation (11). Data here indicated that depolarization and NO mobilized the same intracellular β1 subunit pool but through distinct mechanisms. NO acts through PKG, whereas membrane depolarization functions through $Ca_V1.2$ channels and ROCK. NO increases surface β1 protein in nondepolarized arterial myocytes at resting potentials of ~−60 mV (11), and we showed that NO also stimulated an increase in surface β1 protein at membrane potentials of ~−40 mV, similar to that in arteries at a physiological intravascular pressure of ~60 mmHg (12). In addition to supporting previous findings, these data indicate that at physiological membrane potentials found in pressurized arteries, NO increases surface β1 to stimulate vasodilation (11).

Biochemical and functional data demonstrate that β1 surface abundance in arterial myocytes is controlled by both Rab11A-dependent and Rab11A-independent mechanisms (11). Here, Rab11A knockdown blocked the depolarization-induced increase in both surface β1 subunits and BK channel apparent Ca^{2+} sensitivity but did not abolish diameter responses to BK

channel modulators in pressurized arteries. Because Ca^{2+} spark inhibition in myocytes abolishes vasoregulation by BK channels (35), our data suggest coupling of BK channels to Ca^{2+} sparks when depolarization-induced, Rab11A-dependent β1 subunit trafficking is blocked. We showed that a population of surface β1 subunits existed in close proximity to plasma membrane BKα proteins that were not modulated by Rab11A knockdown or Rab11A mutants or by NO, brefeldin A, nocodazole, nimodipine, or HA1100 (11). The trafficking mechanism controlling the surface abundance of these “basal” β1 subunits is unclear but appears to be voltage-, Rab11A-, Rab4A-, and sGC/PKG-independent [data here and in (10, 11)]. These surface β1 subunits may generate a baseline amount of BK channel coupling to Ca^{2+} sparks in myocytes.

In summary, we showed that membrane depolarization stimulated $Ca_V1.2$ channels, leading to Ca^{2+} influx and the activation of ROCK, which phosphorylated Rab11A, promoting surface trafficking of Rab11A-postive recycling endosomes that delivered β1 subunits to the plasma membrane. The increase in surface β1 enhanced BK channel apparent Ca^{2+} sensitivity and functional BK channel activity in pressurized arteries.

MATERIALS AND METHODS

Chemicals

SNP, DT-2, ODQ, brefeldin A, ryanodine, KN93, LY294002, and NS1619 were purchased from Sigma-Aldrich and were used at a final concentration of 10 μM. Gadolinium chloride was from Sigma-Aldrich and used at a final concentration of 100 μM. Nimodipine (Sigma-Aldrich) was used at 1 μM. HA1100 and BIM II were from Cayman Chemical and were used at a final concentration of 10 μM. Iberiotoxin (Sigma-Aldrich) was used at a concentration of 100 nM. Lithocholate (Steraloids Inc.) was used at a final concentration of 50 μM and was dissolved as previously described (36).

Tissue preparation and smooth muscle cell isolation

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Sprague-Dawley rats (male, 7 to 8 weeks) were used for all experiments and were randomly picked from a lot. Euthanasia was performed by intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed, and cerebral arteries were dissected, cleaned, and placed in chilled oxygenated (21% O_2, 5% CO_2, and 74% N_2) PSS of the following composition: 119 mM NaCl, 4.7 mM KCl, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 11 mM glucose (pH 7.4). Myocytes were enzymatically dissociated from isolated arteries, as previously described (37). All experiments involving dissociated myocytes were completed on the same day of isolation. Human brain samples were obtained after Institutional Review Board approval and written informed consent from patients and in accordance with the guidelines of the Declaration of Helsinki. The samples obtained were from a 13- and 17-year-old males and a 25-year-old female patient who underwent a lobectomy and were reported to have no history of hypertension or stroke.

Transfection of intact cerebral arteries

Rab11A short hairpin vector (shV) and scrambled shV were purchased from OriGene Technologies Inc. Rat rab11A cDNA was obtained from a clone library (GenScript USA Inc.). A dominant-negative mutant of Rab11A with serine-to-asparagine mutation (Rab11AS25N) was generated and subcloned into pcDNA3.1(+) (Life Technologies). $Ca_V1.2$, ROCK1, ROCK2, and scrambled siRNAs were obtained from Life Technologies. Cerebral arteries were transfected using a CUY21Vivo-SQ electroporator (BEX Co. Ltd.). Cerebral arteries were placed in an electroporation chamber in Ca^{2+}-free phosphate-buffered saline containing the vector or siRNA. Tandem pulse electroporation was applied. Arteries were then removed and kept in Ca^{2+}-free phosphate-buffered saline for 10 min, after which they were placed in serum-free Dulbecco′s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/ F-12) supplemented with 1% penicillin-streptomycin (Sigma-Aldrich) for 3 days under standard conditions (21% O_2, 5% CO_2, and 74% N_2; 37°C). At the end of the incubation period, the arteries were washed once in chilled PSS solution and used for surface biotinylation or immunofluorescence resonance energy transfer (immunoFRET) experiments.

Surface biotinylation

Cellular distribution of BKα and β1 proteins in intact cerebral arteries was determined using surface biotinylation at room temperature, as previously described (38). Arteries were incubated for 1 hour in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Pierce) in Hepes-buffered PSS solution containing 134 mM NaCl, 6 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.4 with NaOH) at room temperature, after which arteries were exposed to test conditions at room temperature. To measure reversal of depolarization-induced β1 subunit trafficking, arteries were exposed to 60 mM K^+–containing PSS for 10 min, transferred to 6 mM K^+ PSS for either 1 or 10 min at room temperature, and then biotinylated in chilled solution at 4°C for 1 hour. The reaction was quenched using 100 mM glycine in phosphate-buffered saline followed by washing in phosphate-buffered saline. Biotinylated arteries were homogenized in a lysis buffer consisting of 50 mM tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1% SDS. Total protein was estimated, as previously described (39), to allow normalization for pull-down. Biotinylated proteins were pulled down using monomeric avidin beads (Pierce). The nonbiotinylated supernatant contained the intracellular protein fraction. Biotinylated plasma membrane proteins that were bound to avidin beads were eluted by boiling in Laemmli buffer (with 5% β-mercaptoethanol). Cellular distribution of proteins was analyzed by Western blotting and calculated as a percentage of total protein.

Western blot analysis

A standard 7.5% SDS–polyacrylamide gel was used for separation of proteins, which were then transferred onto nitrocellulose membranes. Blots were physically cut between ~50 and 75 kDa to probe the same protein samples for BKα and β1 without stripping. Membranes were then incubated with a mouse monoclonal antibody recognizing BKα (1:500 dilution; NeuroMab, University of California, Davis), a rabbit polyclonal antibody recognizing BKβ1

(1:500; Abcam), a mouse monoclonal antibody recognizing rab11A (1:500; Abcam), a mouse monoclonal antibody recognizing $Ca_V1.2$ (1:100; NeuroMab), a rabbit monoclonal antibody recognizing ROCK1 (1:500; Cell Signaling), a rabbit polyclonal antibody recognizing ROCK2 (1:500; Cell Signaling), or a mouse monoclonal antibody recognizing active rab11 (1:500; NewEast Biosciences) antibodies overnight at 4°C in tris-buffered saline (TBS) with 0.1% Tween 20 and 5% nonfat dry milk. Proteins were visualized using appropriate horseradish peroxidase–conjugated secondary antibodies (Pierce) and a chemiluminescent detection kit (Pierce). Band intensity was quantified by digital densitometry using Quantity One software (Bio-Rad).

Confocal imaging and immunoFRET

ImmunoFRET was performed similarly, as previously described (40). Myocytes were exposed to 60 mM K^+–containing PSS for 10 min and either fixed or then exposed to 6 mM K^+–containing PSS for 10 min to measure reversal and then fixed. Myocytes were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 for 2 min at room temperature. One hour after incubation in phosphate-buffered saline containing 5% bovine serum albumin, myocytes were treated overnight at 4°C with a mouse monoclonal antibody recognizing BKα (NeuroMab, University of California, Davis) and a rabbit polyclonal antibody recognizing BKβ1 (Abcam). Antibodies were used at a dilution of 1:100 each in phosphate-buffered saline containing 5% bovine serum albumin. After washes in phosphate-buffered saline, cells were incubated at 37°C for 1 hour with secondary antibodies: Alexa 546–conjugated or Alexa 488–conjugated (1:100 dilution; Life Technologies). Coverslips were then washed and mounted on glass slides. For colocalization experiments, Alexa 633–conjugated WGA was first incubated with fixed cells for 10 min, after which myocytes were processed, as described above. Fluorescence images were acquired using a Zeiss LSM Pascal confocal microscope. Alexa 488 and Alexa 546 were excited at 488 and 543 nm, and emissions were collected at 505 to 530 and >560 nm, respectively. A *z*-resolution of ~1 μm was used. For FRET analysis, the following background values were determined: the donor and acceptor signal in the absence of FRET, cross-talk of the donor and acceptor signals into the FRET channel, and the FRET signal from the donor and acceptor channels, respectively. After background subtraction, normalized FRET was calculated on a pixel-by-pixel basis for the entire image using the method of Xia and Liu (41) and Zeiss LSM FRET Macro tool version 2.5. FRET pixel colocalization with WGA was calculated using the Mander′s colocalization plugin for ImageJ (version 1.51h, National Institutes of Health). The RG2B plugin for ImageJ was used to colocalize FRET and WGA pixels in images.

Patch-clamp electrophysiology

Single BK channel currents were recorded in isolated myocytes using the inside-out patch clamp configuration (Axopatch 200B and Clampex 8.2, MDS Analytical Technologies). Myocytes were allowed to settle, and inside-out patches were pulled in 6 mM K^+–containing or 30 mM K^+–containing Hepes-buffered PSS. The composition of the pipette and bath solution during patch recordings was 130 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, 5 mM EGTA, and 1.6 mM HEDTA (pH 7.2) with KOH. Free Ca^{2+} concentration was adjusted to between 1 and 300 μM by adding $CaCl_2$, and free Mg^{2+} was maintained at 1 mM using $MgCl_2$. Free Ca^{2+} concentration in solutions was measured using Ca^{2+}-sensitive (no.

476041, Corning) and reference (no. 476370, Corning) electrodes. Control myocytes did not receive any treatment. In some experiments, HA1100 (10 μM) was applied for 30 min before 30 mM K^+–containing PSS treatment. Inside-out patch experiments were performed at a membrane voltage of −40 mV. Currents were filtered at 1 kHz and digitized at 5 kHz. Analysis was performed offline using Clampfit 9.2 (MDS Analytical Technologies). Data from the Ca^{2+} sensitivity experiments were fit with an unconstrained single Boltzmann function where the midpoint was between the minimal and maximal amplitudes of each fit.

Pressurized artery myography

Endothelium-denuded arterial segments of 1 to 2 mm in length were cannulated in a perfusion chamber (Living Systems Instrumentation) maintained at 37°C and continuously perfused with PSS (pH 7.4) gassed with 21% O_2, 5% CO_2, and 74% N_2. Arterial diameter was measured at 1 Hz using a charge-coupled device camera attached to a Nikon TS100-F microscope and the automatic edge-detection function of IonWizard software (IonOptix). Luminal flow was absent during experiments. Myogenic tone (%) was calculated as follows: $100 \times (1 - D_{active}/D_{passive})$, where D_{active} is the active arterial diameter and $D_{passive}$ is the diameter determined in the presence of Ca^{2+}-free PSS supplemented with 5 mM EGTA.

Statistical analysis

GraphPad Prism version 4.0 and Origin version 6.0 were used for statistical analyses. Values are presented as means ± SE. Student′s *t* test was used for comparing paired and unpaired data from two populations. For multiple-group comparisons, analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test was used. $P < 0.05$ was considered significant. Power analysis was performed to verify that the sample size gave a value of >0.8 if P was >0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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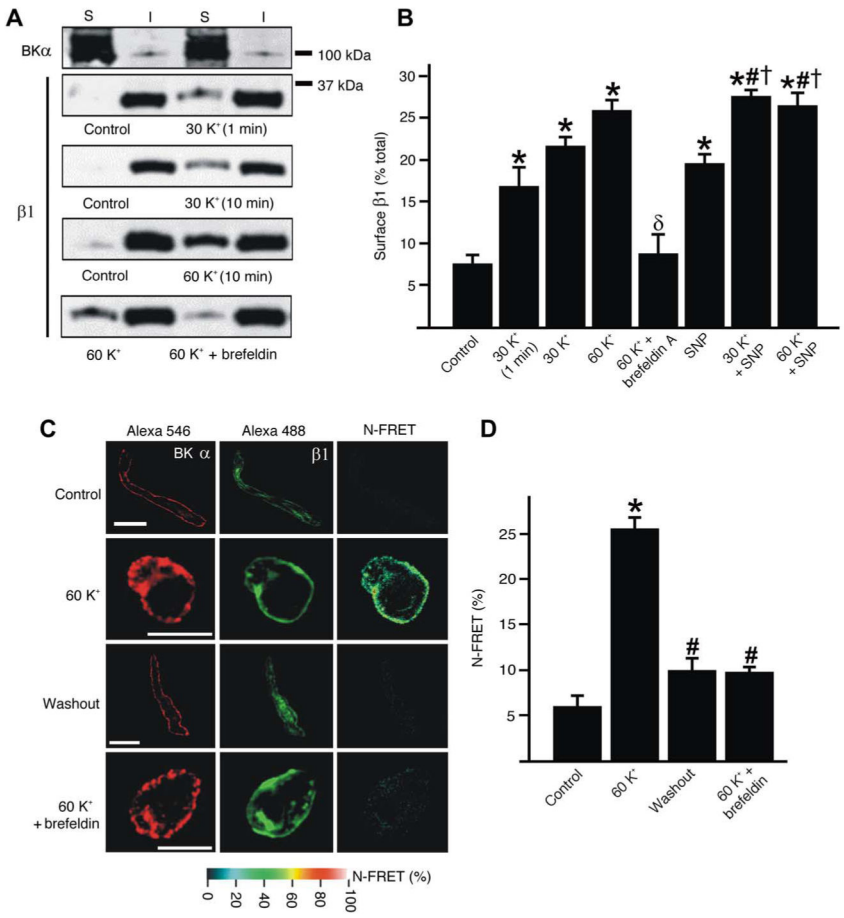

Fig. 1. Depolarization stimulates surface trafficking of BK β1 subunits in arterial myocytes

(**A**) Representative Western blots illustrating surface (S) and intracellular (I) BKα and β1 proteins in rat cerebral arteries obtained using arterial surface biotinylation. (**B**) Mean data, n = 6 to 8 animals for each group; $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to SNP, $^{\dagger}P < 0.05$ compared to 30 K^+, and $^{\delta}P < 0.05$ compared to 60 K^+ alone. All stimuli were applied for 10 min unless stated otherwise. (**C**) Immunofluorescence images of BKα and β1 and the calculated normalized FRET (N-FRET) under each condition in arterial myocytes. Scale bars, 10 μm. (**D**) Mean data for BKα and β1 immunoFRET, n = 6 images for each group obtained from four different animals; $^{*}P < 0.05$ compared to control and $^{\#}P < 0.05$ compared to 60 K^+.

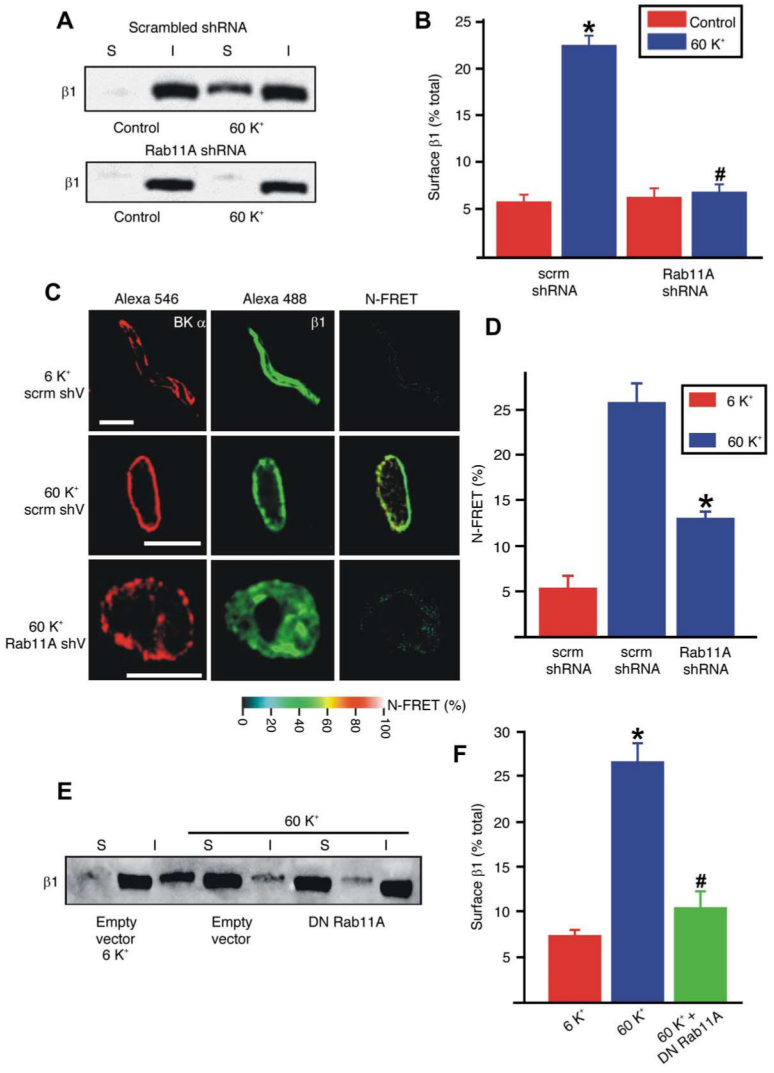

Fig. 2. Rab11A knockdown inhibits depolarization-induced surface trafficking of β1 subunits in arterial myocytes

(**A**) Representative Western blots illustrating surface and intracellular β1 protein in cerebral arteries transfected with scrambled or rab11A shRNA and modulation by 60 K^+ (10 min). (**B**) Mean data, $n = 6$ animals for each group; $^*P < 0.05$ compared to scrambled (scrm) control and $^{\#}P < 0.05$ compared to scrambled 60 K^+. (**C**) Immunofluorescence and immunoFRET images of BKα and β1 in arterial myocytes. Scale bars, 10 μm. (**D**) Mean data, $n = 6$ images for each group obtained from four different animals; $^*P < 0.05$ compared to scrambled shRNA. (**E**) Representative Western blot of β1 protein in depolarized control arteries and arteries expressing dominant-negative (DN) rab11A. (**F**) Mean data, $n = 6$ animals for each group; $^*P < 0.05$ compared to control and $^{\#}P < 0.05$ compared to 60 K^+.

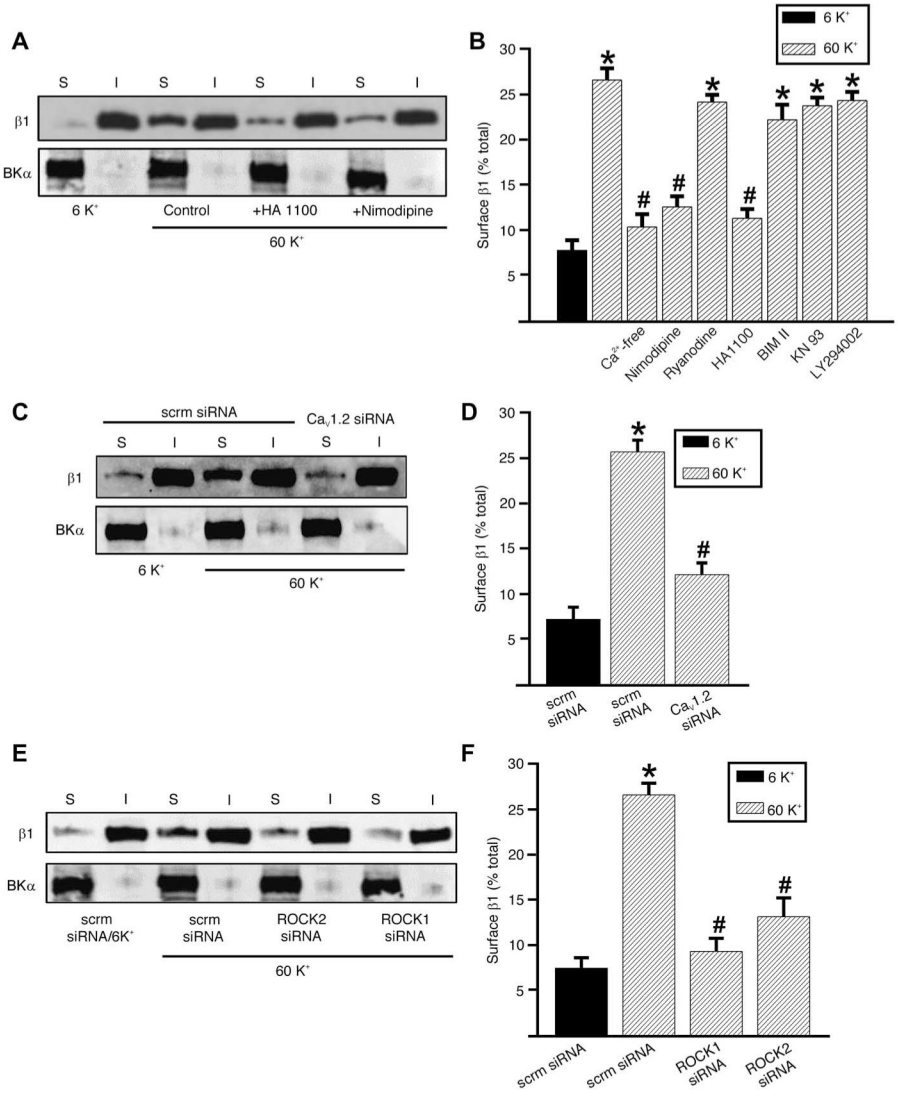

Fig. 3. Extracellular Ca^{2+} removal and $Ca_V1.2$ channel and ROCK inhibitors attenuate depolarization-induced β1 surface trafficking in arteries

(**A**) Representative Western blots illustrating surface and intracellular BKα and β1 protein using arterial surface biotinylation. (**B**) Mean data from biotinylation experiments showing effect of extracellular Ca^{2+} removal and different antagonists on depolarization-induced changes in the surface abundance of β1 protein, $n = 6$ animals for each group; $^{*}P < 0.05$ compared to 6 K^+ and $^{\#}P < 0.05$ compared to 60 K^+. (**C**) Representative Western blots illustrating surface and intracellular BKα and β1 protein from arteries treated with scrambled siRNA or $Ca_V1.2$ siRNA. (**D**) Mean data, $n = 5$ animals for each group. (**E**) Representative Western blots illustrating surface and intracellular BKα and β1 protein from arteries treated with scrambled siRNA or ROCK1 or ROCK2 siRNA. (**F**) Mean data, $n = 6$ animals for each group; $^{*}P < 0.05$ compared to scrambled control and $^{\#}P < 0.05$ compared to 60 K^+.

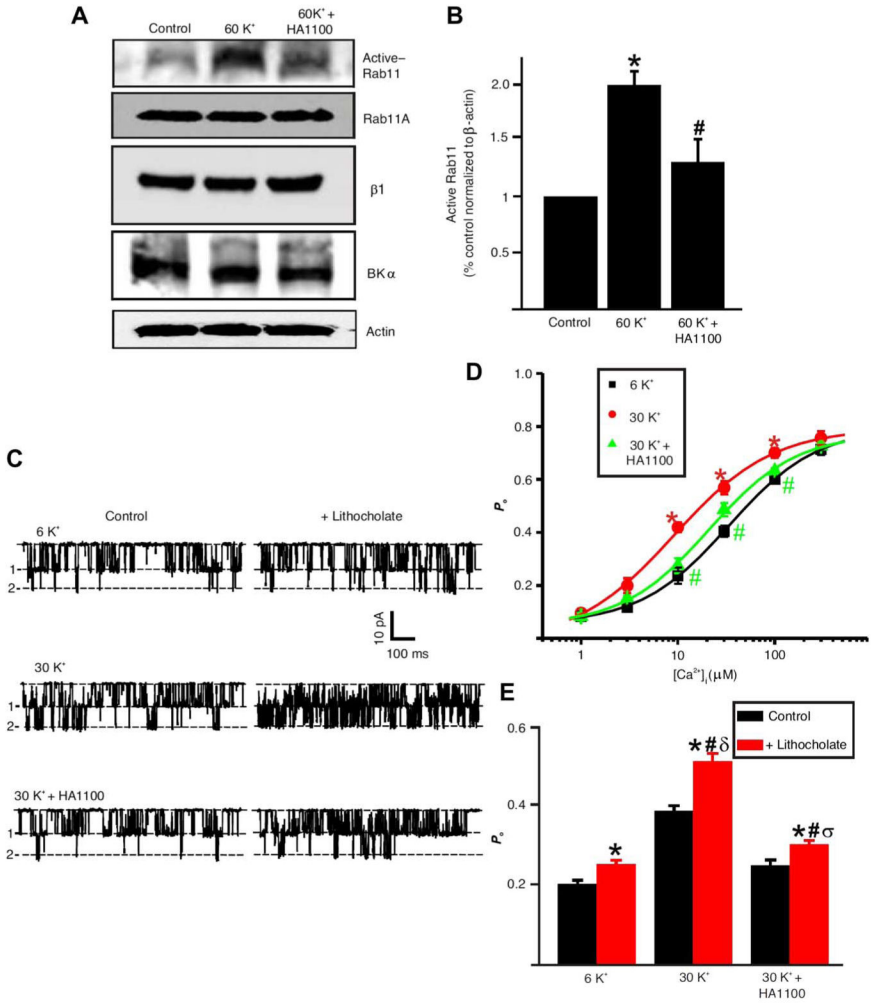

Fig. 4. Depolarization-induced ROCK activation increases BK channel apparent Ca^{2+}-sensitivity in arterial myocytes

(**A**) Representative Western blots illustrating total protein from arteries under control conditions, treated with 60 K^+ or 60 K^+ + HA1100 (10 μM). (**B**) Mean data, $n = 6$ animals for each group; $^*P < 0.05$ compared to control and $^{\#}P < 0.05$ compared to 60 K^+. (**C**) Representative traces of single BK channel activity and activation by lithocholate in the same patches pulled from either control myocytes (6 K^+) or myocytes exposed to 30 K^+ or 30 K^+ + HA1100 (10 μM). Traces are shown from experiments performed with 10 μM free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. (**D**) Mean data, $n = 6$ recordings for each data point; $^*P < 0.05$ compared to 6 K^+ and $^{\#}P < 0.05$ compared to 30 K^+. (**E**) Mean data illustrating lithocholate activation of BK channels with 10 μM free $[Ca^{2+}]_i$, $n = 6$ recordings for each data set obtained from eight different animals; $^*P < 0.05$ compared to 6 K^+ control, $^{\#}P < 0.05$ compared to respective control under the same condition, and $^{\delta}P < 0.05$ compared to 30 K^+ + lithocholate.

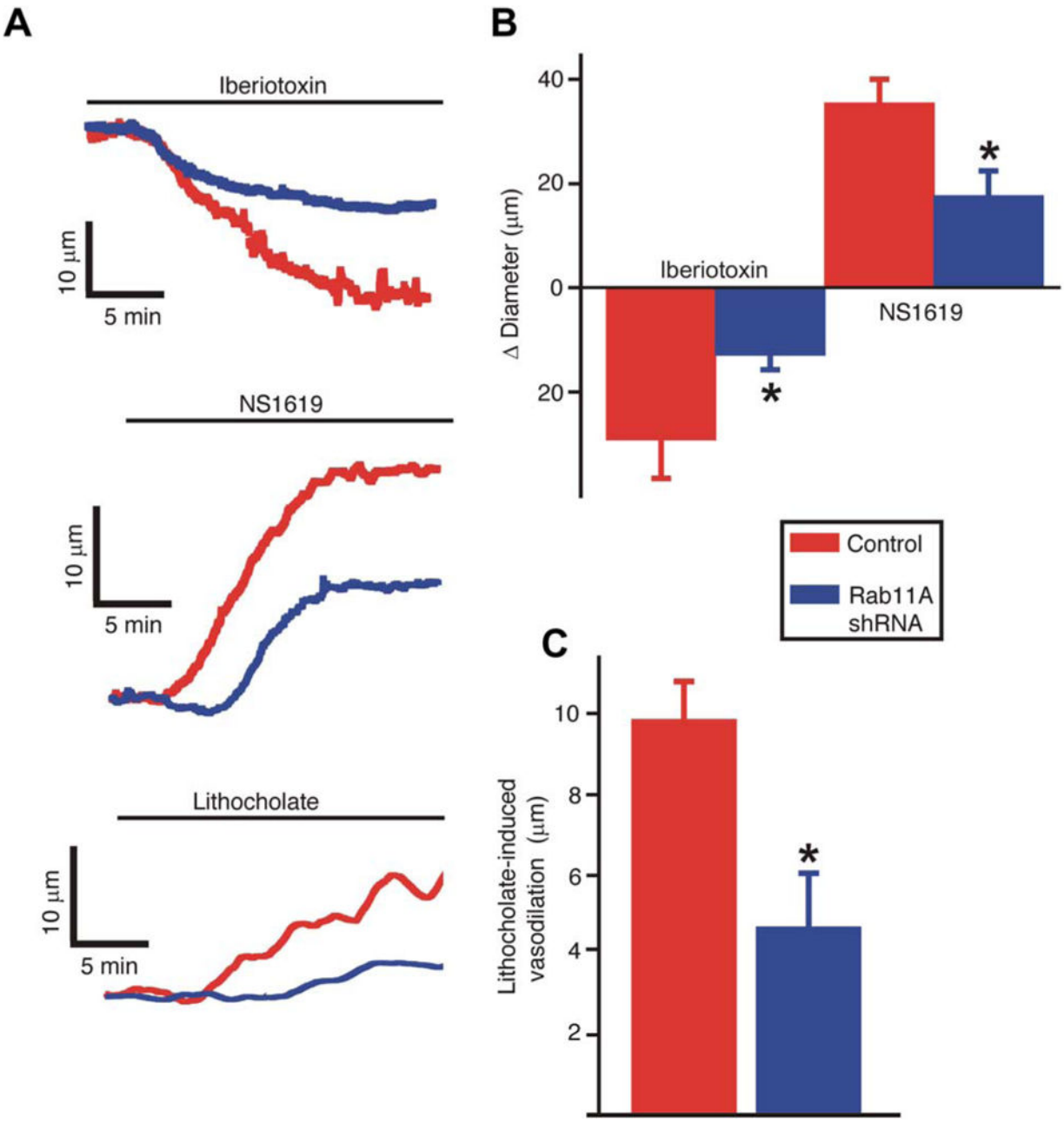

Fig. 5. Rab11A knockdown inhibits BK channels in pressurized arteries

(**A**) Original diameter recordings illustrating constriction in response to iberiotoxin, dilation in response to NS1619, and dilation in response to lithocholate in either control (red trace) or rab11A knockdown (blue trace) arteries. (**B**) Mean data, $n = 6$ animals for each group; $*P < 0.05$ compared to control. (**C**) Mean data illustrating dilation to lithocholate, $n = 6$ animals for each group; $*P < 0.05$ compared to control.