Corrections

PHYSIOLOGY

The authors note that, due to a printer’s error, on page 2362, right column, first full paragraph, line 12 “ODQ, a soluble guanylyl cyclase (sGC) inhibitor, DT-2, a protein kinase G (PKG) inhibitor, or L-NNA, a NOS inhibitor, alone did not alter β1 cellular distribution, but ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), L-NNA (Nω-Nitro-L-arginine) or DT-2 blocked the SNP-induced elevation in membrane β1 (Fig. 1A and B and Fig. S1D).” should instead appear as “ODQ, a soluble guanylyl cyclase (sGC) inhibitor, DT-2, a protein kinase G (PKG) inhibitor, or L-NNA, a NOS inhibitor, alone did not alter β1 cellular distribution, but ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) or DT-2 blocked the SNP-induced elevation in membrane β1 (Fig. 1A and B and Fig. S1D).”

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Dynamic regulation of β1 subunit trafficking controls vascular contractility

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Ion channels composed of pore-forming and auxiliary subunits control physiological functions in virtually all cell types. A conventional view is that channels assemble with their auxiliary subunits before anterograde plasma membrane trafficking of the protein complex. Whether the multisubunit composition of surface channels is fixed following protein synthesis or flexible and open to acute and, potentially, rapid modulation to control activity and cellular excitability is unclear. Arterial smooth muscle cells (myocytes) express large-conductance Ca\(^{2+}\)-activated potassium (BK) channel α and auxiliary β1 subunits that are functionally significant modulators of arterial contractility. Here, we show that native BKα subunits are primarily (~95%) plasma membrane-localized in human and rat arterial myocytes. In contrast, only a small fraction (~10%) of total β1 subunits are located at the cell surface. Immunofluorescence resonance energy transfer microscopy demonstrated that intracellular β1 subunits are stored within Rab11A-positive recycling endosomes. Nitric oxide (NO), acting via cGMP-dependent protein kinase, and CAMP-dependent pathways stimulated rapid (<1 min) anterograde trafficking of β1 subunit-containing recycling endosomes, which increased surface β1 almost threefold. These β1 subunits associated with surface-resident BKα proteins, elevating channel Ca\(^{2+}\) sensitivity and activity. Our data also show that rapid β1 subunit anterograde trafficking is the primary mechanism by which NO activates myocyte BK channels and induces vasodilation. In summary, we show that rapid β1 subunit surface trafficking controls functional BK channel activity in arterial myocytes and vascular contractility. Conceivably, regulated auxiliary subunit trafficking may control channel activity in a wide variety of cell types.

expression of native BK channel subunits in smooth muscle cells (myocytes) of small arteries that regulate regional organ blood flow and systemic blood pressure.

BK channels are expressed in a wide variety of mammalian cell types and regulate functions including arterial contractility, neurotransmission, and endocrine secretion. BK channels are heterotetramers of pore-forming α subunits that can also contain auxiliary β subunits, of which four are known (β1 to -4) (12, 13). β1 subunits are expressed in a tissue-specific manner and modulate BK channel Ca\(^{2+}\) sensitivity and gating properties to customize cellular functionality (14). Arterial myocytes express BK subunits, which elevate BK channel Ca\(^{2+}\) sensitivity into a concentration range that permits functional coupling to local micromolar intracellular Ca\(^{2+}\) transients termed Ca\(^{2+}\) sparks (15). β1 subunits are essential for BK channels to control arterial myocyte membrane potential and contractility and to modulate systemic blood pressure (16, 17). Ca\(^{2+}\) spark to BK channel coupling is weak in β1 subunit knockout mice, leading to membrane depolarization, vasoconstriction, and systemic hypertension (14, 15). Pathological alterations in BK channel β1 subunit expression and function are also associated with cardiovascular diseases, including atherosclerosis, stroke, and hypertension (18). Whether physiological signaling mechanisms can control the α-to-β subunit ratio in BK channels to modulate channel activity and myocyte contractility is unclear.

Results

β1 Subunits Are Primarily Intracellular and Can Be Stimulated to Rapidly Traffic to the Plasma Membrane in Arterial Myocytes. Arterial surface biotinylation was used to measure the cellular distribution

Significance

Plasma membrane ion channels composed of pore-forming and auxiliary subunits regulate physiological functions in virtually all cell types. A conventional view is that ion channels assemble with their auxiliary subunits prior to surface trafficking of the multiprotein complex. Arterial myocytes express large-conductance Ca\(^{2+}\)-activated potassium (BK) channel α and auxiliary β1 subunits that modulate contractility and blood pressure and flow. The data here show that although most BKα subunits are plasma membrane-resident, β1 subunits are primarily intracellular in arterial myocytes. Nitric oxide, an important vasodilator, stimulates rapid surface trafficking of β1 subunits, which associate with, and activate, BK channels, leading to vasodilation. Thus, we show that rapid auxiliary subunit trafficking is a unique mechanism to control functional surface ion channel activity.

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of BKα and β1 subunits in myocytes of intact resistance-size (≈200-µm-diameter) arteries that control regional organ blood flow and systemic blood pressure. In rat cerebral arteries, ≈95.2% of BKα subunits were plasma membrane-localized (Fig. L4 and Fig. S1C), whereas only ≈8.1% of β1 subunits were present at the cell surface (Fig. 1 A and B). A similar distribution of BKα and β1 subunits was observed in rat small mesenteric arteries (Fig. S1B) and in cerebral arteries from an 18-y-old human male collected under an institutional review board-approved protocol (Fig. S2). These data indicate that most BKα subunits are located in the arterial myocyte plasma membrane. In contrast, and contrary to expectations, only a small fraction of total β1 subunits are located at the myocyte surface.

We tested the hypothesis that β1 subunits are mobile proteins and that physiological stimuli can control β1 trafficking to regulate BK channel activity. BK channels are activated by cAMP- and cGMP-dependent protein kinases (PKA and PKG), leading to vasodilation, but involvement of β-subunit trafficking in this response is unclear (17). Surface biotinylation revealed that sodium nitroprusside (SNP) or SIN-1, NO donors, increased mean arterial surface β1 protein ≈2.7- and ≈2.5-fold, respectively, in cerebral arteries (Fig. 1 A and B). Immunofluorescence, Förster resonance energy transfer (FRET) imaging, and communoprecipitation (co-IP) were used to examine BKα and β1 cellular distribution, colocalization, and spatial proximity. The mean Normalized-Förster resonance energy transfer (N-FRET) between BKα- and β1-bound secondary antibodies was ≈7.3% in control myocytes (Fig. 1 C and D). SNP (10 min) increased N-FRET to ≈18.4%, or ≈2.5-fold (Fig. 1 C and D). The Förster coefficient for the Alexa488-Alexa546 FRET pair used is ≈6.3 nm (19), suggesting that surface-trafficked β1 subunits associate with BKα. Co-IP was performed to test the hypothesis that SNP can elevate the macromolecular subunit ratio of β1 to BKα in arterial myocytes. Because of the small size of the resistance-size vessels used in this study, arteries collected from six rats were required for each co-IP experiment. The BKα antibody communoprecipitated β1 protein from arterial lysate (Fig. 1E). SNP increased β1 protein that communoprecipitated with BKα 1.97 ± 0.08-fold (n = 3, 18 rats total; Fig. 1E). These data indicate that surface-trafficked β1 subunits associate with plasma-resident BKα channels in arterial myocytes.

NO-induced plasma membrane trafficking of β1 was rapid, being maximal within 1 min, the shortest time point investigated (Fig. 1 A and B). Carbachol, a muscarinic receptor agonist, which stimulates endothelial cell NO generation, and iloprost, a PGI2 analog that activates myocyte PKA, both increased surface β1 protein in endothelium-intact arteries (Fig. 1B). Similar data were obtained when experiments were performed at either room temperature or 37 °C (Fig. 1A). BKα and β1 were similarly distributed and SNP stimulated β1 subunit surface expression in myocytes of rat mesenteric and cerebral arteries (Figs. S1B and S2). ODQ, a soluble guanylyl cyclase (sGC) inhibitor, DT-2, a protein kinase G (PKG) inhibitor, or 1-NNA, a NOS inhibitor, alone did not alter β1 cellular distribution, but ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one), L-NNA (N_6-Nitro-L-arginine) or DT-2 blocked the SNP-induced elevation in membrane β1 (Fig. 1 A and B and Fig. S1D). Forskolin, an adenylyl cyclase and thus PKA activator, increased β1 surface expression similarly to NO donors (Fig. 1 A and B). These data indicate that both NO-induced, sGC-mediated PKG activation, and adenylyl cyclase-mediated PKA activation stimulate rapid β1 surface expression in arterial myocytes.

### β1 Subunits Are Located in Mobile Rab11A-Positive Recycling Endosomes

Fig. 1. BKα subunits are plasma membrane-localized, whereas β1 subunits are intracellular, in arterial myocytes. (A) Representative Western blots illustrating plasma membrane (M) and intracellular (I) BKα and β1 proteins in rat cerebral arteries obtained using arterial surface biotinylation. Lanes are contiguous in each blot. All reagents were used at a final concentration of 10 µM, except Con A, which was used at 250 µg/mL. (B) Mean data for surface biotinylation experiments (n = 6–8 for each). Final working dilution of all reagents was 10 µM, except Con A, which was used at 250 µg/mL. *P < 0.05 vs. control; †P < 0.05 vs. SNP. (C) Immunofluorescence and immuno-FRET images of BKα and β1 in arterial myocytes. (D) Mean data for BKα and β1 immuno-FRET (control, n = 18; SNP 1 min, n = 8; SNP 10 min, n = 15; SNP 10 min plus brefeldin A, n = 14). SNP was 10 µM. *P < 0.05 compared with control; †P < 0.05 vs. SNP. (Scale bar, 10 µm.) (E) Original Western blots and bar graph (representative of three experiments; 18 rats total) of co-IP experiments indicating that SNP (10 µM, 10 min) elevates β1 protein associated with BKα in cerebral arteries. Lane 1, SNP positive control; lane 2, untreated positive control; lane 3, SNP negative control; lane 4, untreated negative control; lane 5, SNP co-IP; lane 6, untreated co-IP. *P < 0.05 compared with untreated control.
endosomal trafficking with either Rab11A or Rab11B expressed on recycling endosomes (20–26). Physiological functions of Rab11 in native cell types, including arterial myocytes, are unclear. Immunofluorescence experiments demonstrated that β1- and Rab11A-bound antibodies generated a mean N-FRET of ∼32.5% in myocytes, indicating close spatial proximity (Fig. 2 B and C). Vectors overexpressing Rab11A shRNA (Rab11A shV) reduced Rab11A protein by ∼50% but did not alter BKα or β1 expression (Fig. S3 B and C). Rab11A knockdown reduced the mean N-FRET signal between rab11A and β1 from ∼32% in scrambled controls to ∼19.7% (Fig. 2 B and C). Rab11A knockdown also blocked both the SNP-induced elevation in surface β1 and the N-FRET signal between BKα and β1-bound fluorescent antibodies in myocytes (Figs. 2 D and E and 3.4 B and D). Overexpression of a constitutively active Rab11A mutant (Rab11A-Q70L) increased surface β1 ∼1.6 fold, whereas a dominant negative Rab11A mutant (Rab11A-S25N) inhibited SNP-induced β1 surface trafficking in arteries (Fig. 3 C and D). In contrast, Rab11B knockdown (by ∼70%; Fig. S4 A and B) did not alter total or surface-localized BKα or β1 protein in control or SNP-treated arteries (Fig. S4 C and D). Immunofluorescence resonance energy transfer (immuno-FRET) experiments indicated that BKα and Rab11A were not localized (Fig. S34), in stark contrast to the close spatial proximity of β1 and Rab11A. Furthermore, NO, forskolin, brefeldin A or Rab11A knockdown did not alter BKα total protein or cellular distribution (Figs. S1 C and D and S3 B and C). These data indicate that NO stimulates rapid Rab11A-mediated anterograde trafficking of β1 subunits in arterial myocytes. In contrast, BKα is trafficked by a Rab11A- and Rab11B-independent mechanism. These data raised the possibility that regulated anterograde trafficking of β1 subunits is a functional mechanism to control surface BK channel activity in arterial myocytes.

Stimulated Surface Trafficking of β1 Subunits Elevates BK Channel Ca2+ Sensitivity. β1 subunits increase BK channel Ca2+ sensitivity (14, 27). Patch-clamp electrophysiology was used to examine the regulation of BK channels by an elevation in surface β1 subunits in arterial myocytes. Inside-out patches were pulled from myocytes that had been exposed to a bath solution either without (control) or with SNP (10 μM, 10 min). BK channel activity was then measured in membrane patches in the absence of SNP at −40 mV, a physiological voltage. The mean apparent dissociation constant (Kd) for Ca2+ of BK channels from control myocytes was ∼25 μM, with a maximum Popen of ∼0.80 (Fig. 4B). In patches pulled from SNP-treated myocytes, BK channel mean Kd for Ca2+ was ∼11 μM or 2.3-fold higher, without an alteration in maximal Popen (Brefeldin A treatment of myocytes blocked the SNP-induced elevation in BK channel Ca2+ sensitivity (Fig. 4B). These data indicate that SNP stimulates surface trafficking of β1 subunits that associate with BKα subunits and increase Ca2+ sensitivity.

BK channel regulation by lithocholate, a selective activator of β1 subunit-containing BK channels, was also studied (28). We tested the hypothesis that lithocholate would be a more effective activator of BK channels that contain additional SNP-trafficked β1 subunits. Lithocholate increased BK channel mean Popen in inside-out patches pulled from both control cells and SNP-treated myocytes (Fig. 4C). Importantly, lithocholate elevated mean Popen ± 0.07 ± 0.01 in control myocytes and 0.12 ± 0.02 in SNP-treated myocytes, or 1.71-fold more, consistent with the hypothesis being tested (Fig. 4C). These data indicate that NO-stimulated surface trafficking of β1 subunits elevates BK channel Ca2+ sensitivity and BK channel activation by a β1 ligand. When combined with other data in this study, these data indicate that NO increases the amount of functional β1 subunits associated with BK channels.

β1 Surface Trafficking in Arterial Myocytes Dilates Cerebral Arteries. The physiological function of regulated β1 subunit trafficking was studied using pressurized (60 mmHg) endothelium-denuded cerebral arteries. SNP (10 μM) dilated arteries on average by ∼23 μm (Fig. 5 A and B). Iberiotoxin alone constricted arteries by ∼21 μm and reduced SNP-induced vasodilation by ∼65%, indicating that BK channel activation is the primary mechanism underlying NO-induced vasodilation (Fig. 5B). Brefeldin A alone did not modify myogenic tone or iberiotoxin- or 60 mM K+-induced vasoconstriction but reduced SNP-induced vasodilation by ∼74% (Fig. S5C and Fig. 5 A and B). Consistent with these findings, brefeldin A reduced carbachol-induced vasodilation in endothelium-intact arteries by ∼50% (Fig. S5B). Rab11A knockdown did not alter myogenic tone but reduced SNP-induced vasodilation by ∼60%, compared with scrambled controls (Fig. 5 C and D). Lithocholate diluted control arteries on average by ∼6 μm (Fig. 5 E and F). SNP increased mean lithocholate-induced vasodilation to ∼24 μm, or fourfold (Fig. 5 E and F). These data demonstrate that NO stimulates rapid Rab11A-mediated β1 subunit surface trafficking, leading to BK channel activation and vasodilation.

Discussion
Whether the subunit composition of ion channels is fixed following protein synthesis and shutting to the plasma membrane or is flexible and open to physiological modulation to control ion channel activity and tissue function was unclear. Here, we demonstrate that distinct pathways control BK channel α and β1 subunit trafficking in arterial myocytes. We also show that BKα subunits are primarily plasma membrane-localized, whereas a large proportion of β1 subunits reside in highly mobile Rab11A-positive recycling endosomes in arterial myocytes. PKG activation stimulates rapid anterograde trafficking of β1 subunits, which associate with surface BK channels, leading to channel activation and vasodilation. These data describe a unique mechanism by which ion channel activity is controlled in cells. We show that rapid and regulated anterograde trafficking of β1, a BK channel auxiliary subunit, controls BK channel activity and...
myocyte contractility. This study raises the possibility that regulated and rapid trafficking of auxiliary subunits may be a common mechanism to control the activity of many different ion channels and the physiological functions of a multitude of cell types.

In the absence of exogenous stimuli, a very small proportion of total β1 is surface-localized in arterial myocytes. l-NAME, brefeldin, nocodazole, Rab11A knockdown, and Rab11A mutants alone did not alter basal surface β1 levels, suggesting that endothelial cell-mediated NO release and a sGC+, PKG-, and Rab11A-independent mechanism controls basal surface expression of β1 protein. Carbachol, iloprost, and PKG and PKA (based on data with forskolin) activation increased surface β1 protein. An elevation in surface β1 may have occurred because of stimulation of anterograde trafficking and/or inhibition of subunit internalization. Data with brefeldin, nocodazole, Rab11A knockdown, and Rab11A mutants indicate that stimulated anterograde trafficking is the primary mechanism elevating surface β1. These experiments also suggest that β1-containing Rab11A-positive recycling endosomes directly bud from the ER–Golgi complex in arterial myocytes. shRNA reduced Rab11A protein by half, which abolished SNP-induced β1 surface trafficking but did not ablate the FRET signal between Rab11A and β1. These data suggest that partial Rab11A suppression does not prevent the formation of some β1-containing, Rab11A-positive recycling endosomes but blocks PKG-mediated stimulation of anterograde trafficking. BK channel activation was the primary mechanism mediating NO-induced vasodilation. Iberiotoxin and Rab11A knockdown did not abolish NO-induced vasodilation, supporting evidence that BK channel-independent mechanisms, including the activation of other K⁺ channel types and a reduction in contractile apparatus Ca²⁺ sensitivity also contribute (29). Downstream targets of PKG/PKA activation that stimulate recycling endosome-mediated β1 subunit trafficking were not determined here. PKG and PKA phosphorylate a wide variety of proteins in arterial myocytes (30). Phosphorylation targets may include Rab11A or other regulatory protein(s) that stimulate this anterograde trafficking pathway. In support of this conclusion, NO stimulated β1 trafficking more than the constitutively active Rab11A mutant (Rab11AQ70L), suggesting that PKG acts via proteins other than Rab11A, which amplify β1 anterograde trafficking. Given the number of potential targets for these kinases, regulatory proteins involved were not identified here. In the absence of exogenous stimuli, Con A elevated surface β1, suggesting that β1 recycles. To test the hypothesis that PKA and PKG also elevate surface β1 by inhibiting retrograde trafficking would require identification of the pathway that internalizes this protein in arterial myocytes. Given that the Rab family alone contains more than 50 members, such an investigation was beyond the scope of the current study. Future studies should aim to identify the β1 subunit internalization pathway.

Previous studies have investigated the composition of BK channels formed from recombinant BKα and β1 subunits overexpressed in immortalized cells (31). These experiments suggested that a recombinant BK channel tetramer can contain one to four β1 subunits and that BKα can associate with β1 in a 1:1 ratio (17, 32).

![Fig. 3. Rab11A knockdown inhibits SNP-induced β1 subunit plasma membrane trafficking in arterial myocytes.](image)

![Fig. 4. Stimulated β1 subunit surface expression activates BKCa channels.](image)
These studies also indicated that the αβ1-tetramer ratio can shift BK channel voltage dependence and Ca2+ dependence (31). One focus of our study was to measure whether stimulated anterograde β1 subunit trafficking shifts BK channel properties consistent with an increase in channel-associated β1, leading to a functional response. The data indicate that native BK channel subunit composition is not rigid, but dynamic, and can be rapidly modulated by physiological vasoregulatory stimuli to control activity in arterial myocytes. In myocytes, BK channels are activated by Ca2+ sparks, with β1 subunit knockout attenuating coupling to these local Ca2+ transients (15, 33, 34). Our data indicate that only a small fraction of total β1 is plasma membrane-localized under basal conditions. It is likely that BK channels within the vicinity of Ca2+ sparks contain β1 subunits, although the subunit ratio of these coupled channels is unclear and remains to be determined. Conceivably, other intracellular signals or Rabs may promote basal β1 trafficking, which then provides a baseline level of BK channel coupling to Ca2+ sparks. SNP and forskolin elevate both Ca2+ spark frequency and transient BK current frequency and amplitude in arterial myocytes, inducing vasodilation (35). This kinase-mediated vasodilation is dependent upon BK channel activation, which we show is blocked by inhibition of β1 subunit trafficking. Thus, β1 subunit anterograde trafficking is likely to be a major contributor to the NO-induced, PKG-stimulated increase in transient BK current frequency and amplitude.

Four BK channel β subunit isoforms exist that exhibit tissue-specific expression in cells including those in the brain, kidney, immune system, and adrenal gland (17). Our findings suggest that the composition of the BK channels containing other β subunit isoforms may also be dynamic and subject to rapid and precise regulation to control activity and physiological functions. In addition, many other ion channel pore-forming subunits can traffic to the plasma membrane independently of their auxiliary subunits, including Kv6 (6), KATP (7), GABA (8), and epithelial sodium channel (9). The data here raise the possibility that the subunit composition of many ion channels may be dynamically regulated to alter activity and function in a wide variety of cell types. In summary, our study describes a unique mechanism that controls ion channel activity. We show that regulated and rapid recycling endosome-mediated β1 subunit anterograde trafficking controls BK channel subunit composition and activity in arterial myocytes and vascular contractility.

Materials and Methods

Expanded information can be found in SI Materials and Methods.

Tissue Preparation. Animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Adult male Sprague–Dawley rats (7–9 wk of age) were used for all experiments on rodents. Small (<200-μm-diameter) cerebral and mesenteric arteries were dissected and cleaned of connective tissue. Myocytes were isolated as previously described (36). A human brain sample, from which small (<200-μm-diameter) cerebral arteries were obtained, was obtained after institutional review board approval, written informed consent, and in accordance with the guidelines of the Declaration of Helsinki.

Surface Biotinylation of Intact Arteries. Biotinylation of intact arteries was performed as previously described (2). Arteries were incubated in a mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and Maleimide-PEG2-PEG2-Biotin reagents (Thermo Fisher Scientific) for 1 h. Arteries were washed with 100 mM glycine in PBS for 15 min to remove any unbound biotin. Arteries were then washed in PBS, placed in ice-cold lysis buffer, and homogenized in lysis buffer. Cellular debris was removed by centrifugation. Total protein was then determined to allow normalization for avidin pull down of biotinylated surface proteins. Following pull-down, the supernatant comprised of the non-biotinylated (intracellular) protein fraction. Biotinylated surface proteins were eluted from the avidin beads.

Western Blotting. Surface and intracellular proteins were analyzed using Western blotting [7.5% (wt/vol) SDS polyacrylamide gels] and probed with either mouse monoclonal anti-BKα (1:500; NeuroMab, University of California–San Francisco (Ha), rabbit polyclonal anti-BKα (1:500; Abcam), or mouse monoclonal rab11A (1:500; Abcam) antibodies. Blots were cut at ~50 kDa to allow simultaneous probing for both BKα and β1 subunits. Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Band intensities were analyzed using Quantity One software (Bio-Rad).

Confocal Imaging and Immunofluorescence Resonance Energy Transfer Microscopy. Fixed, permeabilized myocytes were incubated with the same anti-BKα, anti-β1, or anti-rab11A antibodies used for Western blotting experiments or ER-Tracker Green (Life Technologies). Alexa 488- or 546-conjugated secondary antibodies were used. Fluorescence images were acquired using a laser-scanning confocal microscope (LSM5 Pascal; Carl Zeiss). Percentage of weighted colocalization was calculated using Pascal system-embedded software program. For N-FRET analysis, images were background-subtracted and N-FRET was calculated on a pixel-by-pixel basis for the entire image and in regions of interest using the Xia method (37) and Zeiss LSM FRET Macro tool Version 2.5, as previously described (38).

Co-IP. For each experiment, lysate was harvested from cerebral arteries pooled from six rats using ice-cold radioimmunoprecipitation buffer. Co-IP was performed using the Catch and Release V2.0 Coimmunoprecipitation kit (Millipore) according to the manufacturer’s instructions. Briefly, arterial lysate was incubated with control mouse IgG or BKα mouse monoclonal antibody, antibody-affinity ligand, and capture resin in the column provided. The column was centrifuged and washed, and bound proteins were

Fig. 5. SNP-induced vasodilation occurs due to Rab11A-mediated BK channel activation. (A) Original recordings from the same artery illustrating dilation to SNP (10 μM) in control (red trace) and after treatment with brefeldin A (10 μM, 1 h; blue trace). (B) Mean data (experimental number, n = 6–8 for each). *P < 0.05 compared with control; **P < 0.05 vs. brefeldin; *P < 0.05 vs. iberiotoxin. (C) Original recordings illustrating dilation to SNP (10 μM) in arteries transfected with control shV (red) and rab11A shV (blue). (D) Mean data (n = 6 for each). *P < 0.05 vs. control shV. (E) Dilation to lithocholate (45 μM) in control (green) and in the presence of SNP (red). (F) Mean data (n = 6 for each). *P < 0.05 vs. untreated control; **P < 0.05 compared with SNP.
released using denaturing buffer. Boiled eluate was run on a SDS/PAGE gel, and protein samples were analyzed by Western blotting using mouse monoclonal anti-BK (NeuroMab) or rabbit polyclonal anti-BK1 (Abcam) and horseradish peroxidase-conjugated secondary antibodies.

**Rab11A shRNA and Mutant Constructs.** A commercial Rab11A shRNA kit was obtained from OriGene Technologies. Rab11A dominant-negative (Rab11A-Q70L) and constitutively active (Rab11A-Q25N) mutants were generated from rat rab11A cDNA obtained from a clone library (GenScript).

**Transfection of Intact Cerebral Arteries.** shRNA, mutant construct, or empty vectors were transfected into arteries using either reverse permeabilization or electroporation (CU2Y21 Vivo-So electroporator; Bex), as previously described (39).

**Patch-Clamp Electrophysiology.** Single BK channel currents were recorded in inside-out patches from isolated cerebral artery myocytes. The pipette and bath solution both contained: 130 mM KCl, 10 mM Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 1 mM MgCl₂, 5 mM EGTA, 1.6 mM HEDTA (N-2-Hydroxyethyl)ethylenediamine-N,N’,N’-triacetic acid, pH 7.2 with KOH).

Free Ca²⁺ concentration was adjusted to be between 1 and 300 µM by the addition of CaCl₂, with free Mg²⁺ maintained at 1 mM by altering MgCl₂.

Free Ca²⁺ concentration in solutions was measured using Ca²⁺-sensitive (no. 476041; Corning) and reference (no. 476370; Corning) electrodes. Inside-out patch experiments were performed at a membrane voltage of ~−40 mV. BK currents were filtered at 1 kHz and digitized at 5 kHz. Analysis was performed offline using Clampfit 9.2 (MDS Analytical Technologies). Ca²⁺-sensitivity data were fit with an unconstrained single Boltzmann function.

**Pressurized Artery Diameter Measurements.** Experiments were performed using endothelium-denuded rat cerebral arteries cannulated at each end in a perfusion chamber (Living Systems Instrumentation). Arterial diameter was measured at 1 Hz using a CCD camera. Myogenic tone (percentage) was calculated as follows: 100 × (Dmax − Dactive)/Dmax, where Dactive is active arterial diameter, and Dmax is the diameter determined in the presence of Ca²⁺-free physiological saline solution supplemented with 5 mMol EGTA.

**Statistical Analysis.** Values are expressed as means ± SEM. Student t test was used for comparing paired and unpaired data from two populations, and ANOVA with Student–Newman–Keuls post hoc test used for multiple group comparisons. P < 0.05 was considered significant.

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