LRRC26 Is a Functional BK Channel Auxiliary γ Subunit in Arterial Smooth Muscle Cells

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Rationale: Smooth muscle cell (myocyte) large-conductance calcium (Ca\textsuperscript{2+})-activated potassium (BK) channels are functionally significant modulators of arterial contractility. Arterial myocytes express both pore-forming BKα and auxiliary β1 subunits, which increase channel Ca\textsuperscript{2+} sensitivity. Recently, several leucine-rich repeat containing (LRRC) proteins have been identified as auxiliary γ subunits that elevate the voltage sensitivity of recombinant and prostate adenocarcinoma BK channels. LRRC expression and physiological functions in native cell types are unclear.

Objective: Investigate the expression and physiological functions of leucine-rich repeat containing protein 26 (LRRC26) in arterial myocytes.

Methods and Results: Reverse transcription polymerase chain reaction and Western blotting detected LRRC26 mRNA and protein in cerebral artery myocytes. Bioluminescence, immunofluorescence resonance energy transfer microscopy, and coimmunoprecipitation indicated that LRRC26 was located in close spatial proximity to, and associated with, plasma membrane BKα subunits. LRRC26 knockdown (RNAi) reduced total and surface LRRC26, but did not alter BKα or β1, proteins in arteries. LRRC26 knockdown did not alter Ca\textsuperscript{2+} sparks but reduced BK channel voltage sensitivity, which decreased channel apparent Ca\textsuperscript{2+} sensitivity and transient BK current frequency and amplitude in myocytes. LRRC26 knockdown also increased myogenic tone over a range (40–100 mm Hg) of intravascular pressures, and reduced vasoconstriction to iberiotoxin and vasodilation to NS1619, BK channel inhibitors and activators, respectively. In contrast, LRRC26 knockdown did not alter depolarization (60 mmol/L K\textsuperscript{+})–induced vasoconstriction.

Conclusions: LRRC26 is expressed, associates with BKα subunits, and elevates channel voltage- and apparent Ca\textsuperscript{2+} sensitivity in arterial myocytes to induce vasodilation. This study indicates that arterial myocytes express a functional BK channel γ subunit. (Circ Res. 2014;115:423-431.)

Key Words: auxiliary subunit ▪ BK ▪ LRRC26 ▪ muscle, smooth, vascular ▪ potassium channels ▪ vasodilation

Large-conductance calcium (Ca\textsuperscript{2+})-activated potassium (BK) channels are expressed in a wide variety of cell types, where these proteins control multiple physiological functions.\textsuperscript{1-5} Arterial smooth muscle cell (myocyte) BK channels regulate membrane potential, which modulates the activity of voltage-dependent Ca\textsuperscript{2+} channels and intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}).\textsuperscript{6} BK channel inhibition leads to an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and vasoconstriction, whereas channel activation reduces [Ca\textsuperscript{2+}]\textsubscript{i}, leading to vasodilation.\textsuperscript{6-8} Genetic ablation of the BK channel pore-forming α subunit leads to vasoconstriction and hypertension, demonstrating the essential nature of these proteins to physiological control of regional organ blood flow and systemic blood pressure.\textsuperscript{9}

Pore-forming BK channel α (Slo) subunits (Slo) can form heteromultimers with auxiliary β subunits, of which 4 isoforms (β1–β4) have been identified.\textsuperscript{10-11} In arterial myocytes, Slo1 is the principal BKα subunit, with β1 the molecular and functional β subunit isoform.\textsuperscript{12} β1 subunits elevate BK channel apparent Ca\textsuperscript{2+} sensitivity and enhance coupling to Ca\textsuperscript{2+} sparks, which are local micromolar intracellular Ca\textsuperscript{2+} transients that occur because of ryanodine receptor–mediated sarcoplasmic reticulum Ca\textsuperscript{2+} release.\textsuperscript{7,14-18} A single Ca\textsuperscript{2+} spark can activate multiple nearby BK channels, leading to a transient BK current. Similar to BKα subunit knockout, β1 subunit ablation reduces BK channel activity in arterial myocytes, elevates arterial contractility, and increases systemic blood pressure.\textsuperscript{14,15,19}

Recent studies have identified leucine-rich repeat containing proteins (LRRC) as a novel family of BK channel auxiliary γ subunits.\textsuperscript{20,21} LRRC proteins are structurally distinct from β subunits and are characterized by the consensus sequence: LXXLXLXX\textsubscript{y}/XL, where X is any amino acid residue and L can be leucine, phenylalanine, isoleucine, or valine.\textsuperscript{22} Four LRRC proteins (LRRC26, 38, 52, and 55) have been described that each elevate BK channel voltage sensitivity although to

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differing degrees. Of these 4 LRRC proteins, LRRC26 produced the largest negative shift (≈−153 mV) in the voltage dependence of recombinant BKα channels expressed in HEK293 cells. LRRC26 also left shifted the voltage dependence of BK channels in human prostate adenocarcinoma (LNCaP) cells. LRRC25, which is enriched in testis, shifted recombinant Slo3 activation to lower pH and voltages. Real-time polymerase chain reaction (PCR) of whole-organ lysates suggested that LRRC proteins exhibit tissue-specific expression although which individual cell types express LRRC proteins is unclear. Similarly, the physiological function of LRRC proteins in native cell types, including arterial myocytes, is uncertain. Such an investigation is appropriate given the functional significance of BK channels in a wide variety of cells and their physiological and pathological involvement in the cardiovascular system.

Here, we explored LRRC26 expression and function in cerebral artery myocytes. LRRC26 transcript and protein were detected in arterial myocytes with the majority of protein located at the plasma membrane. LRRC26 was located in close spatial proximity to BK channel α subunits. In the presence of physiological [Ca++]i, selective LRRC26 knockdown reduced BK channel voltage and apparent Ca++ sensitivity, which inhibited transient BK currents. LRRC26 knockdown also increased pressure-induced vasoconstriction (myogenic tone) and resistance-size arteries. Importantly, this study indicates for the first time that arterial myocytes express a functional BK channel α subunit.

### Methods

Expanding Methods are available in the Online Data Supplement. All animal protocols were reviewed and approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Male Sprague–Dawley rats (8 weeks) were euthanized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed and placed in an ice-cold (4°C) physiological saline solution consisting of (in mmol/L): 6 KCl, 112 NaCl, 24 NaHCO3, 1.2 MgSO4, 1.2 KH2PO4, 1.8 CaCl2, and 10 glucose, which was gassed with 21% O2-5% CO2-74% N2 to pH 7.4. Resistance-size arteries were carefully dissected away from the brain and the connective tissue removed. Where appropriate, cerebral artery myocytes were enzymatically dissociated, as previously described.

#### Polymerase Chain Reaction

Total RNA was extracted from either whole arteries or 21% O2-5% CO2-74% N2 to pH 7.4. Resistance-size arteries. Isolated selected arterial myocytes using TRIzol (Life Technologies) or the Absolutely RNA Nanoprep kit (Stratagene), respectively. First-strand cDNA was generated from 1 to 5 ng of total RNA using Protoscript M-MULV (New England Biolabs). PCR was performed on first-strand cDNA using primers sequences shown in Online Table I. PCR products were separated on 1.5% agarose gels.

#### Protein Analysis

Samples were separated on SDS-PAGE and transferred onto nitrocellulose membranes that were then incubated with goat polyclonal anti-LRRC26 (Santa Cruz Biotechnology), rabbit polyclonal anti-BKα (Abcam), mouse monoclonal anti-BKα (Neuromab; University of California at Davis), or mouse monoclonal antiaction (Millipore). After incubation with their respective secondary antibodies, membranes were developed using a chemiluminescence detection kit (Pierce) and imaged with a Kodak In Vivo F Pro Imaging System (Carestream Molecular Imaging). Band densitometry was analyzed using Quantity One software (Bio-Rad). LRRC26, β1, and BKα band densities were normalized to actin.

#### Surface Biotinylation

Arteries were incubated with EZ-Link Sulfo-NHS-PEG2-Biotin and EZ-Link Maleimide-PEG2-Biotin (Pierce). Free biotin was quenched by washing the arteries in PBS with glycine. Biotinylated arteries were homogenized, centrifuged, and the supernatant collected. After protein estimation, the sample was incubated with avidin beads (Monomeric Avidin Agarose; Pierce) and the supernatant (nonbiotin-bound proteins) was set aside. Biotinylated proteins were eluted from the avidin beads. Western blotting was used to determine the relative distribution of surface (biotinylated) and intracellular (nonbiotinylated) fractions.

#### Immunofluorescence and Immunofluorescence Resonance Energy Transfer Microscopy

Isolated myocytes were plated, fixed, and permeabilized. For colocalization experiments, cells were blocked (BSA) and incubated with goat polyclonal anti-LRRC26 antibody (Santa Cruz Biotechnology). Cells were then incubated with antigen Alexa 488 secondary antibody. Myocytes were incubated with Alexa 546–tagged wheat germ agglutinin (Life Technologies). Images were acquired using a laser-scanning confocal microscope (LSM Pascal; Carl Zeiss). Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505 to 530 and 5260 nm, respectively. Weighted colocalization was determined with the LSM FRET Macro tool (version 2.5; Carl Zeiss).

For immunofluorescence resonance energy transfer, myocytes were fixed and incubated with one of the following primary antibodies: goat polyclonal anti-LRRC26 (Santa Cruz Biotechnology), mouse monoclonal anti-BKα (Neuromab, University of California at Davis), or rabbit polyclonal anti-–transient receptor potential melastatin 4 cation channel (Thermo Fisher Scientific). Cells were then incubated with the following secondary antibodies: antigoat Alexa 488 or antigoat Alexa 546 (LRRC26), antimouse Alexa 546 (BKα), or antirabbit Alexa 488 (transient receptor potential melastatin 4 cation channel). Fluorescence images were acquired using a laser-scanning confocal microscope. Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505 to 530 and 5260 nm, respectively. Images were background subtracted, and N-FRET was calculated using the Xia method and LSM FRET Macro tool (version 2.5; Carl Zeiss).

#### Communoprecipitation

For each experiment, lystate was harvested from arteries pooled from 6 rats using ice-cold Radio-Immunoprecipitation buffer. Communoprecipitation was performed using the Catch and Release version 2.0 Co-immunoprecipitation kit (Millipore) as per the manufacturer’s protocol. Briefly, arterial lystate was incubated with control mouse IgG or BKα mouse monoclonal antibody, antibody affinity ligand, and the capture resin in the column provided. Bound proteins were released and run on a SDS-PAGE. Protein samples were analyzed by Western blotting using mouse monoclonal anti-BKα (Neuromab) or goat polyclonal anti-LRRC26 (Santa Cruz) and horseshadish peroxidase-conjugated secondary antibodies, as previously described.

#### LRRC26 Knockdown

Cerebral arteries were placed in an electroporation chamber (Bex) with either control or LRRC26-specific siRNAs (Life Technologies).
Arteries were transfected using an electroporator (CUV21 Vivo-SQ electroporator; Bex Co Ltd) and stored at 37°C in DMEM-F12 50/50 (HEPES-free) culture medium supplemented with 1% penicillin-streptomycin for 48 to 72 hours before use.

**Electrophysiology**

Single BK channel or transient BK currents were recorded at room temperature in isolated myocytes using the inside-out or whole cell patch-clamp configurations, respectively. An Axopatch 200B amplifier and Clampex 8.2 (Molecular Devices) were used to record currents. For inside-out patch-clamp, the pipette and bath solutions both contained (in mmol/L) the following: 130 KCl, 10 HEPES, 5 EGTA, 1.6 HEDTA, 1 MgCl₂, and 10 μmol/L free Ca²⁺ (pH 7.2). Free Ca²⁺ was adjusted between 1 and 300 μmol/L, and free Mg²⁺ concentration maintained at 1 mmol/L with CaCl₂ and MgCl₂, respectively. Free Ca²⁺ concentration was calculated using WEBMAXC Standard software. BK channel activity (NPₒ) was calculated using the following equation: NPₒ = Σ (tᵢ / tᵢ + tᵢₜ), where tᵢ is the relative open time (time open/time total) for each channel level. Open probability (Pₒ) was calculated by dividing NPₒ by the total number of channels. Voltage- and apparent Ca²⁺-sensitivity experiments were performed offline using Clampfit 9.2 (MDS Analytic Technologies). BK channel activity (NPₒ) was calculated using the following equation: NPₒ = Σ (tᵢ / tᵢ + tᵢₜ), where tᵢ is the relative open time (time open/time total) for each channel level. Open probability (Pₒ) was calculated by dividing NPₒ by the total number of channels. Voltage- and apparent Ca²⁺-sensitivity experiments were performed offline using Clampfit 9.2 (MDS Analytic Technologies).

**Confocal Ca²⁺ Imaging**

Intracellular Ca²⁺ signals were imaged in myocytes of cerebral arteries using fluo-4 AM and a Noran Oz laser-scanning confocal microscope, as previously described.²³

**Pressurized Artery Myography**

Middle cerebral artery segments were cannulated in a perfusion chamber (Living Systems Instrumentation) and continuously perfused with physiological saline solution. Intravascular pressure was controlled through a reservoir system and monitored with a pressure transducer. Wall diameter was measured using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix). Myogenic tone (%) was calculated as: 100 × (1 – D_active/D_passive), where D_active is active arterial diameter and D_passive is the passive arterial diameter determined by the application of Ca²⁺-free physiological saline solution supplemented with 5 mmol/L EGTA.

**Statistical Analysis**

Data are expressed as mean±SE. An independent sample t test was used to determine if a significant difference existed between group means. The criterion for statistical significance was the same for all tests (α=0.05).

**Results**

**LRRC26 mRNA and Protein Are Present in Arterial Myocytes**

Reverse transcription PCR was performed to examine LRRC26 message in cerebral arteries and pure, acutely isolated cerebral artery myocytes. Primers amplified LRRC26 transcript from both whole cerebral artery cDNA and isolated arterial myocyte cDNA (Figure 1A and 1B). To examine the specificity of the myocyte cDNA, primers to myosin heavy chain 11, a smooth muscle marker, platelet-endothelial cell adhesion molecule-1, an endothelial cell marker, and aquaporin-4, an astrocyte marker, were used. Only primers to myosin heavy chain 11 amplified transcripts from arterial myocyte cDNA (Figure 1B). Thus, the arterial myocyte cDNA was pure and not contaminated with cDNA from other vascular wall cell types.

Western blotting using an LRRC26 antibody detected an ≈42-kDa band in cerebral artery lysate, a molecular weight consistent with that of glycosylated LRRC26 (Figure 1C).³ The antigenic peptide for the LRRC26 antibody abolished the ≈42-kDa protein band (Online Figure 1A). These data indicate that LRRC26 mRNA and protein are expressed in arterial myocytes.

**LRRC26 Is Plasma Membrane Localized and Located in Close Spatial Proximity to BKα Subunits in Arterial Myocytes**

Cellular distribution of LRRC26 was studied using surface biotinylation and immunofluorescence microscopy. Arterial surface biotinylation revealed that ≈82% of total LRRC26 was plasma membrane localized (Figure 2A). Similarly, confocal imaging followed by weighted colocalization analysis indicated that 84±4% of LRRC26 is colocalized with wheat germ agglutinin, a plasma membrane marker, in arterial myocytes (Figure 2B, n=6). These data indicate that the vast majority of LRRC26 is plasma membrane localized in arterial myocytes.

To investigate the hypothesis that LRRC26 is a BK channel auxiliary subunit, immunofluorescence resonance energy transfer

**Figure 1. Leucine-rich repeat containing protein 26 (LRRC26) is expressed in arterial myocytes. A,** Original gel image indicating that reverse transcription polymerase chain reaction (RT-PCR) amplified transcripts for LRRC26 in intact cerebral arteries. **B,** RT-PCR amplified transcripts for LRRC26 and myosin heavy polypeptide 11 (Myh11), a myocyte marker, in isolated cerebral artery myocytes. Endothelial cell (platelet-endothelial cell adhesion molecule-1 [PECAM-1]) and astrocyte (aquaporin-4 [AQP4]) markers were not amplified in the same cDNA. **C,** Western blot indicating that a LRRC26 antibody detected a ≈42-kDa protein in cerebral artery lysate.
microscopy and coimmunoprecipitation were performed. Alexa Fluor488 and Alexa Fluor546-tagged secondary antibodies bound to LRRC26 and BKα primary antibodies, respectively, generated N-FRET of 20±2% in isolated arterial myocytes (Figure 2C; n=8). In contrast, the same fluorescent secondary antibodies to LRRC26 and transient receptor potential melastatin 4 cation channel primary antibodies generated N-FRET of only 5±1%, which is consistent with background (Figure 2C; n=5). The antigenic peptide abolished immunofluorescence produced by the LRRC26 antibody (Online Figure IB). The selectivity of the BKα and transient receptor potential melastatin 4 cation channel antibodies used has been previously established.28 Given that the Förster coefficient of the Alexa Fluor pair used for these experiments is ≈6.3 nm, data indicate that LRRC26 is located in close spatial proximity to BKα subunits.

Coimmunoprecipitation was used to test the hypothesis that LRRC26 and BKα subunits are located in the same macromolecular complex in arterial myocytes. Because of the small size of the resistance-size arteries used in this study, arteries collected from n=6 rats were required for each experiment. The BKα antibody coimmunoprecipitated both BKα and LRRC26 protein from arterial lysate (Figure 2D). These data indicate that LRRC26 is primarily plasma membrane localized, located in close spatial proximity to BKα, and coimmunoprecipitates with BKα in arterial myocytes.

**LRRC26 Knockdown Reduces BK Channel Voltage and Apparent Ca2+ Sensitivity in Arterial Myocytes**

To study physiological functions of LRRC26 in arterial myocytes, expression was inhibited using RNA interference (RNAi). LRRC26-specific siRNA reduced both total and surface arterial LRRC26 protein by ≈47% and 48%, respectively, but did not alter total or surface BKα or β1 subunit expression (Figure 3A–3F).

BK channel properties were examined using patch-clamp electrophysiology. Channels were measured in inside-out patches pulled from myocytes isolated from arteries treated with either control siRNA or LRRC26 siRNA. BK channel activity was measured with physiological free [Ca2+]i of 10 μmol/L. In control myocyte patches, the mean half-maximal voltage of activation (V1/2) for BK channels was ≈−20 mV with a maximum Po of ≈0.81 (Figure 4B). LRRC26 knockdown increased mean V1/2 to ≈+13 mV or by +33 mV but did not alter maximum Po (Figure 4B). In contrast, LRRC26 knockdown did not alter single BK channel conductance (Online Figure II). These data indicate that native LRRC26 elevates BK channel voltage sensitivity in arterial myocytes.

RNAi was also used to measure the regulation of BK channel apparent Ca2+ sensitivity by LRRC26 at −40 mV, a physiological arterial myocyte membrane potential.4 In inside-out patches pulled from control myocytes, the mean Kd for Ca2+ was ≈32 μmol/L with a maximum Pp of ≈0.77 (Figure 5B). LRRC26 knockdown induced a rightward shift in the Ca2+–response curve increasing the mean Kd for Ca2+ to ≈46 μmol/L. In contrast, LRRC26 knockdown did not alter maximum Pp (control, 0.77; LRRC26 siRNA, 0.70; Figure 5B). β1 subunits elevate BK channel apparent Ca2+ sensitivity in arterial myocytes.14 To examine the possibility that LRRC26 knockdown reduced BK channel activation by β1, we measured responses to lithocholate, a β1 subunit-specific BK channel activator.28 Lithocholate increased BK channel Pp from =0.22 to 0.36, or 1.64-fold in control siRNA-treated myocytes, and from =0.06 to 0.17, or 2.83-fold in LRRC26 siRNA-treated myocytes (Figure 5C). These results indicate that LRRC26 knockdown does not inhibit β1 subunit-mediated BK channel activation. Collectively, these data indicate that LRRC26 elevates BK channel voltage and apparent Ca2+ sensitivity in arterial myocytes.

**LRRC26 Knockdown Inhibits Transient BK Currents, but Does Not Alter Ca2+ Sparks, in Cerebral Artery Myocytes**

To determine LRRC26 involvement on a functional mechanism of BK channel activation, Ca2+ spark–induced transient BK currents were measured in isolated myocytes. At a physiological voltage of −40 mV, LRRC26 knockdown reduced mean

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**Figure 2.** Leucine-rich repeat containing protein 26 (LRRC26) is primarily located in the plasma membrane and in close spatial proximity to large-conductance calcium (Ca)2+-activated potassium (BK) α subunits in arterial myocytes. A, Representative Western blot and mean data of surface biotinylation experiments indicating that the majority of LRRC26 protein is located in the plasma membrane in cerebral arteries (n=6). B, Representative confocal images illustrating that LRRC26 colocalizes with wheat germ agglutinin (WGA), a plasma membrane marker (n=6). Scale bar, 10 μm. C, Confocal images illustrating pixel overlay and N-fluorescence resonance energy transfer for indicated primary antibodies. D, Western blot illustrating that BKα antibodies coimmunoprecipitate BKα and LRRC26 proteins in arterial lysate. Co-IP indicates coimmunoprecipitation; IC, intracellular fraction; TRPM4, transient receptor potential melastatin 4 cation channel.
transient BK current frequency from ≈0.70 to 0.20 Hz or by ≈71% (Figure 6B). At the same voltage, LRRC26 knockdown also decreased mean BK current amplitude from ≈19.0 to 10.7 pA or by ≈44% (Figure 6C). Similar data were obtained at 0 mV, where LRRC26 knockdown reduced transient BK current frequency and amplitude by ≈55% and 36%, respectively (Figure 6B and 6C). In contrast to effects on transient BK currents, LRRC26 knockdown did not alter Ca2+ spark frequency or amplitude in myocytes of intact cerebral arteries (Online Figure III). These data suggest that LRRC26 knockdown attenuates BK channel coupling to Ca2+ sparks, which reduces transient BK current frequency and amplitude in arterial myocytes.

**LRRC26 Regulates Functional BK Channel Activity and Arterial Contractility**

Physiological functions of LRRC26 were measured using cannulated arteries pressurized to between 20 and 100 mm Hg. LRRC26 knockdown increased myogenic tone at intravascular pressures between 40 and 100 mm Hg (Figure 7B). LRRC26 knockdown reduced vasoconstriction (at 60 mm Hg) induced by iberiotoxin, a BK channel inhibitor, from ≈22.3 in control to ≈10.8 µm or by ≈52% (Figure 7C and 7E). In contrast, LRRC26 knockdown did not alter membrane depolarization-induced (60 mmol/L K+) vasoconstriction (Online Figure IV), which was larger than that of iberiotoxin (Figure 7C and 7E). LRRC26 knockdown also did not alter passive arterial diameter (data at 60 mm Hg: control, 284±8 µm; n=13 and LRRC26 knockdown, 272±7 µm; n=11). These data support that constriction to iberiotoxin in LRCC26 knockdown arteries is not attenuated because of a reduction in vasocontractile range but because of reduced BK channel function. In support of this conclusion, LRRC26 knockdown reduced vasodilation to NS1619, a BK channel activator, from ≈35.6 µm in control to ≈24.3 µm or by ≈32% (Figure 7D and 7E). These data indicate that LRRC26 knockdown reduces BK channel voltage and apparent Ca2+ sensitivity, leading to a reduction in transient BK currents and an elevation in intravascular pressure-induced vasoconstriction.

**Discussion**

Here, we investigated for the first time LRRC expression and functionality in arterial myocytes. Our data indicate that LRRC26 is present in arterial myocytes, where it is primarily located in the plasma membrane and associated with BK channel α subunits. LRRC26 knockdown decreased native BK channel voltage and apparent Ca2+ sensitivity and reduced transient BK current frequency and amplitude. LRRC26 knockdown also increased myogenic tone and reduced functional BK channel activity. Taken together, these data indicate that LRRC26 elevates BK channel voltage and apparent Ca2+ sensitivity, inducing vasodilation. Thus, LRRC26 is a BK channel auxiliary γ subunit in arterial myocytes.
LRRC26 was first identified in immunopurified BK channel complexes from LNCaP cells using liquid chromatography/tandem mass spectroscopy. Coimmunoprecipitation experiments using BKα and LRRC26 antibodies for pulldown were performed in LNCaP cells and recombinant expression systems and demonstrated an association between the BK channel and LRRC26. These data in addition to the profound shift in BK channel voltage sensitivity induced by LRRC26 led to designation of LRRC26 as a BK channel γ auxiliary subunit, terminology now used by others. Several members of the LRRC Elron subfamily have since been identified as BK channel γ (γ1–4) subunits that can exhibit tissue-specific expression and function. LRRC26 message was detected in whole salivary gland, prostate, trachea, thyroid gland, thymus, colon, fetal brain, and aorta. Of the 4 LRRC isoforms studied in aorta, LRRC26 message was highest, with low expression of LRRC38 and little to no expression of LRRC52 and 55. LRRC proteins were not measured. Similarly, which aortic cell type(s) expressed LRRCs was not determined. Aorta is a conduit artery that does not regulate peripheral vascular resistance. It was unclear whether resistance-size arteries that control organ blood pressure and flow express LRRC proteins. We detected LRRC26 mRNA in both intact cerebral arteries and isolated pure cerebral artery myocytes. Western blotting using

Figure 5. Leucine-rich repeat containing protein 26 (LRRC26) knockdown decreases large-conductance calcium (Ca2+)—activated potassium (BK) channel apparent Ca2+ sensitivity in arterial myocytes. A, Representative BK channel recordings from inside-out patches pulled from control siRNA- or LRRC26 siRNA-treated arterial myocytes with 1, 10, or 30 μmol/L free Ca2+ at −40 mV. B, Mean data. Experimental numbers are (from left to right) control siRNA: 8, 11, 15, 7, 5; LRRC26 siRNA: 5, 4, 12, 7, 10, 11. Data are fit with a Boltzmann function. C, Lithocholate (150 μmol/L) activates BK channels in patches from control siRNA or LRRC26 siRNA-treated myocytes (−40 mV, 10 μmol/L [Ca2+]). *P<0.05 vs control siRNA. #P<0.05 vs same condition before lithocholate.

Figure 6. Leucine-rich repeat containing protein 26 (LRRC26) knockdown inhibits transient large-conductance calcium (Ca2+)—activated potassium (BK) currents in arterial myocytes. A, Representative recordings of transient BK currents recorded in control siRNA- and LRRC26 siRNA—treated arterial myocytes at −40 and 0 mV. B and C, Mean data. Experimental numbers are (from left to right) control siRNA: 8, 9, 11, 15, 7; LRRC26 siRNA: 5, 11. Data are fit with a Boltzmann function. B, Lithocholate (150 μmol/L) activates BK channels in patches from control siRNA or LRRC26 siRNA-treated myocytes (−40 mV, 10 μmol/L [Ca2+]). *P<0.05 vs control siRNA.
a LRRC26 antibody identified a protein that was abolished by an antigenic peptide at ≈42 kDa, which is consistent with glycosylated LRRC26.21 Arterial biotinylation, immunofluorescence, and immunofluorescence resonance energy transfer indicated that the majority of LRRC26 (>80%) was located at the myocyte surface and in close spatial proximity to plasma membrane–resident BKα subunits. Furthermore, the BK channel antibody coimmunoprecipitated BKα and LRRC26 proteins from arterial lysate suggest that they are located in the same macromolecular complex. These data indicate that native LRRC26 protein is associated with pore-forming BKα subunits in arterial myocytes.

Here, LRRC26 knockdown shifted BK channel $V_{1/2}$ by ≈+33 mV in arterial myocytes. This depolarizing shift occurred within the physiological voltage range of arterial myocytes, which is primarily between −60 and −40 mV.4 Previous studies indicated that LRRC26 overexpression shifted the $V_{1/2}$ of recombinant BKα subunits expressed in HEK293 cells by ≈−153 mV and BK channels in PC3 prostate cancer cells by ≈−136 mV.20,21 LRRC26 knockdown in LNCaP cells, which express atypical BK channels with high voltage sensitivity, right-shifted BK channel $V_{1/2}$ by +134 mV. There are several explanations for the different degrees of shift in $V_{1/2}$ measured in these previous studies and in arterial myocytes. First, LRRC26 knockdown reduced total LRRC26 protein by approximately half in cerebral arteries. Conceivably, complete LRRC26 knockout in arterial myocytes may induce a further decrease in BK channel voltage sensitivity. Second, the HEK293 and PC3 cell experiments previously described compared control BK channel voltage sensitivity with that after LRRC26 overexpression. LRRC26 expression may be lower in arterial myocytes than in these recombinant systems that typically express large amounts of protein. Furthermore, LNCaP cells may express more LRRC26 than arterial myocytes and knockdown may shift BK channel voltage sensitivity to a larger degree than we observed in arterial myocytes. Third, arterial myocytes express β1 auxiliary subunits. LRRC26 modulation of BK channel voltage sensitivity did not require β1, but β1 overexpression blocked the LRRC26-induced voltage-shift in recombinant BK channels expressed in HEK293 cells.20 These data suggested that β1 competes with LRRC26 to modulate BK channel activity.20 In contrast to β1, which induces incremental gating shifts as the number of subunits in the channel complex increases, LRRC26 alters BK channel gating in an all-or-none manner when differing ratios of γ1:BKα RNA are injected in *Xenopus laevis* oocytes.30,31 Our data obtained using litcholocholate show that LRRC26 knockdown did not inhibit β1 subunit-mediated BK channel activation. These data were consistent with those demonstrating that LRRC26 knockdown did not alter surface β1 expression. Recent evidence suggests that only a small fraction of total β1 subunits is associated with BK channels in arterial myocytes.32 Intracellular β1 subunits in arterial myocytes are stored within Rab11A-positive recycling endosomes and stimulated to surface traffic by cGMP- and cAMP-dependent signaling pathways, which elevates BK channel–associated β1 subunits, leading to channel activation.32 Conceivably, endogenous β1 subunits may attenuate the LRRC26-induced elevation in BK channel voltage sensitivity in arterial myocytes although this remains to be determined. Additional studies should investigate the possibility that LRRC26 and β1 subunits interact to modulate BK channel activity in arterial myocytes.

Our data indicate that LRRC26 knockdown reduced BK channel voltage and apparent Ca$^{2+}$ sensitivity, here defined as a
shift in the voltage and Ca\textsuperscript{2+} concentration ranges over which the channel opens, respectively. In contrast, LRRC26 overexpression did not alter the slope of the $V_{1/2}$–[Ca\textsuperscript{2+}] relationship and shifted the $V_{1/2}$ of recombinant BK channels with mutated Ca\textsuperscript{2+} activation sites.\textsuperscript{30} Our data may be explained when taking into account that BK channel apparent Ca\textsuperscript{2+} sensitivity is voltage dependent.\textsuperscript{13} Although LRRC26 may not modulate BK channel Ca\textsuperscript{2+} sensitivity itself, a LRRC26-induced leftward shift in voltage sensitivity indirectly increases activation by Ca\textsuperscript{2+} because Ca\textsuperscript{2+} and voltage dependence are allosterically coupled. Data also suggest that LRRC26 shifts the Ca\textsuperscript{2+} set point, which is typically defined as the free Ca\textsuperscript{2+} concentration required for half-maximal activation at 0 mV. Here, single BK channel apparent Ca\textsuperscript{2+} sensitivity was not determined as a function of voltage but measured at $-40$ mV, a voltage similar to that of arteries at a physiological intravascular pressure of $\approx 60$ mmHg.\textsuperscript{14} Ca\textsuperscript{2+} set points here cannot be calculated according to the standard definition but extrapolation of the current data at $-40$ mV is possible given that $V_{1/2}$ was in the linear portion of the $P_{ca}$–V relationship. At 10 mmol/L [Ca\textsuperscript{2+}], the $V_{1/2}$ of control and LRRC26 knockdown BK channels was $-20$ and $+13$ mV, respectively, a difference of 33 mV. Therefore, Ca\textsuperscript{2+} set points are $<10$ mmol/L [Ca\textsuperscript{2+}], for control BK channels and $>10$ mmol/L [Ca\textsuperscript{2+}] for LRRC26-knockdown channels.

Here, LRRC26 knockdown reduced BK channel apparent Ca\textsuperscript{2+} sensitivity within the micromolar Ca\textsuperscript{2+} concentration range generated by Ca\textsuperscript{2+} sparks.\textsuperscript{17} Ca\textsuperscript{2+} sparks are local intracellular Ca\textsuperscript{2+} transients generated by the opening of sarcoplasmic reticulum ryanodine-sensitive Ca\textsuperscript{2+} release channels.\textsuperscript{7,16–18} A single Ca\textsuperscript{2+} spark activates multiple BK channels producing a transient BK current. Transient BK current frequency is regulated by Ca\textsuperscript{2+} spark frequency, whereas the effective coupling of BK channels to Ca\textsuperscript{2+} sparks controls both transient BK current frequency and amplitude. LRRC26 knockdown did not alter Ca\textsuperscript{2+} sparks but decreased transient BK current frequency and amplitude at both $-40$ and 0 mV. Our data suggest that LRRC26 knockdown reduces BK channel apparent Ca\textsuperscript{2+} sensitivity, thereby attenuating the effective coupling of BK channels to Ca\textsuperscript{2+} sparks, leading to a decrease in transient BK current frequency and amplitude in arterial myocytes. To summarize, data indicate that LRRC26 elevates the effective coupling of BK channels to Ca\textsuperscript{2+} sparks, thereby increasing transient BK current frequency and amplitude in arterial myocytes. Previous studies have demonstrated that $\beta$ subunits also increase BK channel sensitivity to Ca\textsuperscript{2+} sparks in arterial myocytes.\textsuperscript{14,15} In contrast to $\beta$ subunits, which elevate BK channel coupling to Ca\textsuperscript{2+} sparks by directly increasing Ca\textsuperscript{2+} sensitivity, LRRC26 indirectly elevates Ca\textsuperscript{2+} sensitivity by increasing voltage sensitivity. Thus, our data indicate that $\beta$ and $\gamma$ subunits control BK channel Ca\textsuperscript{2+} sensitivity and activity via distinct mechanisms in arterial myocytes. Such multimodal regulation permits fine tuning of BK channel activity.

Intravascular pressure stimulates membrane depolarization, which activates voltage-dependent Ca\textsuperscript{2+} channels, leading to an [Ca\textsuperscript{2+}] elevation and vasocostriction.\textsuperscript{6} Pressure-induced depolarization also activates Ca\textsuperscript{2+} sparks, which stimulate BK channels to partially oppose the myogenic response.\textsuperscript{24,25} We show that LRRC26 knockdown elevated myogenic tone across a wide range of intravascular pressures. Iberiotoxin was a less effective vasocostrictor and NS1619 a weaker vasodilator, indicating that LRRC26 knockdown inhibits functional BK channel activity. Thus, data indicate that LRRC26 activates BK channels to oppose the myogenic response.

Findings of this study should stimulate future research into physiological and pathological functions of BK channel $\gamma$ subunits in arterial myocytes and other cell types. Conceivably, LRRC proteins, including LRRC26, may be expressed in myocytes of vascular beds other than the cerebral circulation, increase BK channel activity, and modulate contractility. $\gamma$ subunit expression and functionality may exhibit regional vascular differences similarly to $\beta$ subunits. For example, cerebral artery myocyte BK channels have a higher $\beta$ to subunit ratio than cremaster artery myocytes, elevating their Ca\textsuperscript{2+} sensitivity.\textsuperscript{36} Because $\gamma$ and $\beta$ subunits interact to modulate BK channels, variable expression of each subunit may fine tune and customize BK channel voltage and Ca\textsuperscript{2+} sensitivity in myocytes of different vascular beds. Hypertension is associated with a decrease in $\beta$ expression and function in arterial myocytes, leading to a reduction in BK channel activity and vasocostriction.\textsuperscript{35,37,38} Conceivably, alterations in $\gamma$ subunits may also contribute to attenuated BK channel activity during vascular disease. Finally, given that BK channels are a potential therapeutic target, $\gamma$ subunits may be a novel molecular target to treat cardiovascular diseases.

In summary, we show that LRRC26 is expressed in cerebral artery myocytes where it is primarily plasma membrane localized and associated with BK$\alpha$ subunits. LRRC26 knockdown reduced BK channel voltage and apparent Ca\textsuperscript{2+} sensitivity within physiological ranges and inhibited transient BK current frequency and amplitude. LRRC26 knockdown also increased myogenic tone and reduced functional BK channel activity. These data indicate that LRRC26 is an arterial myocyte BK channel auxiliary $\gamma$ subunit that elevates voltage and apparent Ca\textsuperscript{2+} sensitivity to induce vasodilation.

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**Disclosures**

None.

**References**

LRRC26 is an Arterial Myocyte γ Subunit

What New Information Does This Article Contribute?

- LRRC26 is expressed in cerebral artery myocytes where it is primarily located in the plasma membrane and associated with BK channel α subunits.
- LRRC26 elevates BK channel voltage and apparent Ca2+ sensitivity and Ca2+ spark–induced transient BK currents in arterial myocytes, inducing vasodilation.
- LRRC26 is a BK channel auxiliary γ subunit in arterial myocytes.

BK channels are formed from pore-forming BK α and auxiliary γ1 subunits, which elevate channel apparent Ca2+ sensitivity. Recent studies have identified LRRC as a novel family of BK channel auxiliary γ subunits although expression and physiological functions in native cell types are unclear. We show that LRRC26 is expressed in arterial myocytes where it is located primarily in the plasma membrane and associated with BK channel α subunits. LRRC26 knockdown decreased native BK channel voltage and apparent Ca2+ sensitivity and reduced Ca2+ spark–induced transient BK current frequency and amplitude. LRRC26 knockdown increased pressure-induced vasoconstriction (myogenic tone) and reduced functional responses to a BK channel activator and inhibitor. Our data indicate that LRRC26 elevates BK channel voltage and apparent Ca2+ sensitivity in arterial myocytes, inducing vasodilation. The identification of LRRC26 as a functional BK channel γ subunit should promote the study of novel mechanisms of vascular control by this protein and pathological involvement in cardiovascular diseases.
LRRC26 Is a Functional BK Channel Auxiliary γ Subunit in Arterial Smooth Muscle Cells
Kirk W. Evanson, John P. Bannister, M. Dennis Leo and Jonathan H. Jaggar

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SUPPLEMENTAL MATERIAL

EXPANDED METHODS

All animal protocols were reviewed and approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Male Sprague-Dawley rats (8 weeks) were euthanized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed and placed in an ice-cold (4°C) physiological saline solution (PSS) consisting of (in mmol/L): 6 KCl, 112 NaCl, 24 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.8 CaCl₂, and 10 glucose, which was gassed with 21% O₂-5% CO₂-74% N₂ to pH 7.4. Resistance-size arteries were carefully dissected away from the brain and the connective tissue removed. Where appropriate, cerebral artery myocytes were enzymatically dissociated, as previously described.¹

PCR

Total RNA was extracted from either whole arteries or ~200-300 isolated selected arterial myocytes using TRIzol (Life Technologies) or the Absolutely RNA Nanoprep kit (Stratagene), respectively. First-strand cDNA was generated from 1-5 ng of total RNA using Protoscript M-MULV (New England Biolabs). PCR was performed on first-strand cDNA using primers sequences shown in Supplemental Table 1. PCR was performed as follows: 93°C for 3 minutes then 40 cycles of 93°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute with a final extension step of 72°C for 7 minutes. PCR products were separated on 1.5% agarose gels.

Protein Analysis

Arteries were homogenized in lysis buffer, centrifuged for 8 minutes at 6,000 g, and the supernatant stored at -20°C. Laemmli buffer was added and the samples boiled for 3 minutes prior to loading onto the gel. Samples (40 μg) were separated on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were incubated with either goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology), rabbit polyclonal anti-BK β1 (1:500, Abcam), mouse monoclonal anti-BKα (1:500, Neuromab, UC Davis), or mouse monoclonal antiax-in (1:10000, Millipore). For antigenic peptide experiments, membranes were incubated with goat polyclonal anti-LRRC26 (1:100) and antigenic peptide (20 μg/ml). Following incubation with their respective secondary antibodies, membranes were developed using a chemiluminescent detection kit (Pierce) and imaged with a Kodak In Vivo F Pro Imaging System (Carestream Molecular Imaging). Band densitometry was analyzed using Quantity One software (Bio-Rad). LRRC26, β1, and BKα band densities were normalized to actin.

Surface Biotinylation

Arteries were incubated on a rocker for 1 hour in a solution consisting of phosphate buffered saline (PBS) with 1 mg/ml each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin (Pierce). Free biotin was quenched by washing the arteries in PBS with 100 mmol/L glycine. Biotinylated arteries were homogenized in a lysis buffer (50 mmol/L tris HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% triton X, 0.1% SDS), centrifuged for 8 minutes at 6,000 g, and the supernatant collected. Following protein estimation, the sample (1 μl beads / μg protein) was incubated with avidin beads (Monomeric Avidin Agarose, Pierce) for 1 hour on a rocker. The sample was centrifuged at 13,000 g and the supernatant (nonbiotin-bound proteins) set aside. The beads were washed 3 times, and then Laemml buffer (2.5% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% β-mercaptoethanol in 100 mmol/L Tris-HCl; pH 6.8) was added to both the beads and the nonbiotin-bound protein lysate. Samples were then boiled for 3 minutes to elute the biotinylated proteins from the avidin beads and to denature the proteins for analysis. The biotinylated sample was centrifuged at 13,000 g and the supernatant removed.
and transferred to a new tube. Western blotting was used to determine the relative distribution of surface (biotinylated) and intracellular (nonbiotinylated) fractions.

**Immunofluorescence and ImmunoFRET Microscopy**

Isolated myocytes were plated on poly-L-lysine-coated coverslips, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. For colocalization experiments, cells were blocked with PBS containing 5% bovine serum albumin (BSA) and incubated with goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology) overnight at 4°C. For antigenic peptide experiments, cells were incubated with goat polyclonal anti-LRRC26 (1:100) and antigenic peptide (20 µg/ml). Cells were washed with PBS and incubated with anti-goat Alexa 488 secondary antibody for 1 hour. Myocytes were incubated with Alexa 546-tagged wheat germ agglutinin (1:100, Life Technologies). After washing, coverslips were secured to slides with mounting media (1:1 glycerol:PBS) and images acquired using a laser scanning confocal microscope (LSM Pascal, Carl Zeiss). Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505-530 and ≥ 560 nm, respectively. Weighted colocalization was determined with the LSM FRET Macro tool (v2.5, Carl Zeiss).

For immunoFRET, myocytes were fixed and incubated with one of the following primary antibodies: goat polyclonal anti-LRRC26 (1:100, Santa Cruz Biotechnology), mouse monoclonal anti-BKα (1:100, Neuromab, UC Davis) or rabbit polyclonal TRPM4 (1:100, Thermo Fisher Scientific) overnight at 4°C. Cells were then incubated for 1 hour with the following secondary antibodies: anti-goat Alexa 488 or anti-goat Alexa 546 (LRRC26), anti-mouse Alexa 546 (BKα), or anti-rabbit Alexa 488 (TRPM4). After washing, coverslips were dried and mounted onto glass slides. Fluorescence images were acquired using a laser-scanning confocal microscope. Alexa 488 and 546 secondary antibodies were excited at 488 and 543 nm with emission detected at 505-530 and ≥ 560 nm, respectively. Images were background-subtracted, and N-FRET calculated on a pixel-by-pixel basis for the entire image and in regions of interest (within the boundaries of the cell) using the Xia method and LSM FRET Macro tool (v2.5, Carl Zeiss). The Förster co-efficient for the Alexa Fluor pair is ~ 6.3 nm.

**Co-Immunoprecipitation**

For each experiment, lysate was harvested from cerebral arteries pooled from 6 rats using ice-cold Radio-Immunoprecipitation (RIPA) buffer, giving ~700 µg total protein. Co-immunoprecipitation was performed using the Catch and Release V2.0 Co-immunoprecipitation kit (Millipore) as per the manufacturer’s protocol. Briefly, arterial lysate was incubated with control mouse IgG or BKα mouse monoclonal antibody (1 µg/µL) with 10 µL of antibody affinity ligand and 0.5 mL of the capture resin in the column provided at 4 °C overnight. The column was then centrifuged at 5000 rpm for 30 sec and the flow through discarded. The capture resin was washed twice with the wash buffer provided and bound proteins released with 70 µL of denaturing buffer. The eluate was boiled for 3 min and run on a SDS-PAGE gel. Protein samples were analyzed by Western blotting using mouse monoclonal anti-BKα (NeuroMab) or goat polyclonal anti-LRRC26 (Santa Cruz) and horseradish peroxidase-conjugated secondary antibodies, as previously described.

**LRRC26 Knockdown**

Cerebral arteries were placed in an electroporation chamber (Bex) containing 200 µl of PBS with either control or LRRC26-specific siRNAs (Life Technologies) for 5 minutes. The arteries were transfected using an electroporator (CUY21Vivo-SQ electroporator, Bex Co. Ltd.) and stored at 37°C in DMEM-F12 50/50 (HEPES-free) culture medium supplemented with 1% penicillin-streptomycin for 48-72 hours prior to use. Arteries permeabilized using electroporation or reverse permeabilization develop similar levels of myogenic tone.
Electrophysiology

Single BK channel or transient BK currents were recorded in isolated myocytes using the inside-out or perforated cell patch-clamp configurations, respectively. An Axopatch 200B amplifier and Clampex 8.2 (Molecular Devices) were used to record currents. For inside-out patch-clamp, the pipette and bath solutions both contained (in mmol/L): 130 KCl, 10 HEPES, 5 EGTA, 1.6 HEDTA, 1 MgCl₂, and 10 µmol/L free Ca²⁺ (pH 7.2). Free Ca²⁺ was adjusted to between 1 and 300 µmol/L and free Mg²⁺ concentration maintained at 1 mmol/L with CaCl₂ and MgCl₂, respectively. Free Ca²⁺ concentration was calculated using WEBMAXC Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm) and measured using Ca²⁺-sensitive (no. 476041; Corning) and reference (no. 476370; Corning) electrodes. To measure channel voltage-sensitivity, 300 ms voltage pulses between -100 and +100 mV were applied in 20 mV increments using a holding potential of -40 mV. BK channel apparent Ca²⁺-sensitivity was measured at a steady voltage of -40 mV. For the single-channel experiments, the bath solution (in mmol/L): 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose (pH 7.4). The pipette solution contained (in mmol/L): 140 KCl, 1.9 MgCl₂, 0.037 CaCl₂, 10 HEPES, 0.1 EGTA, and 2 Na₂ATP (pH 7.2). For all patch-clamp experiments, data were digitized at 5 kHz and filtered at 1 kHz. Analyses for voltage- and apparent Ca²⁺-sensitivity experiments were performed offline using Clampfit 9.2 (MDS Analytical Technologies). BK channel activity (NPₒ) was calculated using the following equation: NPₒ = Σ (t₁ + t₂…tᵢ), where tᵢ is the relative open time (time open / total time) for each channel level. Open probability (Pₒ) was calculated by dividing NPₒ by the total number of channels. The total number of channels in a patch was determined by applying a voltage of +100 mV with 1 mmol/L free Ca²⁺ in the bath solution. Voltage- and apparent Ca²⁺-sensitivity data were fit with the Boltzmann function: Y = Pₒₘᵦᵣ₉ X represents voltage or Ca²⁺, and slope represents the steepness of the curve. Transient BK currents were analyzed offline using a custom software program provided by Drs. M.T. Nelson and A.D. Bonev (University of Vermont). The criterion for a transient BK current was defined as a current equal to or greater than three times the single channel amplitude.

Confocal Ca²⁺ imaging

Cerebral artery segments were cannulated and incubated in the dark with fluo-4 AM (10 µmol/L) (Molecular Probes, Invitrogen, Eugene, OR) and 0.05% Pluronic F-127 (Molecular Probes, Invitrogen, Eugene, OR) for 1 h followed by a 30-minute wash. Intracellular Ca²⁺ signals in smooth muscle cells were imaged using a Noran Oz laser-scanning confocal microscope with a 60X water-immersion objective (NA 1.2) by illuminating with 488-nm light and collecting emitted light >500 nm. Sequential images (256 x 240 pixels, 56.3 x 52.8 µm) of each region of the arterial wall containing ~8-10 smooth muscle cells were recorded every 16.6 ms (i.e., at 60 Hz) for 10 seconds. In each artery, 2-3 regions that contained different smooth muscle cells were scanned under each condition. Ca²⁺ spark frequency and amplitude was analyzed offline using custom software written with IDL 5.3 kindly provided by Dr. M. T. Nelson (University of Vermont). The full 10 second acquisition period of each image stack was analyzed to measure Ca²⁺ sparks which were detected by dividing fluorescence (F) in an area 1.54 µm × 1.54 µm (7 x 7 pixels, 2.37 µm²) in each image by a baseline fluorescence (Fₒ) that was determined by averaging 10 images without Ca²⁺ spark activity. A Ca²⁺ spark was defined as a local rapid increase in F/Fₒ>1.2. Ca²⁺ spark frequency was obtained from multiple regions of the same artery and then averaged, giving data for each artery. Multiple arterial Ca²⁺ spark frequency values were then averaged, generating mean data with standard errors. In results, n (experimental number) refers to the number of arteries from which mean data were obtained.
Pressurized Artery Myography
Middle cerebral artery segments were cannulated in a perfusion chamber (Living Systems Instrumentation) maintained at 37°C and continuously perfused with PSS. Intravascular pressure was controlled through a reservoir system and monitored with a pressure transducer. Wall diameter was measured using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix) by acquiring data at 1 Hz. Myogenic tone (%) was calculated as: 100 x (1 – D_{active} / D_{passive}), where D_{active} is active arterial diameter and D_{passive} is the passive arterial diameter determined by the application of Ca^{2+}-free PSS supplemented with 5 mmol/L EGTA. Myogenic tone was assessed over a range of intravascular pressures (20-100 mmHg) with D_{active} and D_{passive} obtained at each pressure to generate a pressure-response curve.

Statistical analysis
Data are expressed as mean ± SE. An independent samples t-test was used to determine if a significant difference existed between group means. The criterion for statistical significance was the same for all tests (α = 0.05).
Online Figure I. LRRC26 antigenic peptide specifically blocks Western blot and immunofluorescence detection of LRRC26. A-B: Western blot (A) and confocal images (B) illustrating the effect of the antigenic peptide to block LRRC26 detection using a LRRC26-specific antibody. Scale Bar=10 μm. AP (antigenic peptide), DIC (differential interference contrast)
Online Figure II. LRRC26 knockdown did not affect single BK channel conductance. Mean data for control (262±5 pS, n=10) and LRRC26 (260±5 pS, n=7) siRNA-treated myocytes.
Online Figure III. LRRC26 knockdown did not alter Ca\textsuperscript{2+} sparks in arterial myocytes. A: confocal images demonstrating average fluo-4 AM fluorescence in control siRNA- and LRRC26 siRNA-treated arterial myocytes. B: representative traces of Ca\textsuperscript{2+} sparks that occurred at labeled areas in panel A. C: mean data of Ca\textsuperscript{2+} spark frequency and amplitude (control siRNA: n=6, LRRC26 siRNA: n=6).
Online Figure IV. LRRC26 knockdown did not affect 60 mmol/L K⁺-induced constriction at 60 mmHg (control siRNA: n=6, LRRC26 siRNA: n=7).
## Online Table I

Primer sequences used for RT-PCR

<table>
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<th>Transcripts</th>
<th>Primers</th>
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<tr>
<td>LRRC26</td>
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<td>Forward</td>
<td>5'-CTGCTATACCTAGTCCTGCG-3'</td>
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<td>Reverse</td>
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<td>Myosin heavy chain 11</td>
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SUPPLEMENTAL REFERENCES


