Endothelin-1 Stimulates Vasoconstriction Through Rab11A Serine 177 Phosphorylation

Xue Zhai, M. Dennis Leo, Jonathan H. Jaggar

**Rationale:** Large-conductance calcium-activated potassium channels (BK) are composed of pore-forming BKα and auxiliary β1 subunits in arterial smooth muscle cells (myocytes). Vasoconstrictors, including endothelin-1 (ET-1), inhibit myocyte BK channels, leading to contraction, but mechanisms involved are unclear. Recent evidence indicates that BKα is primarily plasma membrane localized, whereas the cellular location of β1 can be rapidly altered by Rab11A-positive recycling endosomes. Whether vasoconstrictors regulate the multisubunit composition of surface BK channels to stimulate contraction is unclear.

**Objective:** Test the hypothesis that ET-1 inhibits BK channels by altering BKα and β1 surface trafficking in myocytes, identify mechanisms involved, and determine functional significance in myocytes of small cerebral arteries.

**Methods and Results:** ET-1, through activation of PKC (protein kinase C), reduced surface β1 abundance and the proximity of β1 to surface BKα in myocytes. In contrast, ET-1 did not alter surface BKα, total β1, or total BKα proteins. ET-1 stimulated Rab11A phosphorylation, which reduced Rab11A activity. Rab11A serine 177 was identified as a high-probability PKC phosphorylation site. Expression of a phosphorylation-incapable Rab11A construct (Rab11A S177A) blocked the ET-1-induced Rab11A phosphorylation, reduction in Rab11A activity, and decrease in surface β1 protein. ET-1 inhibited single BK channels and transient BK currents in myocytes and stimulated vasoconstriction via a PKC-dependent mechanism that required Rab11A S177. In contrast, NO-induced Rab11A activation, surface trafficking of β1 subunits, BK channel and transient BK current activation, and vasodilation did not involve Rab11A S177.

**Conclusions:** ET-1 stimulates PKC-mediated phosphorylation of Rab11A at serine 177, which inhibits Rab11A and Rab11A-dependent surface trafficking of β1 subunits. The decrease in surface β1 subunits leads to a reduction in BK channel calcium-sensitivity, inhibition of transient BK currents, and vasoconstriction. We describe a unique mechanism by which a vasoconstrictor inhibits BK channels and identify Rab11A serine 177 as a modulator of arterial contractility. ([Circ Res. 2017;121:650-661. DOI: 10.1161/CIRCRESAHA.117.311102.)

**Key Words:** cell membrane ■ endothelins ■ muscle cells ■ protein kinase C ■ serine
Novelty and Significance

What Is Known?

• BK channels composed of pore-forming BKα and auxiliary J1 subunits are expressed in arterial smooth muscle cells and regulate contractility.

• J1 subunit anterograde trafficking is controlled by Rab11A—a Rab GTPase—and surface abundance of the J1 subunit can be increased by several stimuli that activate BK channels, including NO and membrane depolarization, leading to vasodilation.

• Whether vasoconstrictors modulate the surface trafficking of J1 subunits to inhibit BK channels is unclear.

What New Information Does This Article Contribute?

• We show that endothelin-1—a potent vasoconstrictor—stimulates PKC, which phosphorylates Rab11A at serine 177, reducing Rab11A activity and Rab11A-dependent surface trafficking of J1 subunits.

• The endothelin-1-induced decrease in surface J1 subunits inhibits BK channels, resulting in vasoconstriction.

Nonstandard Abbreviations and Acronyms

AUC area under the curve
BIM bisindolylmaleimide
BK large-conductance calcium-activated potassium
Ca2+ calcium
ET-1 endothelin-1
FRET Förster resonance energy transfer
PKC protein kinase C
Po open probability
SNP sodium nitroprusside
WT wild type

The membrane current (I) generated by a population of ion channels, such as BK, is determined by their open probability (Po), the number of channels (N), and single channel current (i). Recent evidence indicates that in resting arterial myocytes, only a small proportion of total J1 subunits are present at the plasma membrane, with most J1 protein located intracellularly within Rab11A-positive recycling endosomes. NO stimulates rapid (<1 minute) PKG (protein kinase G)-mediated surface trafficking of intracellular J1 subunits, which associate with BKα to increase apparent Ca2+ sensitivity, thereby elevating Po. Stimulated J1 subunit trafficking in myocytes is a principal mechanism by which NO promotes cerebral vasodilation. Membrane depolarization also activates BK channels by stimulating Rho kinase-mediated J1 subunit trafficking in human and rat arterial myocytes. It is unclear whether vasoconstrictors inhibit BK channels and stimulate contraction by reducing the surface abundance of J1 subunits and their association with BKα. If such mechanisms exist, the processes by which vasoconstrictors decrease surface levels of J1 would be important to determine.

Here, we investigated the regulation of arterial myocyte J1 subunit trafficking by ET-1. We show that ET-1 stimulates PKC (protein kinase C)-mediated phosphorylation of Rab11A at serine 177, which inhibits Rab11A- and Rab11A-mediated surface trafficking of J1 subunits. This mechanism reduces BK channel Po and transient BK currents, leading to vasoconstriction. These data describe a unique mechanism by which a vasoconstrictor inhibits BK channels in arterial myocytes and stimulates contraction.

Methods

Tissue Preparation

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Male Sprague-Dawley rats (7–8 weeks) were euthanized with intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was removed, cleaned, and placed in a HEPES (N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid)-buffered physiological saline solution (PSS) containing (in mmol/L) 134 NaCl, 6 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4). Cerebral arteries (middle cerebral, cerebellar, and posterior cerebral) were dissected from the brain, and myocytes were isolated using enzymes, as described previously.

Arterial Surface Biotinylation

Intact arteries were biotinylated as described previously. Briefly, arteries were incubated in HEPES-buffered PSS containing 1 mg/mL EZ-link sulfo-NHS-LC-C-biotin and 1 mg/mL EZ-link maleimide-PEG2-biotin reagents (Pierce) for 1 hour. Arteries were then washed with 100 mmol/L glycine in phosphate-buffered saline to remove unbound biotin. Biotinylated arteries were homogenized in lysis buffer containing 1% Triton X-100. Total protein was determined to normalize for avidin pull-down of biotinylated surface proteins. After pull down, biotinylated proteins were bound to the avidin beads, and the supernatant contained the nonbiotinylated (intracellular) protein fraction. Biotinylated surface proteins were eluted from the avidin beads.

Both protein fractions were analyzed using Western blotting.

Western Blotting

Protein lysates were boiled with Laemmli buffer (Bio-Rad), separated on sodium dodecyl sulfate polyacrylamide gels, and transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk and then probed with mouse monoclonal anti-BKα (1:500 dilution; Neuromab, UC Davis), rabbit polyclonal anti-BKβ1 (1:500; Abcam), rabbit monoclonal anti-rab11A (1:500; Cell Signaling),
rabbit polyclonal antiphosphoserine (1:500; Millipore), or mouse monoclonal anti-α-BKβ1 (NewEast Biosciences) antibodies overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescent detection kit, the blots were imaged with a Kodak In Vivo F Pro Imaging System. Band intensity was quantified using Quantity One software (Bio-Rad).

**Immunofluorescence and Immuno-Förster Resonance Energy Transfer Microscopy**

Isolated myocytes were exposed to agents and then fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with 5% bovine serum albumin and then incubated with mouse monoclonal anti-BKα (Neuromab, UC Davis) and rabbit polyclonal anti-BKβ1 (Abcam) antibodies overnight at 4°C. After washing with phosphate-buffered saline, cells were incubated for 1 hour with Alexa 546- or Alexa 488-conjugated secondary antibodies (1:100 dilution; Life Technologies). Fluorescence images were acquired using a laser-scanning confocal microscope (LSM5 Pascal; Carl Zeiss). For N-Förster resonance energy transfer (FRET) analysis, images were background-subtracted and N-FRET calculated on a pixel-by-pixel basis using the Xia method and Zeiss LSM FRET Macro tool (version 2.5).

**Transfection of Intact Cerebral Arteries**

A Rab11A sequence with a serine 177-to-alanine mutation (Rab11A S177A) was generated and subcloned into pcDNA3.1(++; GenScript USA, Inc, Piscataway, NJ). Empty pcDNA3.1 vectors, pcDNA3.1 encoding Rab11A, or pcDNA3.1 encoding Rab11A S177A were transfected into arteries using electroporation (CUY21Vivo-SQ electroporator; Bex), as described previously. Arteries were then placed in serum-free DMEM (Dulbecco’s Modified Eagle’s Medium)-F12 media supplemented with 1% penicillin-streptomycin for 3 days before use.

**HEK293 Cell Culture and Transfection**

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions (21% O₂, 5% CO₂; 37°C). Cells were transiently transfected with pcDNA3.1(+) encoding full-length BKβ1 (2 μg) or empty vector (2 μg) using Effectene (Qiagen). Cells were lysed 48 hours post-transfection. Protein concentration was calculated as described previously. Cell lysates were run on 7.5% sodium dodecyl sulfate polyacrylamide gels, and blots were probed for β1 and actin.

**Immunoprecipitation**

Proteins were pulled down from arterial lysate using the Catch and Release, version 2.0, immunoprecipitation kit (Millipore) per the manufacturer’s instructions. Samples were incubated with Rab11A primary antibody, capture resin, and antibody affinity ligand in columns. The columns were centrifuged and washed. Bound proteins were released using elution buffer and then analyzed using Western blotting.

**Patch-Clamp Electrophysiology**

Single BK channels or transient BK currents were recorded in isolated myocytes using the inside-out or perforated patch-clamp configurations, respectively. For inside-out patch-clamp, myocytes were allowed to settle in a recording chamber before exposure to combinations of ET-1 (1 hour), bisindolylmaleimide (BIM; 1 hour), and sodium nitroprusside (SNP; 10 minutes) before patch excision. Inside-out patches were pulled, and agents were washed out so that channel activity was measured in the absence of these agents. For inside-out recordings, the pipette and bath solution both contained (in mmol/L) 130 KCl, 10 HEPES, 5 EGTA (ethyleneglycolbis(2-amino-ethylheterer)-N,N',N'-tetraacetic acid), 1.6 HEDTA (N,N'-bis(2-hydroxyethyl) ethylenediaminetetraacetic acid), 1 MgCl₂, and 10 μmol/L free Ca²⁺ (pH 7.2). Transient BK currents were measured using the amphotericin B perforated-patch configuration. For perforated patch-clamp, the bath solution contained (in mmol/L) 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4), and the pipette solution contained (in mmol/L) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA (pH 7.2). Currents were filtered at 1 kHz, digitized at 5 kHz, and analyzed using Clampfit 10.3 (MDS Analytic Technologies).

**Pressurized Artery Myography**

Artery segments (1–2 mm length) were cannulated in a perfusion chamber (Living Systems Instrumentation, St. Albans, VT) and continuously perfused at 37°C PSS which contained 112 NaCl, 4.8 KCl, 24 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 10 glucose, gassed with mixture of 21% O₂, 5% CO₂, and 74% N₂. The endothelium was denuded by introducing an air bubble into the lumen for ≈1 minute followed by wash with PSS. Arterial diameter was measured at an intravascular pressure of 60 mmHg, which was monitored using a pressure transducer. Arterial diameter was measured using a charge-coupled device camera attached to a Nikon TS100-F microscope and the edge-detection function of IonWizard (Ionoptix, Milton, MA). Myogenic tone (%) was calculated as 100×(1–D/Vmax), where D/Vmax is active arterial diameter, and Vmax is the diameter determined in Ca²⁺-free PSS supplemented with 5 mmol/L EGTA.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism v4.0 and Origin v6.0. Values are presented as mean±SEM. Student’s t test was used to compare paired and unpaired data from 2 populations. ANOVA with Student–Newman–Keuls post hoc test was used for multiple group comparisons. P<0.05 was considered significant.

**Results**

**ET-1 Inhibits NO- and Depolarization-Induced Surface Trafficking of β1 Subunits Via PKC Activation**

To measure the cellular distribution of BKα and β1 subunits in myocytes of intact arteries, we used biotinylation—a method previously validated to label surface but not intracellular proteins. Data indicated that BKα subunits were primarily present in the plasma membrane, whereas β1 was mostly intracellular in resting resistance-size cerebral arteries (Figure 1A and 1C). ET-1 alone did not change the surface abundance of either BKα or β1 subunits (Figure 1A and 1B). To further study ET-1 modulation of subunits, SNP—an NO donor—was first applied to stimulate surface trafficking of β1, as demonstrated previously. SNP increased surface β1 protein 2.6-fold but did not alter surface BKα protein (Figure 1A and 1B). ET-1 inhibited the SNP-induced increase in surface β1 protein (Figure 1A and 1B). Changes in surface β1 were associated with corresponding shifts in intracellular β1, suggesting β1 protein redistributed from an intracellular compartment to the surface (Online Figure 1A). SNP and ET-1 did not alter total β1 protein, indicating that β1 subunits were not degraded (Online Figure 1B). SNP and ET-1 did not alter BKα distribution or total protein (Figure 1C; Online Figure 1C). These data suggest that ET-1 inhibits NO-induced surface trafficking of β1 subunits in arterial myocytes.

Many vasoconstrictors, including ET-1, stimulate Gβ/γ in myocytes, leading to an increase in diacylglyceryl, which activates PKC. To examine mechanisms by which ET-1 decreased β1 subunit trafficking, BIM—a PKC inhibitor—was used. BIM abolished the ET-1–induced inhibition of β1 subunit surface trafficking, essentially restoring SNP-induced stimulation of surface β1 protein (Figure 1A and 1B). In contrast, BIM did not alter surface β1 protein when applied alone (Figure 1A). BIM also did not change surface BKα when applied alone or together with ET-1 (Figure 1A). These data suggest that ET-1 activates PKC, which inhibits surface trafficking of β1 subunits in arterial myocytes.
FRET imaging followed by N-FRET analysis was used to measure BKα and β1 subunit colocalization and spatial proximity in isolated cerebral artery myocytes. In control, the majority of BKα subunits were localized at the cell periphery, whereas β1 subunits were primarily intracellular. N-FRET between BKα and β1 subunit-bound secondary antibodies in control was low, as demonstrated previously11 (Figure 2A and 2B). SNP increased N-FRET ≈2.8-fold with FRET located at the cell surface (Figure 2A and 2B). ET-1 inhibited the ability of SNP to increase N-FRET, and this inhibitory effect was blocked by BIM (Figure 2A and 2B). These data suggest that ET-1 activates PKC, which inhibits β1 subunit surface trafficking, thereby blocking plasma membrane coassembly of β1 with BKα subunits in arterial myocytes.

Experiments were performed to investigate whether ET-1 inhibition of β1 subunit surface trafficking is specific to NO or a more general inhibitory mechanism. Membrane depolarization also stimulates an increase in surface β1 subunits, leading to BK channel activation.12 Membrane depolarization (30 mmol/L K+) increased surface β1 protein 3.70-fold but did not alter surface BKα in cerebral arteries (Figure 3A and 3B). ET-1 inhibited the depolarization-induced increase in surface β1 protein (Figure 3A and 3B). BIM reduced the ET-1–induced inhibition of β1 subunit surface trafficking by ≈60% (Figure 3A and 3B). These data suggest that ET-1 stimulation of PKC inhibits both NO- and depolarization-induced surface trafficking of β1 subunits in arterial myocytes.

Control experiments were performed to examine both the arterial biotinylation procedure and the β1 subunit antibodies. Reprobing blots from arterial biotinylation experiments indicated that cytochrome C—a mitochondrial protein—was detected only in the intracellular (ie, nonbiotinylated) fraction. The β1 antibody did not identify a protein in lysate from mock-transfected HEK293 cells but identified a ≈34 kDa protein in cells expressing recombinant β1, which was of a similar molecular mass to that of arterial β1 protein (Online Figure II). These
experiments provide additional support for previous evidence that the biotinylation procedure does not label intracellular proteins and that the $\beta_1$ subunit antibodies detect $\beta_1$ protein.

**ET-1 Inhibits Rab11A Through Phosphorylation at Serine 177**

NO stimulates Rab11A-positive recycling endosomes to deliver $\beta_1$ subunits to the plasma membrane. To examine the molecular mechanism by which ET-1–stimulated PKC inhibits $\beta_1$ trafficking, we measured Rab11A activation in cerebral arteries using Western blotting and a monoclonal antibody that recognizes Rab11-GTP but not Rab11-GDP. SNP increased active Rab11A $\approx$ 2.62-fold (Figure 4A and 4B). ET-1 reduced active Rab11A to $\approx$ 28.4% of that in SNP alone (Figure 4A and 4B). These data suggest that NO activates Rab11A, and ET-1 inhibits Rab11A in arterial myocytes.
The mechanism by which PKC reduces Rab11A activity may occur either directly or indirectly through other signaling intermediates. We tested the hypothesis that PKC directly phosphorylates Rab11A to reduce activity. Analysis of the Rab11A amino acid sequence (NetPhosK 1.0) revealed 5 potential PKC phosphorylation sites, of which Ser177 had the highest probability. We constructed a Rab11A S177A mutant to study physiological functions of serine 177 in β1 subunit trafficking. To investigate the hypothesis that ET-1 phosphorylates Rab11A S177, cerebral arteries were transfected with vectors encoding either wild-type (WT) Rab11A or Rab11A S177A. Vectors encoding WT Rab11A or Rab11A S177A did not alter levels of BKα, β1, or actin proteins. Arteries expressing either WT Rab11A or Rab11A S177A were exposed to ET-1 or nothing (control) after which Rab11A protein was pulled down and run on Western blots. ET-1 did not alter the amount of total Rab11A in arteries expressing either WT Rab11A or Rab11A S177A (Figure 4F; Online Figure III). Reprobing blots with an antiphosphoserine antibody indicated that in the absence of ET-1, basal Rab11A phosphorylation was similar in arteries expressing either Rab11A WT or Rab11A S177A. ET-1 increased Rab11A phosphorylation ≈227% in arteries expressing WT Rab11A. In contrast, ET-1 increased Rab11A phosphorylation only ≈138% in arteries expressing Rab11A S177A or ≈30% of that in ET-1–treated arteries expressing Rab11A WT (Figure 4F and 4G). These data indicate that ET-1 phosphorylates serine 177 in Rab11A, leading to Rab11A inhibition in arterial myocytes.

**ET-1 Inhibits β1 Subunit Surface Trafficking Through Rab11A Serine 177**

Experiments were performed to investigate the regulation of β1 trafficking by Rab11A S177 in arterial myocytes. SNP increased surface β1 protein similarly in mock and Rab11A S177A groups (Figure 5A and 5B). In the mock group, ET-1 reversed the SNP-induced increase in β1 subunit surface protein (Figure 5A and...
5B). Rab11A S177A expression blocked the ability of ET-1 to inhibit β1 subunit surface expression (Figure 5A and 5B). Confocal imaging of isolated cerebral artery myocytes illustrated that Rab11A S177A expression prevented the ET-1–induced reduction in N-FRET between BKα and β1-subunit bound secondary antibodies (Figure 5C and 5D). Taken together, these data suggest that ET-1–induced PKC acts through Ser177 on Rab11A, leading to a reduction in Rab11A activity and inhibition of β1 subunit surface trafficking in arterial myocytes.

ET-1 Inhibits BK Channels Through PKC-Mediated Phosphorylation of Rab11A

To investigate BK channel regulation by ET-1, patch-clamp electrophysiology was performed. Isolated arterial myocytes were exposed to nothing (control), SNP, ET-1+SNP, or SNP+ET-1+BIM. Inside-out patches were pulled from myocytes and agents washed out with bath solution to measure channel properties under the same experimental conditions and to remove any direct effects of pharmacological modulators on activity. BK channel activity was measured with 10 μmol/L free intracellular Ca2+ concentration at either −40 mV—a physiological voltage—or +80 mV to stimulate maximal activation. In excised patches from control myocytes, mean BK channel Po was ≈ 0.20 (Figure 6A and 6C). SNP treatment of myocytes increased BK channel mean Po to ≈ 0.47, or ≈ 2.35-fold (Figure 6A and 6C). ET-1 reduced SNP-induced BK channel activation to ≈ 40.0% of that stimulated by SNP alone (Figure 6A and 6C). BIM blocked the ET-1–induced reduction in BK channel Po (Figure 6A and 6C). SNP, ET-1+SNP, or BIM+ET-1+SNP did not alter BK channel maximal Po or the mean number of channels in patches (1.3±0.2, 1.5±0.3, 1.2±0.2, and 1.3±0.3 channels in control, SNP, ET-1+SNP, and BIM+ET-1+SNP, respectively; P<0.05; Figure 6C). When combined with the biochemical data here, these data indicate that ET-1 activates PKC, which inhibits surface trafficking of β1 subunits, leading to a decrease in BK channel Po.

Given that Rab11A S177A expression attenuated ET-1 inhibition of β1 surface expression, we measured BK channel regulation by this mutant construct in arterial myocytes. The mean Po of BK channels in mock-transfected cells treated with...
ET-1+SNP was similar to that nontransfected, fresh-isolated cells in the same experimental condition (Figure 6B and 6C). Rab11A S177A expression blocked the ability of ET-1 to inhibit BK channels (Figure 6B and 6C). Rab11A S177A did not alter BK channel maximal Po or the number of channels in patches (2.2±0.1 and 2.3±0.2 channels in mock and Rab11A S177A, respectively; \( P > 0.05 \); Figure 6C). These data suggest that ET-1 inhibits BK channels through Rab11A S177 in arterial myocytes.

ET-1 Inhibits Transient BK Currents Through Rab11A S177

In arterial myocytes, BK channels are activated by Ca\(^{2+}\) sparks.\(^4\) A single Ca\(^{2+}\) spark activates multiple nearby BK channels, leading to a transient BK current. To examine the functional relevance of Rab11A S177 on Ca\(^{2+}\) spark-induced BK channel activation, transient BK currents were recorded in isolated arterial myocytes at a steady holding potential of −40 mV. Mean area under the curve (AUC) was calculated for each transient BK current (Figure 7A; inset). SNP similarly increased mean transient BK current AUC in mock-transfected cells and in cells expressing Rab11A S177A, by \( \approx 2.46 \)- and \( \approx 2.42 \)-fold, respectively (Figure 7C). In mock-transfected cells, ET-1 attenuated the SNP-induced increase in mean transient BK current AUC (Figure 7A and 7C). In contrast, Rab11A S177A did not alter frequency modulation of transient BK currents by SNP or ET-1 (Figure 7D). These data suggest that ET-1 reduces transient BK current AUC through Rab11A S177. In contrast, Rab11A S177 does not modulate transient BK current frequency in arterial myocytes.

ET-1 Stimulates Vasoconstriction Through Rab11A S177 in Pressurized Arteries

The functional significance of Rab11A-mediated β1 subunit trafficking was studied in pressurized, myogenic (60
mm Hg) arteries. SNP similarly dilated mock-transfected and Rab11A S177A-transfected cerebral arteries (Figure 8A and 8C). ET-1 applied in the continued presence of SNP stimulated vasoconstriction in both mock-transfected and Rab11A S177A-transfected arteries. However, in the presence of SNP, ET-1–induced vasoconstriction in Rab11A S177A arteries was ≈42.5% less than in mock arteries under the same condition (Figure 8A and 8C). Rab11A S177A expression also reduced vasoconstriction to ET-1 (1–10 nmol/L) alone to between 41.2 and 57.5% of controls (Figure 8B and 8C). In contrast, Rab11A S177A did not alter myogenic tone at 60 mm Hg or depolarization-induced vasoconstriction (60 mmol/L K+; Figure 8D). These data indicate that Rab11A S177 contributes to ET-1–induced vasoconstriction.

**Discussion**

Here, we describe a unique mechanism by which NO and ET-1 modulate BK channel activity in myocytes to control arterial contractility. We show that ET-1 reduces Rab11A activity through PKC-mediated phosphorylation of S177, which reduces anterograde trafficking of β1, leading to a decrease in plasma membrane β1 protein. In contrast, NO activates Rab11A, leading to an increase in surface β1 protein. The ET-1–induced reduction in surface β1 protein inhibits BK channels and transient BK currents. This unique differential mechanism by which NO and ET-1 regulate Rab11A activity controls arterial contractility.

Previous studies have shown that NO, through PKG activation, and membrane depolarization, via Rho kinase 1 and 2 activation, stimulate anterograde trafficking of Rab11A-positive recycling endosomes, which deliver intracellular β1 subunits to the plasma membrane in arterial myocytes.11,12 In both of these mechanisms, these additional surface β1 subunits associate with plasma membrane-resident BKα subunits, leading to channel activation and vasodilation.11 Whether vasocostrictors regulated the surface abundance of β1 subunits to modulate BK channel activity was unclear. In resting arterial myocytes, only a small proportion of total β1 protein is located at the cell surface.11 To study potential modulation of surface β1 subunits by ET-1, we used SNP or depolarization to first stimulate surface trafficking of β1 protein. Our data indicate that ET-1 inhibits both NO- and depolarization-induced surface trafficking of β1 subunits. In arterial myocytes, ET-1 activates Gq/11, which in turn stimulates phospholipase C to produce diacylglycerol and inositol-1, 4, 5-triphosphate from phosphatidylinositol-4, 5-bisphosphate.19 Diacylglycerol activates PKC, which can phosphorylate many different protein targets.21 Here, BIM inhibited the ability of ET-1 to block both NO- and depolarization-induced β1 subunit surface trafficking, supporting involvement of PKC. In contrast, ET-1, SNP, or depolarization did not alter surface BKα protein, consistent with previous evidence that β1 and BKα surface traffic through distinct mechanisms.12,22 Confocal imaging confirmed that NO stimulated spatial association of surface BKα and β1 subunits and that ET-1 blocked this effect. BIM applied in the
presence of ET-1 restored the ability of NO to stimulate close spatial association of BKα and β1. These data indicate that ET-1 stimulates PKC, which inhibits β1 subunit surface trafficking. Through this mechanism, ET-1 reduces the amount of β1 protein that is associated with plasma membrane BKα channels.

Rab proteins facilitate protein trafficking between different cellular compartments.23,24 Previous studies indicated that Rab11A and β1 subunits are located in close spatial proximity in arterial myocytes.11 Rab11A knockdown inhibited NO-induced surface trafficking of β1 subunits in arterial myocytes.11 NO acts through PKG activation, although mechanisms involved were unclear. Several possibilities existed, including that PKG directly activates Rab11A, PKG indirectly activates Rab11A, or that PKG does not alter Rab11A activity to stimulate β1 surface delivery. Here, our data show for the first time that NO increases Rab11 activity in arterial myocytes, indicating that PKG-mediated stimulation of Rab11A is the mechanism involved. We also show that ET-1 reduces Rab11A activity, demonstrating that differential regulation of Rab11A activity controls surface β1 trafficking. To our knowledge, this is also the first time that PKC has been shown to inhibit Rab11A activity. PKC may have directly phosphorylated Rab11A to reduce activity or modulated an upstream protein(s) that inhibits Rab11A. To test these possibilities, we performed sequence analysis, which revealed 5 putative PKC phosphorylation sites, with S177 the highest probability location. To explore the functional significance of S177, leading to a reduction in Rab11A activity. Rab11A S177A expression also prevented ET-1 from reducing surface β1 protein. In contrast, Rab11A S177A did not alter the NO-induced increase in β1 surface protein, indicating that NO activates Rab11A through an S177-independent mechanism. These data also suggest that PKC and PKG act by independently modulating Rab11A activity; PKG does not act by inhibiting the PKC-mediated mechanism and PKC does not act through inhibition of the PKG-mediated mechanism. Supporting this finding is evidence that phorbol myristate acetate—a PKC activator—stimulated recombinant Rab11 phosphorylation in HeLa cells.25 Also, serotonin (5-HT) stimulated sequestration of recombinant 5-HT receptors through a PKC-dependent mechanism into a Rab11-positive compartment in HEK293 cells.26 In summary, our data indicate that ET-1 stimulates PKC-mediated phosphorylation of Rab11A serine 177, leading to a decrease in Rab11A activity and a reduction in surface β1 subunits in arterial myocytes.

Vasoregulatory stimuli can act through protein kinases to regulate BK channels in arterial myocytes.27-33 ET-1 inhibits whole-cell BK currents in rat renal artery myocytes, but signaling mechanisms involved were not investigated.23 Previous studies have shown that that PKA (protein kinase A) and PKG increase the activity of BK channels in inside-out membrane patches from arterial myocytes.34-36 Similarly, PKC inhibits BK channels in excised membrane patches.37,38 PKA, PKG, and PKC also modulate BK channel activity indirectly by modulating the frequency of Ca2+ sparks generated by sarcoplasmic reticulum ryanodine-sensitive Ca2+-release channels.4 Our study is the first to show that a vasoconstrictor inhibits BK channels by reducing anterograde trafficking of β1 subunits, thereby decreasing the surface abundance of these auxiliary proteins. Data here show that the NO-induced elevation in surface β1 stimulates an increase in BK channel P, that is retained on patch excision.11 Similarly, the PKC-induced decrease in BK channel P is maintained in excised patches. BIM
and Rab11A S177A abolished BK channel inhibition by ET-1, supporting other data here that PKC and Rab11A S177 phosphorylation block β1 trafficking.

Ca2+ sparks activate BK channels in arterial myocytes.4 To investigate the involvement of Rab11A S177 in BK channel modulation by Ca2+ sparks, transient BK currents were recorded in isolated myocytes. Virtually all Ca2+ sparks activate a transient BK current in rat cerebral artery myocytes, indicating that transient BK current frequency is determined by Ca2+ spark frequency.4 In contrast, the current generated by a transient BK is determined by the effective coupling of BK channels to Ca2+ sparks, a relationship determined by BK channel Ca2+ sensitivity.4 SNP increased both transient BK current frequency and AUC, supporting other data here that PKC and Rab11A S177A abolished BK channel inhibition by ET-1, supporting data published previously.39

ET-1 inhibited the NO-induced elevation in transient BK current frequency or AUC, supporting other results here that S177 is not a phosphorylation site for PKG and indicating that the construct used did not cause nonspecific modification of this signaling pathway. In control myocytes, ET-1 inhibited the NO-induced elevation in transient Ca2+ current frequency and mean AUC. Rab11A S177A blocked the ET-1–induced decrease in mean transient BK current AUC. S177A did not alter the ET-1–mediated decrease in transient BK current frequency, suggesting that Ca2+ spark frequency was not modified by the expression of this construct. Our data indicate that ET-1 inhibits BK channel P, and transient BK currents through phosphorylation at Rab11A S177. These results also indicate that Rab11A S177 is a novel target for blocking vasoconstrictor-induced BK channel inhibition.

ET-1 constricts arteries in several tissues through mechanisms that include PKC-mediated myosin light chain kinase phosphorylation.40–43 Here, data indicate that Rab11A S177A expression attenuated ET-1–induced vasoconstriction over a range of concentrations. Inhibition of ET-1–induced vasoconstriction occurred either in the presence or absence of SNP supporting other results here that ET-1–induced vasoconstriction occurs because of Rab11A S177 phosphorylation. This finding is consistent with both data published previously and results here suggesting that intravascular pressure-induced membrane depolarization stimulates β1 surface trafficking and that ET-1 inhibits this mechanism, producing vasoconstriction.43 In contrast, Rab11A S177A did not alter depolarization (60 mmol/L K+)–induced vasoconstriction or myogenic tone, indicating that Rab11A S177A is not a nonspecific inhibitor of vasoconstriction. Collectively, these results show that ET-1 blocks β1 subunit surface trafficking by both membrane depolarization and NO, which inhibits BK channels, producing vasoconstriction.

In summary, we show that ET-1 activates PKC, which phosphorylates Rab11A S177, leading to a decrease in Rab11A activity and a reduction in β1 subunit surface trafficking. The ET-1–induced reduction in β1 subunit surface delivery inhibits BK channels and transient BK currents, leading to vasoconstriction. In contrast, NO activates Rab11A and increases surface β1 subunits through a Rab11A S177–independent mechanism. In summary, we show that differential regulation of Rab11A by ET-1 and NO controls BK channels and contractility in arterial myocytes.

Acknowledgments
We thank Dr Alejandro Dopico for his comments on this article.

Sources of Funding
National Heart, Lung, and Blood Institute grants to J.H. Jaggar (HL67061, HL110347, and HL133256), and American Heart Association Scientist Development Grant to M.D. Leo (15SDG22680019).

Disclosures
None.

References


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Xue Zhai, M. Dennis Leo and Jonathan H. Jaggar

Circ Res. 2017;121:650-661; originally published online July 10, 2017;
doi: 10.1161/CIRCRESAHA.117.311102
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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Data Supplement (unedited) at:
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**Supplemental material**

**Expanded Methods**

**Tissue preparation**

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Male Sprague-Dawley rats (7-8 weeks) were euthanized with intraperitoneal injection of sodium pentobarbital (150mg/kg). The brain was removed, cleaned and placed in a HEPES-buffered physiological saline solution containing (in mmol/L): 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4). Cerebral arteries (middle cerebral, cerebellar, posterior cerebral) were dissected from the brain and myocytes were isolated using enzymes, as previously described.¹

**Arterial surface biotinylation**

Intact arteries were biotinylated as previously described.² ³ Briefly, arteries were incubated in HEPES-buffered physiological saline solution containing 1 mg/ml EZ-Link Sulfo-NHS-LC-LC-Biotin and 1 mg/ml EZ-Link Maleimide-PEG2-Biotin reagents (Pierce) for 1h. Free biotin was quenched by washing the arteries in PBS with 100 mmol/L glycine. Arteries were then washed in PBS to remove any residual glycine solution. Biotinylated arteries were homogenized in lysis buffer containing 1% Triton X-100. Total protein was determined to normalize for avidin pull-down of biotinylated surface proteins. After pulldown, the sample was centrifuged at 13,000g, biotinylated proteins were bound to the avidin beads and the supernatant contained the nonbiotinylated (intracellular) protein fraction. The beads were washed three times with PBS and Laemmli buffer was added to both the beads and intracellular protein lysate. Samples were then boiled for three minutes. Biotinylated surface proteins were eluted from the avidin beads. Both protein fractions were analyzed using Western blotting.

**Western blotting**

Protein lysates were boiled with Laemmli buffer (Bio-Rad), separated on SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk then probed with mouse monoclonal anti-BKα (1:500 dilution, Neuromab, UC Davis), rabbit polyclonal anti-BKβ1 (1:500, Abcam), rabbit monoclonal anti-rab11A (1:500, Cell Signaling), rabbit polyclonal anti-phosphoserine (1:500, Millipore) or mouse monoclonal anti-active rab11 (1:500, NewEast Biosciences) antibodies overnight at 4°C. After incubating with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescent detection kit, the blots were imaged with a Kodak In Vivo F Pro Imaging System. Band intensity was quantified using Quantity One software (Bio-Rad).

**Immunofluorescence and immuno-FRET microscopy**

Isolated myocytes were exposed to agents and then plated on poly-L-lysine-coated coverslips. Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with 5% bovine serum albumin (BSA) and then incubated with mouse monoclonal anti-BKα (Neuromab, UC Davis) and rabbit polyclonal anti-BKβ1 (Abcam) antibodies overnight at 4°C. After washing with PBS, cells were incubated for one hour with Alexa 546- or Alexa 488-conjugated secondary antibodies (1:100 dilution; Life Technologies). After washing, coverslips were mounted onto slides with 1:1 glycerol: PBS media. Fluorescence images were acquired using a laser-scanning confocal microscope (LSM5 Pascal; Carl Zeiss). Alexa 488 and Alexa 546 were excited at 488 and 543 nm and emission was collected at 505-530 and ≥560 nm, respectively. For N-FRET analysis, images were background-subtracted and N-FRET calculated on a pixel-by-pixel basis using the Xia method⁴ and Zeiss LSM FRET Macro tool (Version 2.5).

**Transfection of Intact Cerebral Arteries**

A Rab11A sequence with a serine 177-to-alanine mutation (Rab11A S177A) was generated and subcloned into pcDNA3.1(+) (GenScript USA Inc., Piscataway, NJ). Empty pcDNA3.1 vectors, pcDNA3.1
encoding Rab11A, or pcDNA3.1 encoding Rab11A S177A were transfected into arteries using electroporation (CUY21Vivo-SQ electroporator; Bex), as previously described. Briefly, cerebral arteries were placed in an electroporation chamber containing Ca\(^{2+}\)-free PBS with either Rab11A WT, Rab11A S177A or empty vectors. Tandem-pulse electroporation was applied to arteries in the chamber. Arteries were then placed in serum-free DMEM-F12 media supplemented with 1% penicillin-streptomycin for 3 days before use.

**HEK293 cell culture and transfection**

HEK293 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under standard culture conditions (21% O\(_2\)-5% CO\(_2\); 37°C). Cells were transiently transfected with pcDNA3.1(+) encoding full-length β1 (2 μg) or empty vector (2 μg) using Effectene (Qiagen). Cells were lysed 48 hrs post-transfection. Protein concentration was calculated as previously described. Cell lysates were run on 7.5% SDS polyacrylamide gels and blots were probed for β1 and actin.

**Immunoprecipitation**

For each experiment, lysate was harvested from arteries from six rats using ice-cold radioimmunoprecipitation buffer. Proteins were pulled down from arterial lysate using the Catch and Release version 2.0 immunoprecipitation kit (Millipore) per the manufacturer’s instructions. Samples were incubated with Rab11A primary antibody, 0.5ml capture resin and 10μl antibody affinity ligand in columns overnight. The columns were centrifuged at 5000 rpm and the flow-through discarded. The capture resin was washed twice. Bound proteins were released using denaturing buffer and then boiled for 3 min. Protein samples were analyzed using Western blotting with an anti-rab11A antibody (1:500, Cell Signaling). The blots were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed with an anti-phosphoserine antibody (1:500, Millipore).

**Patch Clamp Electrophysiology**

Single BK channels or transient BK currents were recorded in isolated myocytes using the inside-out or perforated patch-clamp configurations, respectively. Currents were recorded using an Axopatch 200B amplifier and Clampex 10.3. For inside-out patch-clamp, myocytes were allowed to settle in a recording chamber before exposure to combinations of ET-1 (1 hr), BIM (1 hr) and SNP (10 min) prior to patch excision. Inside-out patches were pulled and agents then washed out so that channel activity was measured in the absence of these agents using holding potentials of -40 or +80 mV. For inside-out recordings, the pipette and bath solution both contained (in mmol/L): 130 KCl, 10 HEPES, 5 EGTA, 1.6 HEDTA, 1 MgCl\(_2\), and 10 μmol/L free Ca\(^{2+}\) (pH 7.2). Transient BK currents were measured using the amphotericin B perforated-patch configuration at a steady voltage of -40 mV. For perforated patch-clamp, the bath solution contained (in mmol/L): 134 NaCl, 6 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, and 10 glucose (pH 7.4) and the pipette solution contained (in mmol/L): 110 potassium aspartate, 30 KCl, 1 MgCl\(_2\), 10 HEPES, and 0.05 EGTA (pH 7.2). Currents were filtered at 1 kHz, digitized at 5 kHz and analyzed using Clampfit 10.3 (MDS Analytical Technologies).

**Pressurized artery myography**

Artery segments (1-2 mm length) were cannulated in a perfusion chamber (Living Systems Instrumentation, St. Albans, VT) and continuously perfused with 37°C physiological saline solution (PSS) which contained: 112 NaCl, 4.8 KCl, 24 NaHCO\(_3\), 1.8 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\) and 10 glucose, gassed with mixture of 21% O\(_2\), 5% CO\(_2\) and 74% N\(_2\). The endothelium was denuded by introducing an air bubble into the lumen for ~1 minute followed by wash with PSS. Arterial diameter was measured at an intravascular pressure of 60 mmHg, which was monitored using a pressure transducer. Arterial diameter was measured using a CCD camera attached to a Nikon TS100-F microscope and the edge-detection function of IonWizard (Ionoptix, Milton, MA). Myogenic tone (%) was calculated as: 100
\times (1 - \frac{D_{\text{active}}}{D_{\text{passive}}}), \text{ where } D_{\text{active}} \text{ is active arterial diameter and } D_{\text{passive}} \text{ is the diameter determined in Ca}^{2+}\text{-free PSS supplemented with 5 mmol/L EGTA.}

**Statistical analysis**
Statistical analyses were performed using GraphPad Prism v4.0 and Origin v6.0. Values are presented as mean ± SEM. Student’s t-test was used to compare paired and unpaired data from two populations. ANOVA with Student-Newman-Keuls post hoc test was used for multiple group comparisons. P < 0.05 was considered significant.
Supplemental Figure Legends

Online Figure I. SNP, ET-1 and BIM do not alter total protein of β1 and BKα after surface biotinylation. (A) Mean data for intracellular β1 and BKα protein (n=6 for each). *p<0.05 vs. control, # p<0.05 vs. SNP, † p<0.05 vs. ET-1+SNP. (B) Mean data for total β1 protein compared to control (n=6 for each). (C) Mean data for total BKα protein compared to control (n=6 for each).

Online Figure II. Representative Western blot illustrating that the β1 subunit antibody detects β1 protein in HEK 293 cells transfected with a pcDNA3.1-BKβ1 expression vector, but not in cells transfected with empty vector.

Online Figure III. ET-1 does not change total Rab11A protein in arteries transfected with either Rab11A WT or Rab11A S177A. Mean data of total Rab11A protein expression in ET-1-treated cerebral arteries transfected with Rab11A WT or Rab11A S177A compared to non-ET-1-treated Rab11A WT groups (n=6).
Supplemental References