

# Type 1 IP<sub>3</sub> receptors activate BK<sub>Ca</sub> channels via local molecular coupling in arterial smooth muscle cells

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Plasma membrane large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channels and sarcoplasmic reticulum inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptors (IP<sub>3</sub>Rs) are expressed in a wide variety of cell types, including arterial smooth muscle cells. Here, we studied BK<sub>Ca</sub> channel regulation by IP<sub>3</sub> and IP<sub>3</sub>Rs in rat and mouse cerebral artery smooth muscle cells. IP<sub>3</sub> activated BK<sub>Ca</sub> channels both in intact cells and in excised inside-out membrane patches. IP<sub>3</sub> caused concentration-dependent BK<sub>Ca</sub> channel activation with an apparent dissociation constant (*K*<sub>d</sub>) of ~4 μM at physiological voltage (−40 mV) and intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>; 10 μM). IP<sub>3</sub> also caused a leftward-shift in BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity and reduced the *K*<sub>d</sub> for free [Ca<sup>2+</sup>]<sub>i</sub> from ~20 to 12 μM, but did not alter the slope or maximal P<sub>o</sub>. BAPTA, a fast Ca<sup>2+</sup> buffer, or an elevation in extracellular Ca<sup>2+</sup> concentration did not alter IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. Heparin, an IP<sub>3</sub>R inhibitor, and a monoclonal type 1 IP<sub>3</sub>R (IP<sub>3</sub>R1) antibody blocked IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. Adenophostin A, an IP<sub>3</sub>R agonist, also activated BK<sub>Ca</sub> channels. IP<sub>3</sub> activated BK<sub>Ca</sub> channels in inside-out patches from wild-type (IP<sub>3</sub>R1<sup>+/+</sup>) mouse arterial smooth muscle cells, but had no effect on BK<sub>Ca</sub> channels of IP<sub>3</sub>R1-deficient (IP<sub>3</sub>R1<sup>-/-</sup>) mice. Immunofluorescence resonance energy transfer microscopy indicated that IP<sub>3</sub>R1 is located in close spatial proximity to BK<sub>Ca</sub> α subunits. The IP<sub>3</sub>R1 monoclonal antibody coimmunoprecipitated IP<sub>3</sub>R1 and BK<sub>Ca</sub> channel α and β1 subunits from cerebral arteries. In summary, data indicate that IP<sub>3</sub>R1 activation elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity through local molecular coupling in arterial smooth muscle cells.

## INTRODUCTION

Large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) channels are expressed in a wide variety of cell types and consist of pore-forming α subunits and auxiliary β1–4 subunits that modify channel function (Lu et al., 2006). In arterial smooth muscle cells, β1 subunits are the principal molecular and functional BK<sub>Ca</sub> channel auxiliary subunit isoform (Brenner et al., 2000; Plüger et al., 2000). Arterial smooth muscle cell BK<sub>Ca</sub> channel activation causes membrane hyperpolarization, leading to a reduction in voltage-dependent Ca<sup>2+</sup> channel activity, a decrease in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and vasodilation (Davis and Hill, 1999; Jaggar et al., 2000). In contrast, BK<sub>Ca</sub> channel inhibition causes membrane depolarization, which activates voltage-dependent Ca<sup>2+</sup> channels, leading to an [Ca<sup>2+</sup>]<sub>i</sub> elevation and vasoconstriction.

Global cytosolic [Ca<sup>2+</sup>]<sub>i</sub> is typically 100–300 nM, whereas BK<sub>Ca</sub> channels are sensitive to micromolar [Ca<sup>2+</sup>]<sub>i</sub> (Jaggar et al., 2000; Pérez et al., 2001). In arterial smooth muscle cells, BK<sub>Ca</sub> channels are activated by localized micromolar

[Ca<sup>2+</sup>]<sub>i</sub> transients termed Ca<sup>2+</sup> sparks (Nelson et al., 1995; Jaggar et al., 2000). Ca<sup>2+</sup> sparks are generated by the concerted opening of several SR ryanodine-sensitive Ca<sup>2+</sup> release (RyR) channels (Nelson et al., 1995; Jaggar et al., 2000). Ca<sup>2+</sup> spark-induced BK<sub>Ca</sub> currents induce membrane hyperpolarization and vasodilation.

SR inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-gated Ca<sup>2+</sup> release channels are also expressed in many different cell types, including arterial smooth muscle (Thrower et al., 2001; Morel et al., 2003; Foskett et al., 2007; Zhao et al., 2008; Zhou et al., 2008). In native and cultured vascular smooth muscle cells, IP<sub>3</sub> receptor (IP<sub>3</sub>R) activation stimulates propagating intracellular Ca<sup>2+</sup> waves and elevates global [Ca<sup>2+</sup>]<sub>i</sub> (Lee et al., 2002; Lamont and Wier, 2004; Wilkerson et al., 2006; Zhao et al., 2008). Three different IP<sub>3</sub>R isoforms (1–3) have been identified, each of which is encoded by a different gene (Ross et al., 1992; Blondel et al., 1993). Type 1 IP<sub>3</sub>Rs are the principal molecular and functional IP<sub>3</sub>R isoform mediating agonist and IP<sub>3</sub>-induced intracellular Ca<sup>2+</sup> signals in aortic and cerebral artery smooth muscle cells (Zhao et al., 2008; Zhou et al., 2008). IP<sub>3</sub>R2 also contributes to acetylcholine-induced

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Abbreviations used in this paper: BK<sub>Ca</sub>, large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; coIP, coimmunoprecipitation; immuno-FRET, immunofluorescence resonance energy transfer; IP<sub>3</sub>, 1,4,5-trisphosphate; [IP<sub>3</sub>]<sub>i</sub>, intracellular IP<sub>3</sub> concentration; IP<sub>3</sub>R, IP<sub>3</sub> receptor.

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Ca<sup>2+</sup> oscillations in cultured portal vein smooth muscle cells (Morel et al., 2003). IP<sub>3</sub>R1 activation stimulates propagating intracellular Ca<sup>2+</sup> waves and causes an increase in global [Ca<sup>2+</sup>]<sub>i</sub> (Zhao et al., 2008). IP<sub>3</sub>R1 activation also stimulates a nonselective cation current (I<sub>CaI</sub>) via an SR Ca<sup>2+</sup> release-independent mechanism in cerebral artery smooth muscle cells (Xi et al., 2008; Zhao et al., 2008). This cation current occurs due to physical coupling of IP<sub>3</sub>R1 to TRPC3 channels, is primarily due to Na<sup>+</sup> influx, and leads to membrane depolarization, voltage-dependent Ca<sup>2+</sup> channel activation, and a global [Ca<sup>2+</sup>]<sub>i</sub> elevation (Xi et al., 2008; Adebisi et al., 2010). IP<sub>3</sub>R1-mediated SR Ca<sup>2+</sup> release and TRPC3 channel activation both elevate [Ca<sup>2+</sup>]<sub>i</sub>, leading to vasoconstriction (Xi et al., 2008; Zhao et al., 2008).

IP<sub>3</sub>R-mediated SR Ca<sup>2+</sup> release activates BK<sub>Ca</sub> channels in basilar artery smooth muscle cells (Kim et al., 1998). Given that IP<sub>3</sub>Rs directly activate nearby TRPC3 channels in arterial smooth muscle cells (Xi et al., 2008; Zhao et al., 2008; Adebisi et al., 2010), we studied the mechanisms by which IP<sub>3</sub> and IP<sub>3</sub>Rs modulate BK<sub>Ca</sub> channels. We tested the hypothesis that IP<sub>3</sub> activates BK<sub>Ca</sub> channels via an SR Ca<sup>2+</sup> release-independent mechanism. Our data indicate that IP<sub>3</sub> activates BK<sub>Ca</sub> channels both in intact cells and excised membrane patches where SR Ca<sup>2+</sup> release cannot occur. IP<sub>3</sub> elevated BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity, required IP<sub>3</sub>R1 activation, and was absent in IP<sub>3</sub>R1-deficient (IP<sub>3</sub>R1<sup>-/-</sup>) cells. Our data also indicate that IP<sub>3</sub>R1 is located in close spatial proximity to BK<sub>Ca</sub> channels and coimmunoprecipitates with BK<sub>Ca</sub> channel α and β1 subunits. This study identifies a novel signaling mechanism whereby IP<sub>3</sub>R1 activation stimulates nearby BK<sub>Ca</sub> channels. Since IP<sub>3</sub>Rs and BK<sub>Ca</sub> channels are broadly expressed, this coupling mechanism may exist in a wide variety of different cell types.

## MATERIALS AND METHODS

### Tissue preparation and cell isolation

All animal protocols were reviewed and approved by the University of Tennessee Animal Care and Use Committee. Male Sprague-Dawley rats (6–8 wk) and wild-type and IP<sub>3</sub>R1<sup>-/-</sup> mice (3 wk) were euthanized by intraperitoneal injection of 150 mg/kg sodium pentobarbital. Generation of the IP<sub>3</sub>R1 knockout mice will be described in a future publication. In brief, exon 5 of IP<sub>3</sub>R1 was flanked by two lox P sites, and IP<sub>3</sub>R1-floxed mice were generated by homologous recombination. IP<sub>3</sub>R1-floxed mice were subsequently crossed with protamine Cre mice (O’Gorman et al., 1997) to generate conventional IP<sub>3</sub>R1 knockout mice in which exon 5 is deleted globally and no IP<sub>3</sub>R1 protein can be detected (see Fig. 5 A). The phenotype of the global knockout IP<sub>3</sub>R1 mice generated is the same as that previously published for global loss of function of IP<sub>3</sub>R1 mice (Matsumoto et al., 1996). Brains were removed after the rats and mice were euthanized. Aorta was also collected from mice. Tissues were placed into ice-cold (4°C), oxygenated (21% O<sub>2</sub>, 5% CO<sub>2</sub>), physiological saline solution containing (in mM): 119 NaCl, 4.7 KCl, 24 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>,

0.023 EDTA, and 11 glucose. Posterior cerebral and cerebellar arteries (~50–200 μm in diameter) were dissected from the brains and cleaned of connective tissue. Arterial smooth muscle cells were enzymatically dissociated from cerebral arteries as described previously, maintained at 4°C, and used within 8 h (Cheravov and Jaggard, 2006). Mouse aorta was used only for Western blotting experiments.

### Patch clamp electrophysiology

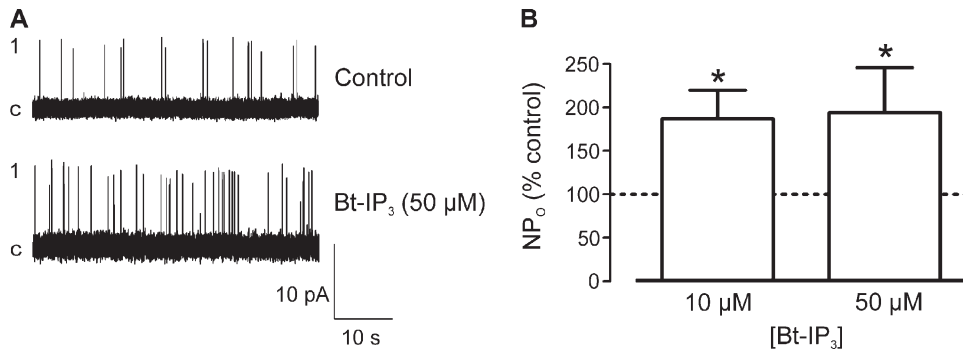
Single BK<sub>Ca</sub> channel currents were recorded in isolated cerebral artery smooth muscle cells using either the cell-attached or inside-out patch clamp configuration (Axopatch 200B and Clampex 8.2; MDS Analytical Technologies). For cell-attached patch, the pipette and bath solution contained (in mM): 130 KCl, 10 HEPES, 1 MgCl<sub>2</sub>, 5 EGTA, 1.6 HEDTA, and 10 μM free Ca<sup>2+</sup>, pH 7.2 with KOH. For inside-out patches, the same pipette and bath solutions were used, except for experiments measuring BK<sub>Ca</sub> channel Ca<sup>2+</sup> sensitivity, where free Ca<sup>2+</sup> concentration was adjusted to between 1 and 300 μM by the addition of CaCl<sub>2</sub> and free Mg<sup>2+</sup> maintained at 1 mM by adjustment of MgCl<sub>2</sub>. Where indicated, equimolar EGTA was substituted for BAPTA, a fast Ca<sup>2+</sup> chelator, in both the pipette and bath solutions. Free Ca<sup>2+</sup> concentration in solutions was measured using a Ca<sup>2+</sup>-sensitive (no. 476041; Corning) and reference (no. 476370; Corning) electrode. Cell-attached and inside-out patch experiments were performed at membrane voltages of +60 and -40 mV, respectively. BK<sub>Ca</sub> currents were filtered at 1 kHz and digitized at 5 kHz. Analysis was performed offline using Clampfit 9.2 (MDS Analytical Technologies).

### Western blotting

Mouse aorta or rat cerebral artery proteins were separated using 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were cut so that the same lysate could be probed for several different molecular weight proteins. Membranes were incubated with mouse monoclonal anti-IP<sub>3</sub>R1 (NeuroMab), mouse monoclonal anti-BK<sub>Ca</sub> α (NeuroMab), or rabbit polyclonal anti-BK<sub>Ca</sub> β1 (Abcam) primary antibodies overnight at 4°C in Tris-buffered solution (TBS) containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk. After washing with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, followed by washing with TBS-T. Membranes were then developed using enhanced chemiluminescence (GE Healthcare), and digital images were obtained using a Kodak FX Pro imaging system.

### Immunofluorescence resonance energy transfer (immuno-FRET)

Isolated cells were allowed to adhere to poly-L-lysine-coated coverslips. Cells were then fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and treated with the following primary antibodies: mouse monoclonal anti-IP<sub>3</sub>R1 (clone L24/18; NeuroMab) and either rabbit polyclonal anti-BK<sub>Ca</sub>α (Abcam) or rabbit polyclonal anti-TRPM4 (Thermo Fisher Scientific), each at a dilution of 1:100. After washing, cells were incubated with the following secondary antibodies: Cy3-conjugated donkey anti-mouse for IP<sub>3</sub>R1 (Jackson ImmunoResearch Laboratories, Inc.) and Cy2-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) for BK<sub>Ca</sub>α or TRPM4. After washing, coverslips were dried and mounted onto glass slides. Fluorescence images were acquired using a laser-scanning confocal microscope (LSM Pascal; Carl Zeiss, Inc.). Cy2 and Cy3 were excited at 488 and 543 nm, and emission was collected at 505–530 and ≥560 nm, respectively. Negative controls were prepared by omitting primary antibodies. Images were background-subtracted, and N-FRET was calculated on a pixel-by-pixel basis for the entire image and in regions of interest (within the boundaries of the cell) using the



**Figure 1.** IP<sub>3</sub> activates BK<sub>Ca</sub> channels in cerebral artery smooth muscle cells. (A) Original recordings obtained from the same cell illustrating activation of BK<sub>Ca</sub> channel by Bt-IP<sub>3</sub> at +60 mV. (B) Mean data (*n*: control, 5; 10 μM Bt-IP<sub>3</sub>, 5; 50 μM Bt-IP<sub>3</sub>, 5). \*, *P* < 0.05.

Xia method (Xia and Liu, 2001) and LSM FRET Macro tool (v2.5; Carl Zeiss, Inc.).

#### Coimmunoprecipitation (coIP)

Arterial lysate was harvested from cerebral arteries pooled from ~15 rats using ice-cold lysis buffer (Thermo Fisher Scientific), giving ~1.5 mg of total protein. coIP was done using the Thermo Fisher Scientific Co-Immunoprecipitation kit. The IP<sub>3</sub>R1 antibody was first immobilized for 2 h using coupling resin (AminoLink Plus; Thermo Fisher Scientific). The resin was then washed and incubated with arterial lysate overnight. After incubation, the resin was again washed and protein was eluted using elution buffer. Non-denaturing sample buffer (Thermo Fisher Scientific) was added to the eluate and boiled. Negative controls received the same treatment, except that the coupling resin was replaced with control agarose resin that is not amine-reactive. Samples were analyzed using Western blotting with mouse monoclonal anti-IP<sub>3</sub>R1, mouse monoclonal anti-BK<sub>Ca</sub> α, or rabbit polyclonal anti-BK<sub>Ca</sub> β1 primary antibodies, and horseradish peroxidase-conjugated secondary antibodies.

#### Statistical analysis

BK<sub>Ca</sub> channel activity (NP<sub>o</sub>) was calculated from continuous gap-free data using the following equation:  $NP_o = \sum (t_1 + t_2 \dots t_i)$ , where  $t_i$  is the relative open time (time open/total time) for each channel level. Open probability ( $P_o$ ) was calculated by dividing NP<sub>o</sub> by channel number (*N*). The total number of channels in inside-out patches was determined by introducing 1 mM of free Ca<sup>2+</sup> into the

bath solution at the end of each experiment. BK<sub>Ca</sub> channel IP<sub>3</sub> sensitivity data and relationships between BK<sub>Ca</sub> channel open probability ( $P_o$ ) and free Ca<sup>2+</sup> concentration were fit with a Boltzmann function:  $Y = P_{o,min} + [(P_{o,max} - P_{o,min}) / (1 + \exp[(K_{1/2} - X) / \text{slope}])]$ . Values are expressed as mean ± SE. Student's *t* test and repeated measures analysis of variance with Student-Newman-Keuls post-hoc test were used for comparing paired or unpaired data and multiple datasets, as appropriate. *P* < 0.05 was considered significant.

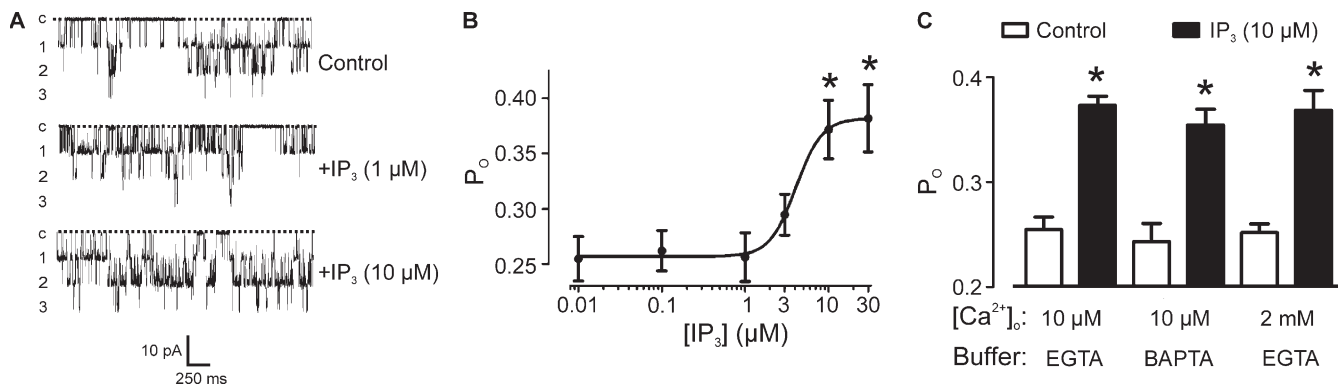
#### Online supplemental material

Data in Fig. S1 demonstrate that antigenic peptides abolish fluorescent labeling by BK<sub>Ca</sub> channel α and TRPM4 channel antibodies. Also shown are differential interference contrast images of the same cells imaged for fluorescence. Fig. S1 is available at <http://www.jgp.org/cgi/content/full/jgp.201010453/DC1>.

## RESULTS

### IP<sub>3</sub> activates BK<sub>Ca</sub> channels in cerebral artery smooth muscle cells

BK<sub>Ca</sub> channel regulation by IP<sub>3</sub> was first measured in intact arterial smooth muscle cells using the cell-attached configuration of the patch clamp technique. Bt-IP<sub>3</sub>, a membrane-permeant IP<sub>3</sub> analogue, at concentrations of 10 and 50 μM, increased mean BK<sub>Ca</sub> channel activity



**Figure 2.** IP<sub>3</sub> activates BK<sub>Ca</sub> channels in excised inside-out membrane patches. (A) Original recordings from the same inside-out patch illustrating concentration-dependent BK<sub>Ca</sub> channel activation by IP<sub>3</sub> at -40 mV. (B) Mean data fit with a Boltzmann function. IP<sub>3</sub> increased BK<sub>Ca</sub> channel  $P_o$  with an apparent  $K_{1/2}$  of  $4.1 \pm 1.3$  μM, a slope of  $2.8 \pm 1.9$ , and a maximal  $P_o$  of  $0.38 \pm 0.02$ . Experimental numbers are ([IP<sub>3</sub>]): 0.01 μM, 4; 0.1 μM, 4; 1 μM, 4; 3 μM, 5; 10 μM, 5; 30 μM, 5. (C) Mean data illustrating that buffering Ca<sup>2+</sup> with BAPTA or elevating pipette (extracellular) Ca<sup>2+</sup> from 10 μM to 2 mM does not alter IP<sub>3</sub> (10 μM)-induced BK<sub>Ca</sub> channel activation (*n*: 10 μM [Ca<sup>2+</sup>]<sub>o</sub>/10 μM [Ca<sup>2+</sup>]<sub>i</sub> (EGTA/EGTA); control, 4; IP<sub>3</sub>, 4; 10 μM [Ca<sup>2+</sup>]<sub>o</sub>/10 μM [Ca<sup>2+</sup>]<sub>i</sub> (BAPTA/BAPTA); control, 4; IP<sub>3</sub>, 4; 2 mM [Ca<sup>2+</sup>]<sub>o</sub>/10 μM [Ca<sup>2+</sup>]<sub>i</sub> (none/EGTA) control, 3; IP<sub>3</sub>, 3). \*, *P* < 0.05.

(NP<sub>o</sub>) ~1.8- and 1.9-fold, respectively, at +60 mV (Fig. 1, A and B).

Next, we investigated whether IP<sub>3</sub> has effects on BK<sub>Ca</sub> channels that are independent of plasma membrane Ca<sup>2+</sup> influx or intracellular Ca<sup>2+</sup> release. BK<sub>Ca</sub> channel regulation was studied in excised inside-out membrane patches at a physiological steady membrane voltage of -40 mV, which is similar to that of cerebral arteries pressurized to 60 mmHg (Knot and Nelson, 1998). In inside-out patches exposed to symmetrical 10 μM of free [Ca<sup>2+</sup>]<sub>i</sub>, 10 μM IP<sub>3</sub> increased mean BK<sub>Ca</sub> channel open probability (P<sub>o</sub>) from ~0.24 to 0.37, or 1.54-fold (Fig. 2, A and B). In contrast, IP<sub>3</sub> did not alter single BK<sub>Ca</sub> channel amplitude (pA: control, 8.5 ± 0.47; IP<sub>3</sub>, 8.0 ± 0.36; n = 5; P > 0.05; Fig. 2 A). IP<sub>3</sub> also caused concentration-dependent BK<sub>Ca</sub> channel activation (Fig. 2 B). Fitting an IP<sub>3</sub> concentration–response curve with a Boltzmann function indicated that IP<sub>3</sub> increased BK<sub>Ca</sub> channel P<sub>o</sub> with an apparent K<sub>d</sub> of ~4.1 μM, a slope of ~2.8, and a maximal P<sub>o</sub> of ~0.38 (Fig. 2 B). IP<sub>3</sub> activated BK<sub>Ca</sub> channels in excised patches for as long as the seal could be maintained (>30 min), indicating that SR Ca<sup>2+</sup> release was unlikely to be responsible for channel activation. In support of this concept, equimolar substitution of bath and pipette EGTA for BAPTA, a fast Ca<sup>2+</sup> chelator, did not alter IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation (Fig. 2 C). Elevating pipette free Ca<sup>2+</sup> from 10 μM to 2 mM also did not alter IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation, indicating that plasma membrane Ca<sup>2+</sup> influx was not responsible for channel activation (Fig. 2 C). Collectively, these data indicate that IP<sub>3</sub> activates BK<sub>Ca</sub> channels via an SR Ca<sup>2+</sup> release-independent mechanism.

#### IP<sub>3</sub> elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity

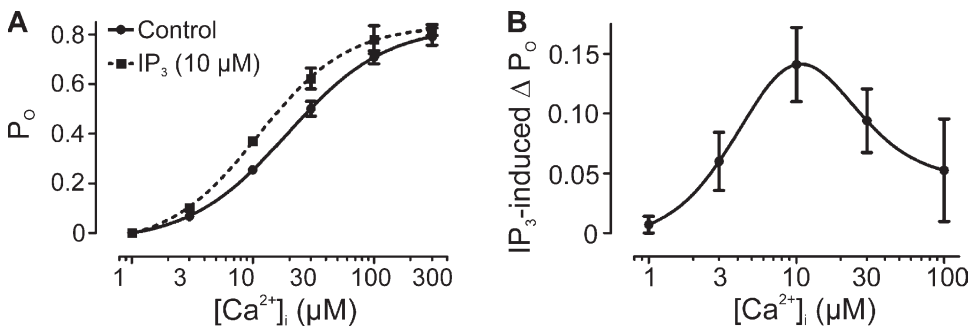
IP<sub>3</sub> regulation of BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity was examined at a steady membrane voltage of -40 mV. In control, mean apparent K<sub>d</sub> for Ca<sup>2+</sup> was ~20 μM, with a slope of ~1.2, and a maximum P<sub>o</sub> of ~0.82 (Fig. 3 A). In the same membrane patches, 10 μM IP<sub>3</sub> decreased the mean K<sub>d</sub> for Ca<sup>2+</sup> to ~12 μM, but did not alter the slope or the maximum P<sub>o</sub> (Fig. 3 A). Relative activation

by IP<sub>3</sub> increased considerably between 1 and 10 μM Ca<sup>2+</sup> (Fig. 3 B). For example, with 1 μM of free Ca<sup>2+</sup>, IP<sub>3</sub> increased mean BK<sub>Ca</sub> channel P<sub>o</sub> 1.14-fold, whereas with 10 μM of free Ca<sup>2+</sup>, IP<sub>3</sub> increased mean P<sub>o</sub> 1.48-fold (Fig. 3 B). At free [Ca<sup>2+</sup>] >10 μM, relative activation by IP<sub>3</sub> became smaller because channel activity was approaching maximal. These data suggest that IP<sub>3</sub> elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity in arterial smooth muscle cells.

#### IP<sub>3</sub>R1 activation mediates IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation

To identify mechanisms by which IP<sub>3</sub> activates BK<sub>Ca</sub> channels, we tested the hypothesis that IP<sub>3</sub>Rs mediate IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. In inside-out patches, 1 mg/ml heparin, an IP<sub>3</sub>R blocker, reversed BK<sub>Ca</sub> channel activation by IP<sub>3</sub> (Fig. 4 A). In contrast, 1 mg/ml heparin alone did not alter mean BK<sub>Ca</sub> channel P<sub>o</sub> (98.9 ± 12.5% of control; n = 5; P > 0.05). These data indicate that IP<sub>3</sub>-induced IP<sub>3</sub>R activation stimulates BK<sub>Ca</sub> channels.

Type 1 IP<sub>3</sub>Rs are the principal molecular isoform expressed in cerebral artery and aortic smooth muscle cells (Zhao et al., 2008; Zhou et al., 2008; Adebisi et al., 2010). Therefore, we studied whether IP<sub>3</sub>R1 mediates IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. In inside-out patches, a monoclonal IP<sub>3</sub>R1 antibody (1:100) reversed IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation (Fig. 4, B and C). In contrast, the IP<sub>3</sub>R1 antibody (1:100) did not alter mean BK<sub>Ca</sub> channel activity when applied alone (94.2 ± 4.9% of control; n = 4; P > 0.05). The addition of heparin in the presence of IP<sub>3</sub> plus IP<sub>3</sub>R1 antibody did not cause any further reduction in BK<sub>Ca</sub> channel P<sub>o</sub> (Fig. 4, B and C). Boiled (95°C for 15 min) IP<sub>3</sub>R1 antibody did not alter IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation (Fig. 4 C). To further examine BK<sub>Ca</sub> channel regulation by IP<sub>3</sub>Rs, we used adenophostin A as an alternate IP<sub>3</sub>R agonist. 1 μM adenophostin A increased BK<sub>Ca</sub> channel activity ~1.60-fold (Fig. 4 D). These data indicate that IP<sub>3</sub>R1 activation is necessary for IP<sub>3</sub>-mediated BK<sub>Ca</sub> channel activation in arterial smooth muscle cells.



**Figure 3.** IP<sub>3</sub> elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity. (A) Mean data illustrating IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation over a range of free Ca<sup>2+</sup> concentrations in inside-out patches at -40 mV. Data are fit with a Boltzmann function constrained to ≥0. Experimental numbers are given after each [Ca<sup>2+</sup>] (μM): control: 1, 8; 3, 6; 10, 6; 30, 6; 100, 8; 300, 8; 10 μM IP<sub>3</sub>: 1, 7; 3, 6; 10, 6; 30, 6; 100, 7;

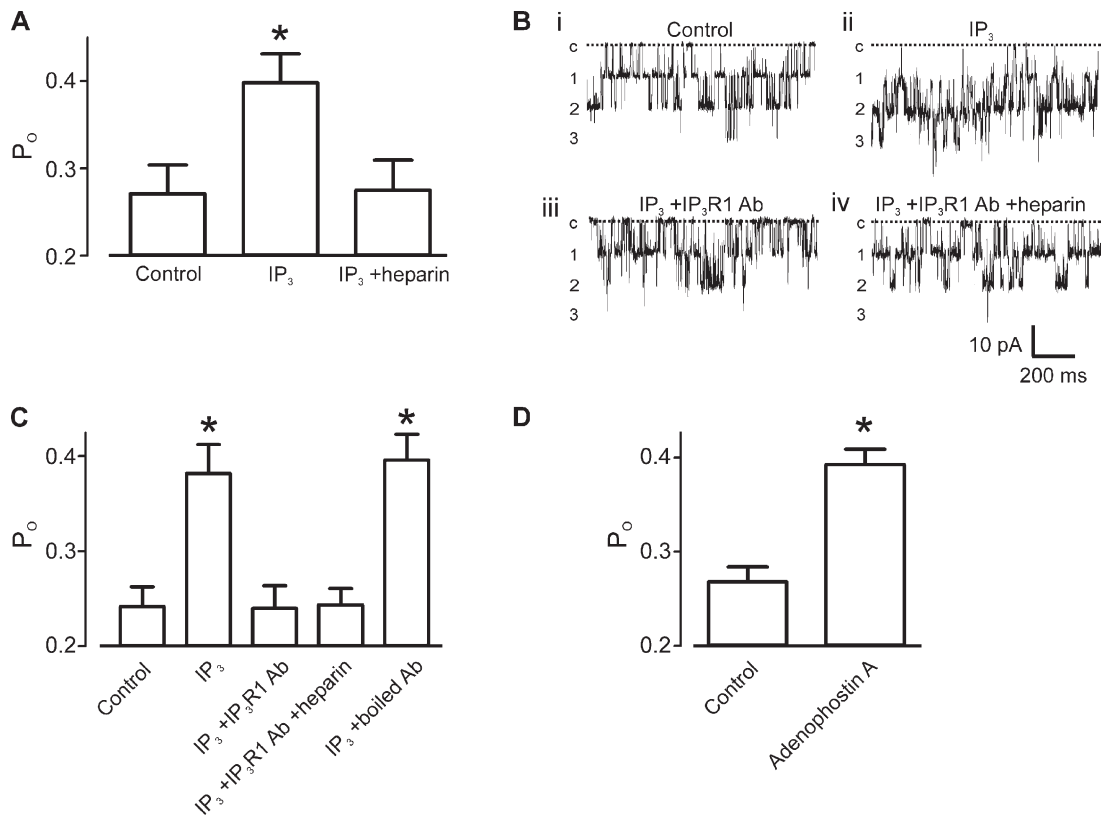
300, 7. (B) Mean data illustrating IP<sub>3</sub>-induced change in BK<sub>Ca</sub> channel P<sub>o</sub> at each [Ca<sup>2+</sup>]. Data are fit with a Gaussian function. In control, the K<sub>d</sub> for Ca<sup>2+</sup> was 20.4 ± 0.9 μM, a slope of 1.2 ± 0.2, and a maximum P<sub>o</sub> of 0.82 ± 0.04. 10 μM IP<sub>3</sub> decreased the mean K<sub>d</sub> for Ca<sup>2+</sup> to 12.4 ± 0.8 μM (P < 0.05), but did not alter the slope (1.3 ± 0.3) or the maximum P<sub>o</sub> (0.83 ± 0.03; P > 0.05 for each).



To further investigate the necessity for IP<sub>3</sub>R1 and to determine whether IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation occurs in another species, we studied BK<sub>Ca</sub> channel regulation in mouse wild-type (IP<sub>3</sub>R1<sup>+/+</sup>) and IP<sub>3</sub>R1 knockout (IP<sub>3</sub>R1<sup>-/-</sup>) cerebral artery smooth muscle cells. Western blotting confirmed that IP<sub>3</sub>R1 was present in IP<sub>3</sub>R1<sup>+/+</sup> mouse aorta, but absent in IP<sub>3</sub>R1<sup>-/-</sup> mouse aorta (Fig. 5 A). BK<sub>Ca</sub> channel P<sub>o</sub> (IP<sub>3</sub>R1<sup>+/+</sup>, 0.30 ± 0.08; IP<sub>3</sub>R1<sup>-/-</sup>, 0.27 ± 0.03; n = 5) and amplitude (pA: IP<sub>3</sub>R1<sup>+/+</sup>, 10.2 ± 0.8; IP<sub>3</sub>R1<sup>-/-</sup>, 11.4 ± 0.8; n = 5) were similar in inside-out patches from IP<sub>3</sub>R1<sup>+/+</sup> and IP<sub>3</sub>R1<sup>-/-</sup> cerebral artery smooth muscle cells exposed to 10 μM Ca<sup>2+</sup> (P > 0.05 for each). IP<sub>3</sub> increased mean BK<sub>Ca</sub> channel P<sub>o</sub> to ~142% of control in inside-out patches from mouse IP<sub>3</sub>R1<sup>+/+</sup> cells (Fig. 5 B). Furthermore, in patches from IP<sub>3</sub>R1<sup>+/+</sup> cells, IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation was reversed by heparin (Fig. 5 B). In contrast, IP<sub>3</sub> or IP<sub>3</sub> plus heparin did not alter BK<sub>Ca</sub> channel P<sub>o</sub> in excised patches from IP<sub>3</sub>R1<sup>-/-</sup> arterial smooth muscle cells (Fig. 5 B). These data indicate that IP<sub>3</sub>R1 is essential for IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation in cerebral artery smooth muscle cells.

#### BK<sub>Ca</sub> channel α subunits colocalize with IP<sub>3</sub>R1

Our data indicate that IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels functionally interact. Therefore, spatial localization of these proteins was studied using immuno-FRET microscopy. Cy2- and Cy3-tagged secondary antibodies targeting primary antibodies bound to IP<sub>3</sub>R1 and BK<sub>Ca</sub> channel α subunits, respectively, generated whole cell N-FRET of 20.4 ± 1.3% (n = 11; Fig. 6 A). In contrast, whole cell N-FRET between IP<sub>3</sub>R1 and TRPM4 channels, which do not colocalize in cerebral artery smooth muscle cells (Adebiyi et al., 2010), was significantly lower at 7.4 ± 1.0% (n = 10; P > 0.05; Fig. 6 A). N-FRET between IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels was observed both at the plasma membrane and intracellularly. Antigenic peptides abolished fluorescent labeling by BK<sub>Ca</sub> and TRPM4 channel antibodies, respectively (Fig. S1). An antigenic peptide was not available for the monoclonal IP<sub>3</sub>R1 antibody, but this antibody detects only IP<sub>3</sub>R1 protein in a Western blot, indicating selectivity (Adebiyi et al., 2010). These data indicate that IP<sub>3</sub>R1 is located in close proximity to plasma membrane BK<sub>Ca</sub> channels in arterial smooth muscle cells.



**Figure 4.** IP<sub>3</sub>R1 mediates IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. (A) Mean data illustrating that heparin reverses IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation (n: control (10 μM Ca<sup>2+</sup>), 5; 30 μM IP<sub>3</sub>, 5; 30 μM IP<sub>3</sub> plus 1 mg/ml heparin, 5). (B) Original recording from the same inside-out patch illustrating control BK<sub>Ca</sub> channel activity (i), IP<sub>3</sub> (30 μM)-induced channel activation (ii), reversal by monoclonal IP<sub>3</sub>R1 antibody (iii, 1:100; clone L24/18; NeuroMab), and no further effect of heparin applied in the continued presence of IP<sub>3</sub>R1 antibody (iv). (C) Mean data illustrating that monoclonal IP<sub>3</sub>R1 antibody inhibits IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation, and that the addition of heparin leads to no further change in activity (control (10 μM Ca<sup>2+</sup>), n = 4; 30 μM IP<sub>3</sub>, n = 4; 30 μM IP<sub>3</sub> plus IP<sub>3</sub>R1 antibody (1:100), n = 4; 30 μM IP<sub>3</sub> plus IP<sub>3</sub>R1 antibody (1:100) plus heparin, n = 4; IP<sub>3</sub> plus boiled IP<sub>3</sub>R1 antibody, n = 4). (D) Mean data illustrating that adenophostin A activates BK<sub>Ca</sub> channels (n: control (10 μM Ca<sup>2+</sup>), 4; 1 μM adenophostin A, 4). \*, P < 0.05.

### BK<sub>Ca</sub> channel $\alpha$ and $\beta$ 1 subunits coimmunoprecipitate with IP<sub>3</sub>R1

coIP was performed to test the hypothesis that IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels are contained within the same macromolecular protein complex. Due to the small size of the resistance (100–200- $\mu$ m diameter), cerebral arteries used in this study, arteries collected from  $\sim$ 15 rats, were required for each coIP experiment. The monoclonal IP<sub>3</sub>R1 antibody coimmunoprecipitated IP<sub>3</sub>R1 with BK<sub>Ca</sub> channel  $\alpha$  and  $\beta$ 1 subunits from cerebral artery lysate (Fig. 6 B). These data indicate that IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels are located within the same macromolecular complex in arterial smooth muscle cells.

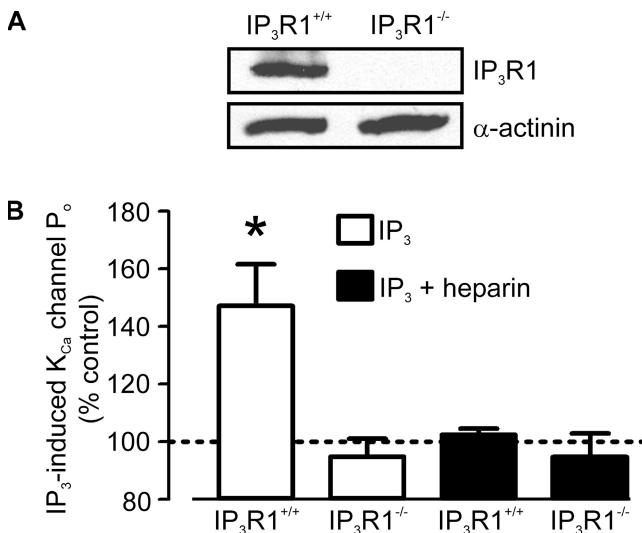
## DISCUSSION

Here, we demonstrate that IP<sub>3</sub>-induced IP<sub>3</sub>R1 activation stimulates BK<sub>Ca</sub> channels via a local SR Ca<sup>2+</sup> release-independent coupling mechanism in cerebral artery smooth muscle cells. Novel findings are that: (a) IP<sub>3</sub> activates BK<sub>Ca</sub> channels in excised membrane patches removed from cytosolic signaling pathways; (b) IP<sub>3</sub> elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity; (c) IP<sub>3</sub>R1 expression and activation are required for IP<sub>3</sub> to stimulate BK<sub>Ca</sub> channels; and (d) IP<sub>3</sub>R1 and BK<sub>Ca</sub> channel subunits are located in close spatial proximity and coimmunoprecipitate. These data identify a novel signaling

mechanism whereby IP<sub>3</sub>R1 channels activate plasma membrane BK<sub>Ca</sub> channels via an SR Ca<sup>2+</sup> release-independent local coupling mechanism in arterial smooth muscle cells. Given that both IP<sub>3</sub>Rs and K<sub>Ca</sub> channels are widely expressed, such communication may occur in other cell types.

Agonist binding to PLC-coupled receptors leads to phosphatidylinositol 4,5-bisphosphate cleavage and an elevation in both diacylglycerol and IP<sub>3</sub> in smooth muscle cells. Diacylglycerol remains membrane bound and stimulates PKC, which phosphorylates a wide variety of target proteins that regulate contractility, including ion channels and the contractile apparatus (Davis and Hill, 1999; Jaggar et al., 2000). Relevant to this study, PKC inhibits arterial smooth muscle cell BK<sub>Ca</sub> channels, leading to membrane depolarization and vasoconstriction (Davis and Hill, 1999; Jaggar et al., 2000). Arteries undergo steady-state changes in membrane potential, with a dynamic range between  $\sim$ -60 and -20 mV (Knot and Nelson, 1998; Davis and Hill, 1999). Here, BK<sub>Ca</sub> channel regulation was studied primarily at a steady voltage of -40 mV, which is the membrane potential of cerebral arteries at a physiological intravascular pressure of 60 mmHg (Knot and Nelson, 1998). IP<sub>3</sub> activated BK<sub>Ca</sub> channels in both intact cells and in excised membrane patches. In excised patches, IP<sub>3</sub> increased BK<sub>Ca</sub> channel activity with an apparent  $K_d$  of  $\sim$ 4  $\mu$ M. Previous studies have estimated global intracellular IP<sub>3</sub> concentration ( $[IP_3]_i$ ) to be 0.1–3  $\mu$ M in unstimulated cells and 1–20  $\mu$ M in agonist-stimulated cells (Finch and Augustine, 1998; Luzzi et al., 1998; Takechi et al., 1998; Patel et al., 1999). Recent studies using fluorescent IP<sub>3</sub> biosensors suggested that receptor agonists elevate global  $[IP_3]_i$  to  $\sim$ 30 nM in cardiac myocytes and up to 700 nM in COS-7 and HSY-EA1 cells (Remus et al., 2006; Tanimura et al., 2009). In arterial myocytes, global  $[IP_3]_i$  is unclear, but local IP<sub>3</sub> gradients higher than global  $[IP_3]_i$  should exist, particularly within the immediate vicinity of PLC where IP<sub>3</sub> is generated. The  $[IP_3]_i$  nearby a target protein such as an IP<sub>3</sub>R will depend on many factors, including PLC activity, proximity of IP<sub>3</sub>Rs to PLC, and IP<sub>3</sub> metabolism. K<sub>Ca</sub> channel IP<sub>3</sub> sensitivity determined here may indicate IP<sub>3</sub> concentrations that would occur nearby IP<sub>3</sub>Rs that are located in close proximity to plasma membrane BK<sub>Ca</sub> channels.

IP<sub>3</sub> acts primarily by relieving Ca<sup>2+</sup> inhibition of IP<sub>3</sub>Rs, thereby permitting Ca<sup>2+</sup>-induced channel activation (Thrower et al., 2001; Foskett et al., 2007). Physiological micromolar  $[Ca^{2+}]_i$  concentrations used here to study K<sub>Ca</sub> channel activity would be expected to attenuate IP<sub>3</sub>-induced IP<sub>3</sub>R activation and may provide one explanation for the micromolar IP<sub>3</sub> sensitivity of BK<sub>Ca</sub> channel activation. IP<sub>3</sub>R IP<sub>3</sub> sensitivity can also vary widely from nanomolar to micromolar depending on multiple factors in addition to  $[Ca^{2+}]_i$ , including IP<sub>3</sub>R isoform, splice variation, which can occur in many regions of the



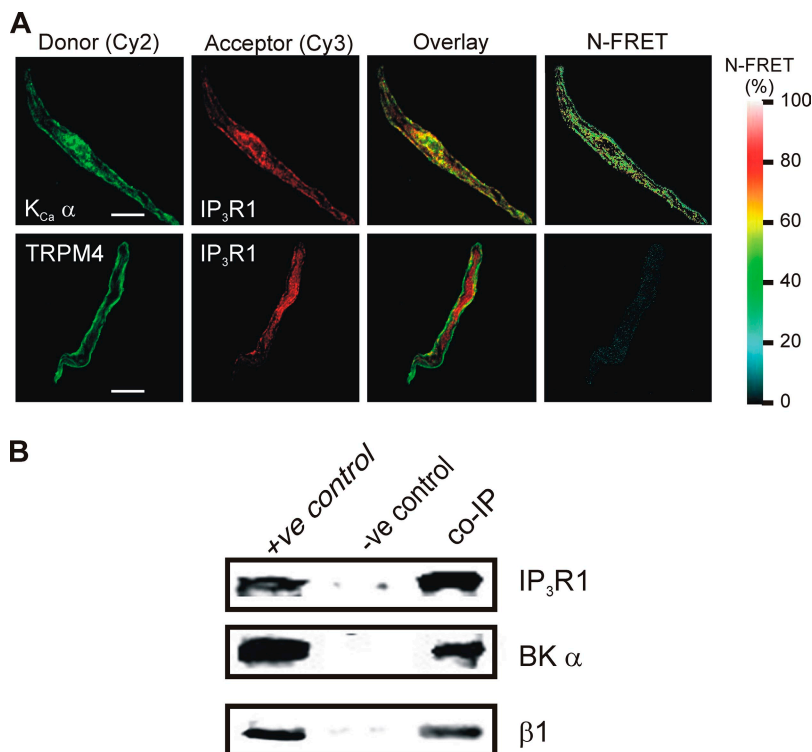
**Figure 5.** Genetic ablation of IP<sub>3</sub>R1 abolishes IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation in cerebral artery smooth muscle cells. (A) Western blot indicating that IP<sub>3</sub>R1 protein ( $\sim$ 270 kD) is present in IP<sub>3</sub>R1<sup>+/+</sup> mouse aorta and absent in IP<sub>3</sub>R1<sup>-/-</sup> aorta. (B) Mean data illustrating that 10  $\mu$ M IP<sub>3</sub> activates BK<sub>Ca</sub> channels in inside-out patches from mouse IP<sub>3</sub>R1<sup>+/+</sup> cerebral artery smooth muscle cells, but not in mouse IP<sub>3</sub>R1<sup>-/-</sup> cerebral artery smooth muscle cells. Mean data also indicate that 1 mg/ml heparin reverses IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation in IP<sub>3</sub>R1<sup>+/+</sup> cells. Membrane voltage was -40 mV. IP<sub>3</sub>R1<sup>-/-</sup>: control,  $n = 5$ ; IP<sub>3</sub>,  $n = 5$ ; IP<sub>3</sub> plus heparin,  $n = 5$ . IP<sub>3</sub>R1<sup>+/+</sup>: control,  $n = 5$ ; IP<sub>3</sub>,  $n = 5$ ; IP<sub>3</sub> plus heparin,  $n = 5$ . \*,  $P < 0.05$ .

protein including the IP<sub>3</sub>-binding domain, and potentially through isoform heterotetramerization (Thrower et al., 2001; Foskett et al., 2007). For example, canine cerebellar IP<sub>3</sub>R1 is sensitive to IP<sub>3</sub> over a broad concentration range and exhibits high (nM) and low (10 μM) affinity IP<sub>3</sub>-binding sites. The IP<sub>3</sub> and Ca<sup>2+</sup> sensitivity of arterial smooth muscle cell IP<sub>3</sub>Rs has not been determined, nor have these channels been cloned. Therefore, deriving mechanistic details regarding IP<sub>3</sub>R communication with K<sub>Ca</sub> channels is difficult until detailed knowledge of smooth muscle cell IP<sub>3</sub>Rs is available, particularly IP<sub>3</sub> and Ca<sup>2+</sup> sensitivity. Our data indicate that under physiological voltage and local [Ca<sup>2+</sup>]<sub>i</sub>, micromolar [IP<sub>3</sub>]<sub>i</sub> is required for IP<sub>3</sub>Rs to activate BK<sub>Ca</sub> channels.

Data indicate that IP<sub>3</sub> does not directly activate BK<sub>Ca</sub> channels. Rather, IP<sub>3</sub>R1 mediates IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. This conclusion is supported by our observation that heparin, an IP<sub>3</sub>R1 antibody, and IP<sub>3</sub>R1 ablation all blocked IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. These data also indicate that functional IP<sub>3</sub>R1 protein is excised together with BK<sub>Ca</sub> channels in inside-out patches. The Förster distance between Cy2 and Cy3 is 5–6 nm. If Cy2 and Cy3 are located in such local proximity, nonradiative dipole–dipole coupling between excited Cy2 (donor) and Cy3 (acceptor) leads to Cy3 emission. Thus, for FRET to occur, IP<sub>3</sub>R1 must be located in very close spatial proximity to BK<sub>Ca</sub> channels. Supporting local interaction between these proteins, coIP data indicated that IP<sub>3</sub>R1 and BK<sub>Ca</sub> channel α and β1 subunits were located in the same

macromolecular complex. In contrast to FRET data indicating close spatial proximity of IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels, immuno-FRET data indicated that IP<sub>3</sub>R1 and TRPM4 channels are not spatially localized in arterial smooth muscle cells, in agreement with a recent report (Adebisi et al., 2010). BK<sub>Ca</sub> channels are also located nearby IP<sub>3</sub>Rs in cultured glioma cells, but in contrast to the observations made here, glioma cell IP<sub>3</sub>Rs activate BK<sub>Ca</sub> channels via Ca<sup>2+</sup> signaling and direct molecular coupling is absent (Weaver et al., 2007). Electron microscopy studies have shown that the SR and plasma membranes can be located in very close proximity (~20 nm) in arterial smooth muscle cells (Devine et al., 1972). Conceivably, IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels may be present within macromolecular complexes that bridge the SR and plasma membranes, allowing local molecular communication between these proteins. Immuno-FRET between IP<sub>3</sub>R1 and BK<sub>Ca</sub> channel α subunits was observed both at the plasma membrane and intracellularly. The physiological function of close localization between IP<sub>3</sub>R1 and intracellular BK<sub>Ca</sub> channels is unclear. Conceivably, BK<sub>Ca</sub> channels may be contained within the SR or Golgi before membrane trafficking, and intracellular FRET may reflect the close proximity of these intracellular BK<sub>Ca</sub> channels to IP<sub>3</sub>R1 located on the SR membrane. Alternatively, IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels may form into a protein complex before membrane trafficking.

Intact SR may have been excised within membrane patches, although SR Ca<sup>2+</sup> would be depleted due to the recording conditions used (no ATP in bath solution).



**Figure 6.** BK<sub>Ca</sub> channel subunits are located in close spatial proximity to IP<sub>3</sub>R1 in cerebral artery smooth muscle cells. (A) Immuno-FRET data illustrating close spatial proximity of IP<sub>3</sub>R1 to BK<sub>Ca</sub> channel α subunits. Cy2- and Cy3-labeled secondary antibodies bound to IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels generate N-FRET. In contrast, secondary antibodies bound to IP<sub>3</sub>R1 and TRPM4 do not generate significant N-FRET. Bars, 10 μm. (B) Monoclonal IP<sub>3</sub>R1 antibody coimmunoprecipitates IP<sub>3</sub>R1 (~270 kD), BK<sub>Ca</sub> channel α (~125 kD), and β1 (~36 kD) subunits from cerebral arteries. Lysate supernatant was used as the input (+ve) control. Beads that have no antibody-binding capacity were used in the negative (–ve) coIP control.

Furthermore, IP<sub>3</sub> activated BK<sub>Ca</sub> channels in patches that had been excised for >30 min and in solutions that contained BAPTA, which would rapidly buffer any Ca<sup>2+</sup> released by SR. Therefore, in inside-out patches studied here, IP<sub>3</sub>R-mediated SR Ca<sup>2+</sup> release cannot underlie IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. Recently, we demonstrated that IP<sub>3</sub>R1 physically and functionally couples to TRPC3, but not TRPC6, channels in cerebral artery smooth muscle cells (Adebiyi et al., 2010). When considering previous observations and those made here, IP<sub>3</sub>R1, TRPC3, and BK<sub>Ca</sub> channels may coassemble within a macromolecular complex that regulates Ca<sup>2+</sup> signaling, membrane potential, and arterial contractility. Conceivably, IP<sub>3</sub>R-induced Ca<sup>2+</sup> influx through plasma membrane TRPC3 channels could elevate submembrane [Ca<sup>2+</sup>]<sub>i</sub> and activate nearby BK<sub>Ca</sub> channels (Adebiyi et al., 2010). Several observations indicate that such a mechanism does not underlie IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation reported here: (a) the driving force for Ca<sup>2+</sup> influx (symmetrical 10 μM Ca<sup>2+</sup>) is weak and unlikely to sufficiently elevate [Ca<sup>2+</sup>]<sub>i</sub> to activate BK<sub>Ca</sub> channels; (b) BK<sub>Ca</sub> channel activation occurred in the presence of BAPTA, which would rapidly buffer any entering Ca<sup>2+</sup>; and (c) elevating pipette free [Ca<sup>2+</sup>] to 2 mM did not alter IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation. In intact cells with physiological Ca<sup>2+</sup> buffers and cation gradients (i.e., 2 mM [Ca<sup>2+</sup>]<sub>o</sub> and 100–300 nM [Ca<sup>2+</sup>]<sub>i</sub>), Ca<sup>2+</sup> influx through TRPC3 channels may contribute to IP<sub>3</sub>R1-induced BK<sub>Ca</sub> channel activation. However, TRPC3 channels are also Na<sup>+</sup> permeant, and the larger driving force for Na<sup>+</sup> influx would limit Ca<sup>2+</sup> influx, reducing any potential Ca<sup>2+</sup> signal to BK<sub>Ca</sub> channels.

BK<sub>Ca</sub> channels exhibited a micromolar K<sub>d</sub> for Ca<sup>2+</sup> similar to that previously determined in rat cerebral artery smooth muscle cells at the same voltage (Pérez et al., 2001). IP<sub>3</sub> caused an elevation in BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity with activation most prominent between 1 and 10 μM [Ca<sup>2+</sup>]<sub>i</sub>. These data indicate that IP<sub>3</sub> will increase BK<sub>Ca</sub> channel sensitivity to local micromolar Ca<sup>2+</sup> gradients, but will not shift BK<sub>Ca</sub> channel Ca<sup>2+</sup> sensitivity into the global nanomolar [Ca<sup>2+</sup>]<sub>i</sub> range. Data here and in a previous study indicate that IP<sub>3</sub>R1 activation both stimulates SR Ca<sup>2+</sup> release and amplifies the sensitivity of nearby BK<sub>Ca</sub> channels to micromolar [Ca<sup>2+</sup>]<sub>i</sub> (Zhao et al., 2008). Therefore, the molecular coupling mechanism likely sensitizes BK<sub>Ca</sub> channels to SR Ca<sup>2+</sup> released from nearby IP<sub>3</sub>Rs.

When considering the physiological function of the local coupling mechanism between IP<sub>3</sub>R1 and BK<sub>Ca</sub> channels, it is important to consider that PLC activation not only elevates IP<sub>3</sub>, but also stimulates PKC. PKC inhibits arterial smooth muscle cell BK<sub>Ca</sub> channels both by reducing Ca<sup>2+</sup> spark frequency and by direct BK<sub>Ca</sub> channel inhibition (Jaggar and Nelson, 2000; Jaggar et al., 2000). Thus, PKC reduces BK<sub>Ca</sub> activation by RYR channels that generate Ca<sup>2+</sup> sparks (Jaggar et al., 2000). However,

vasoconstrictors also activate Ca<sup>2+</sup> waves and elevate global [Ca<sup>2+</sup>]<sub>i</sub> (Jaggar and Nelson, 2000; Mauban et al., 2001; Zhao et al., 2008; Adebiyi et al., 2010). IP<sub>3</sub>R1 activation is essential for vasoconstrictor-induced Ca<sup>2+</sup> waves and contributes to the global [Ca<sup>2+</sup>]<sub>i</sub> elevation (Zhao et al., 2008). Collectively, these studies suggest that PLC-coupled receptor agonists shift control of BK<sub>Ca</sub> channel activity from RYR channels to IP<sub>3</sub>Rs. IP<sub>3</sub>-induced BK<sub>Ca</sub> channel activation would limit PKC-induced BK<sub>Ca</sub> channel inhibition and attenuate the membrane depolarization and vasoconstriction.

The signaling mechanism by which IP<sub>3</sub>R1 elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity was not studied here. Several possibilities exist, including that an IP<sub>3</sub>-induced conformational change in IP<sub>3</sub>R1 may lead to direct interaction with either the BK<sub>Ca</sub> channel α and/or β1 subunit. IP<sub>3</sub>R1 may also activate BK<sub>Ca</sub> channels via an indirect interaction through intermediate proteins, including caveolin-1 (Cheng and Jaggar, 2006; Alioua et al., 2008). A deeper understanding of the molecular mechanisms by which IP<sub>3</sub>Rs stimulate BK<sub>Ca</sub> channels will require further investigation.

In summary, data indicate that IP<sub>3</sub>-induced IP<sub>3</sub>R1 activation elevates BK<sub>Ca</sub> channel apparent Ca<sup>2+</sup> sensitivity through localized molecular coupling in arterial smooth muscle cells. This negative feedback mechanism would limit IP<sub>3</sub>-induced vasoconstriction in cerebral arteries. These data also raise the possibility that local communication between IP<sub>3</sub>Rs and BK<sub>Ca</sub> channels may occur in a wide variety of cell types that express both of these proteins.

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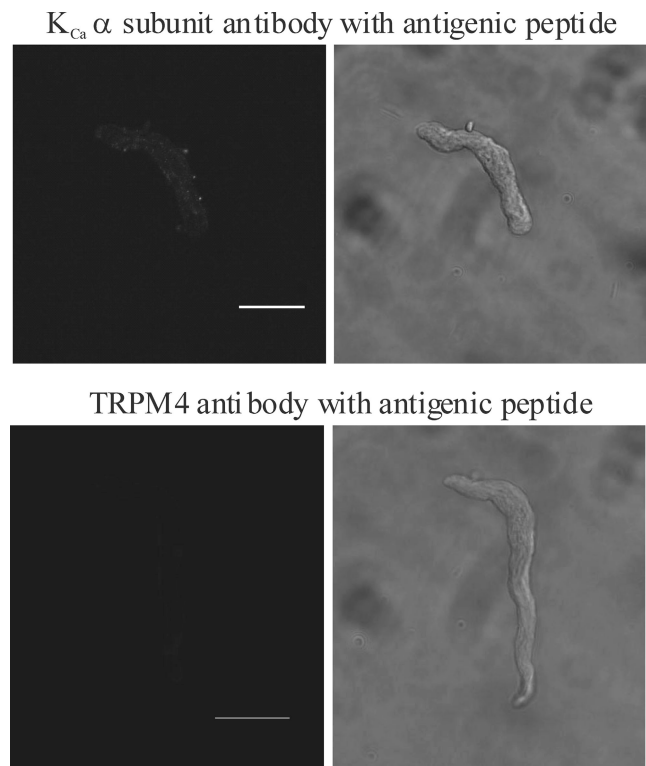
## REFERENCES

- Adebiyi, A., G. Zhao, D. Narayanan, C.M. Thomas-Gatewood, J.P. Bannister, and J.H. Jaggar. 2010. Isoform-selective physical coupling of TRPC3 channels to IP<sub>3</sub> receptors in smooth muscle cells regulates arterial contractility. *Circ. Res.* 106:1603–1612. doi:10.1161/CIRCRESAHA.110.216804
- Alioua, A., R. Lu, Y. Kumar, M. Eghbali, P. Kundu, L. Toro, and E. Stefani. 2008. Slo1 caveolin-binding motif, a mechanism of caveolin-1-Slo1 interaction regulating Slo1 surface expression. *J. Biol. Chem.* 283:4808–4817. doi:10.1074/jbc.M709802200
- Blondel, O., J. Takeda, H. Janssen, S. Seino, and G.I. Bell. 1993. Sequence and functional characterization of a third inositol triphosphate receptor subtype, IP<sub>3</sub>R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. *J. Biol. Chem.* 268:11356–11363.
- Brenner, R., G.J. Pérez, A.D. Bonev, D.M. Eckman, J.C. Kosek, S.W. Wiler, A.J. Patterson, M.T. Nelson, and R.W. Aldrich. 2000.



- Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature*. 407:870–876. doi:10.1038/35038011
- Cheng, X., and J.H. Jaggard. 2006. Genetic ablation of caveolin-1 modifies Ca<sup>2+</sup> spark coupling in murine arterial smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* 290:H2309–H2319. doi:10.1152/ajpheart.01226.2005
- Cherianov, S.Y., and J.H. Jaggard. 2006. TNF- $\alpha$  dilates cerebral arteries via NAD(P)H oxidase-dependent Ca<sup>2+</sup> spark activation. *Am. J. Physiol. Cell Physiol.* 290:C964–C971. doi:10.1152/ajpcell.00499.2005
- Davis, M.J., and M.A. Hill. 1999. Signaling mechanisms underlying the vascular myogenic response. *Physiol. Rev.* 79:387–423.
- Devine, C.E., A.V. Somlyo, and A.P. Somlyo. 1972. Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *J. Cell Biol.* 52:690–718. doi:10.1083/jcb.52.3.690
- Finch, E.A., and G.J. Augustine. 1998. Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. *Nature*. 396:753–756. doi:10.1038/25541
- Foskett, J.K., C. White, K.H. Cheung, and D.O. Mak. 2007. Inositol trisphosphate receptor Ca<sup>2+</sup> release channels. *Physiol. Rev.* 87:593–658. doi:10.1152/physrev.00035.2006
- Jaggard, J.H., and M.T. Nelson. 2000. Differential regulation of Ca<sup>2+</sup> sparks and Ca<sup>2+</sup> waves by UTP in rat cerebral artery smooth muscle cells. *Am. J. Physiol. Cell Physiol.* 279:C1528–C1539.
- Jaggard, J.H., V.A. Porter, W.J. Lederer, and M.T. Nelson. 2000. Calcium sparks in smooth muscle. *Am. J. Physiol. Cell Physiol.* 278:C235–C256.
- Kim, C.J., B.K. Weir, R.L. Macdonald, and H. Zhang. 1998. Erythrocyte lysate releases Ca<sup>2+</sup> from IP<sub>3</sub>-sensitive stores and activates Ca<sup>2+</sup>-dependent K<sup>+</sup> channels in rat basilar smooth muscle cells. *Neurol. Res.* 20:23–30.
- Knot, H.J., and M.T. Nelson. 1998. Regulation of arterial diameter and wall [Ca<sup>2+</sup>] in cerebral arteries of rat by membrane potential and intravascular pressure. *J. Physiol.* 508:199–209.
- Lamont, C., and W.G. Wier. 2004. Different roles of ryanodine receptors and inositol (1,4,5)-trisphosphate receptors in adrenergically stimulated contractions of small arteries. *Am. J. Physiol. Heart Circ. Physiol.* 287:H617–H625. doi:10.1152/ajpheart.00708.2003
- Lee, C.H., R. Rahimian, T. Szado, J. Sandhu, D. Poburko, T. Behra, L. Chan, and C. van Breemen. 2002. Sequential opening of IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channels and SOC during  $\alpha$ -adrenergic activation of rabbit vena cava. *Am. J. Physiol. Heart Circ. Physiol.* 282:H1768–H1777.
- Lu, R., A. Alioua, Y. Kumar, M. Eghbali, E. Stefani, and L. Toro. 2006. MaxiK channel partners: physiological impact. *J. Physiol.* 570:65–72. doi:10.1113/jphysiol.2005.098913
- Luzzi, V., C.E. Sims, J.S. Soughayer, and N.L. Allbritton. 1998. The physiologic concentration of inositol 1,4,5-trisphosphate in the oocytes of *Xenopus laevis*. *J. Biol. Chem.* 273:28657–28662. doi:10.1074/jbc.273.44.28657
- Matsumoto, M., T. Nakagawa, T. Inoue, E. Nagata, K. Tanaka, H. Takano, O. Minowa, J. Kuno, S. Sakakibara, M. Yamada, et al. 1996. Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. *Nature*. 379:168–171. doi:10.1038/379168a0
- Mauban, J.R., C. Lamont, C.W. Balke, and W.G. Wier. 2001. Adrenergic stimulation of rat resistance arteries affects Ca<sup>2+</sup> sparks, Ca<sup>2+</sup> waves, and Ca<sup>2+</sup> oscillations. *Am. J. Physiol. Heart Circ. Physiol.* 280:H2399–H2405.
- Morel, J.L., N. Fritz, J.L. Lavie, and J. Mironneau. 2003. Crucial role of type 2 inositol 1,4,5-trisphosphate receptors for acetylcholine-induced Ca<sup>2+</sup> oscillations in vascular myocytes. *Arterioscler. Thromb. Vasc. Biol.* 23:1567–1575. doi:10.1161/01.ATV.0000089013.82552.5D
- Nelson, M.T., H. Cheng, M. Rubart, L.F. Santana, A.D. Bonev, H.J. Knot, and W.J. Lederer. 1995. Relaxation of arterial smooth muscle by calcium sparks. *Science*. 270:633–637. doi:10.1126/science.270.5236.633
- O’Gorman, S., N.A. Dagenais, M. Qian, and Y. Marchuk. 1997. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA*. 94:14602–14607. doi:10.1073/pnas.94.26.14602
- Patel, S., S.K. Joseph, and A.P. Thomas. 1999. Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium*. 25:247–264. doi:10.1054/ceca.1999.0021
- Pérez, G.J., A.D. Bonev, and M.T. Nelson. 2001. Micromolar Ca<sup>2+</sup> from sparks activates Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels in rat cerebral artery smooth muscle. *Am. J. Physiol. Cell Physiol.* 281:C1769–C1775.
- Plüger, S., J. Faulhaber, M. Fürstenau, M. Löhn, R. Waldschütz, M. Gollasch, H. Haller, F.C. Luft, H. Ehmke, and O. Pongs. 2000. Mice with disrupted BK channel  $\beta$ 1 subunit gene feature abnormal Ca<sup>2+</sup> spark/STOC coupling and elevated blood pressure. *Circ. Res.* 87:E53–E60.
- Remus, T.P., A.V. Zima, J. Bossuyt, D.J. Bare, J.L. Martin, L.A. Blatter, D.M. Bers, and G.A. Mignery. 2006. Biosensors to measure inositol 1,4,5-trisphosphate concentration in living cells with spatiotemporal resolution. *J. Biol. Chem.* 281:608–616. doi:10.1074/jbc.M509645200
- Ross, C.A., S.K. Danoff, M.J. Schell, S.H. Snyder, and A. Ullrich. 1992. Three additional inositol 1,4,5-trisphosphate receptors: molecular cloning and differential localization in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA*. 89:4265–4269. doi:10.1073/pnas.89.10.4265
- Takechi, H., J. Eilers, and A. Konnerth. 1998. A new class of synaptic response involving calcium release in dendritic spines. *Nature*. 396:757–760. doi:10.1038/25547
- Tanimura, A., T. Morita, A. Nezu, A. Shitara, N. Hashimoto, and Y. Tojyo. 2009. Use of fluorescence resonance energy transfer-based biosensors for the quantitative analysis of inositol 1,4,5-trisphosphate dynamics in calcium oscillations. *J. Biol. Chem.* 284:8910–8917. doi:10.1074/jbc.M805865200
- Thrower, E.C., R.E. Hagar, and B.E. Ehrlich. 2001. Regulation of Ins(1,4,5)P<sub>3</sub> receptor isoforms by endogenous modulators. *Trends Pharmacol. Sci.* 22:580–586. doi:10.1016/S0165-6147(00)01809-5
- Weaver, A.K., M.L. Olsen, M.B. McFerrin, and H. Sontheimer. 2007. BK channels are linked to inositol 1,4,5-trisphosphate receptors via lipid rafts: a novel mechanism for coupling [Ca<sup>2+</sup>]<sub>i</sub> to ion channel activation. *J. Biol. Chem.* 282:31558–31568. doi:10.1074/jbc.M702866200
- Wilkinson, M.K., T.J. Heppner, A.D. Bonev, and M.T. Nelson. 2006. Inositol trisphosphate receptor calcium release is required for cerebral artery smooth muscle cell proliferation. *Am. J. Physiol. Heart Circ. Physiol.* 290:H240–H247. doi:10.1152/ajpheart.01191.2004
- Xi, Q., A. Adebisi, G. Zhao, K.E. Chapman, C.M. Waters, A. Hassid, and J.H. Jaggard. 2008. IP<sub>3</sub> constricts cerebral arteries via IP<sub>3</sub> receptor-mediated TRPC3 channel activation and independently of sarcoplasmic reticulum Ca<sup>2+</sup> release. *Circ. Res.* 102:1118–1126. doi:10.1161/CIRCRESAHA.108.173948
- Xia, Z., and Y. Liu. 2001. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. *Biophys. J.* 81:2395–2402. doi:10.1016/S0006-3495(01)75886-9
- Zhao, G., A. Adebisi, E. Blaskova, Q. Xi, and J.H. Jaggard. 2008. Type 1 inositol 1,4,5-trisphosphate receptors mediate UTP-induced cation currents, Ca<sup>2+</sup> signals, and vasoconstriction in cerebral arteries. *Am. J. Physiol. Cell Physiol.* 295:C1376–C1384. doi:10.1152/ajpcell.00362.2008
- Zhou, H., T. Nakamura, N. Matsumoto, C. Hisatsune, A. Mizutani, T. Iesaki, H. Daida, and K. Mikoshiba. 2008. Predominant role of type 1 IP<sub>3</sub> receptor in aortic vascular muscle contraction. *Biochem. Biophys. Res. Commun.* 369:213–219. doi:10.1016/j.bbrc.2007.12.194

## SUPPLEMENTAL MATERIAL

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**Figure S1.** Antigenic peptides abolish fluorescent labeling by  $K_{Ca}$  channel  $\alpha$  and TRPM4 channel antibodies. Also shown are differential interference contrast images of the same cells imaged for fluorescence. Bars, 10  $\mu$ m.