Type 1 IP_3 receptors activate BK_{Ca} channels via local molecular coupling in arterial smooth muscle cells

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Plasma membrane large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels and sarcoplasmic reticulum inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are expressed in a wide variety of cell types, including arterial smooth muscle cells. Here, we studied BK_{Ca} channel regulation by IP_3 and IP_3Rs in rat and mouse cerebral artery smooth muscle cells. IP_3 activated BK_{Ca} channels both in intact cells and in excised inside-out membrane patches. IP_3 caused concentration-dependent BK_{Ca} channel activation with an apparent dissociation constant (K_d) of ~4 µM at physiological voltage (−40 mV) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; 10 µM). IP_3 also caused a leftward-shift in BK_{Ca} channel apparent Ca^{2+} sensitivity and reduced the K_d for free $[Ca^{2+}]_i$ from ~20 to 12 µM, but did not alter the slope or maximal P_o. BAPTA, a fast Ca^{2+} buffer, or an elevation in extracellular Ca^{2+} concentration did not alter IP_3-induced BK_{Ca} channel activation. Heparin, an IP_3R inhibitor, and a monoclonal type 1 IP_3R (IP_3R1) antibody blocked IP_3-induced BK_{Ca} channel activation. Adenophostin A, an IP_3R agonist, also activated BK_{Ca} channels. IP_3 activated BK_{Ca} channels in inside-out patches from wild-type ($IP_3R1^{+/+}$) mouse arterial smooth muscle cells, but had no effect on BK_{Ca} channels of IP_3R1-deficient ($IP_3R1^{-/-}$) mice. Immunofluorescence resonance energy transfer microscopy indicated that IP_3R1 is located in close spatial proximity to BK_{Ca} α subunits. The IP_3R1 monoclonal antibody coimmunoprecipitated IP_3R1 and BK_{Ca} channel α and β1 subunits from cerebral arteries. In summary, data indicate that IP_3R1 activation elevates BK_{Ca} channel apparent Ca^{2+} sensitivity through local molecular coupling in arterial smooth muscle cells.

INTRODUCTION

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) 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_{Ca} channel auxiliary subunit isoform (Brenner et al., 2000; Plüger et al., 2000). Arterial smooth muscle cell BK_{Ca} channel activation causes membrane hyperpolarization, leading to a reduction in voltage-dependent Ca^{2+} channel activity, a decrease in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and vasodilation (Davis and Hill, 1999; Jaggar et al., 2000). In contrast, BK_{Ca} channel inhibition causes membrane depolarization, which activates voltage-dependent Ca^{2+} channels, leading to an $[Ca^{2+}]_i$ elevation and vasoconstriction.

Global cytosolic $[Ca^{2+}]_i$ is typically 100–300 nM, whereas BK_{Ca} channels are sensitive to micromolar $[Ca^{2+}]_i$ (Jaggar et al., 2000; Pérez et al., 2001). In arterial smooth muscle cells, BK_{Ca} channels are activated by localized micromolar $[Ca^{2+}]_i$ transients termed Ca^{2+} sparks (Nelson et al., 1995; Jaggar et al., 2000). Ca^{2+} sparks are generated by the concerted opening of several SR ryanodine-sensitive Ca^{2+} release (RYR) channels (Nelson et al., 1995; Jaggar et al., 2000). Ca^{2+} spark–induced BK_{Ca} currents induce membrane hyperpolarization and vasodilation.

SR inositol 1,4,5-trisphosphate (IP_3)-gated Ca^{2+} 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_3 receptor (IP_3R) activation stimulates propagating intracellular Ca^{2+} waves and elevates global $[Ca^{2+}]_i$ (Lee et al., 2002; Lamont and Wier, 2004; Wilkerson et al., 2006; Zhao et al., 2008). Three different IP_3R 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_3Rs are the principal molecular and functional IP_3R isoform mediating agonist and IP_3-induced intracellular Ca^{2+} signals in aortic and cerebral artery smooth muscle cells (Zhao et al., 2008; Zhou et al., 2008). IP_3R2 also contributes to acetylcholine-induced

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Abbreviations used in this paper: BK_{Ca}, large-conductance Ca^{2+}-activated K^+; [Ca2+]$_i$, intracellular Ca^{2+} concentration; coIP, coimmunoprecipitation; immuno-FRET, immunofluorescence resonance energy transfer; IP_3, 1,4,5-trisphosphate; $[IP_3]_i$, intracellular IP_3 concentration; IP_3R, IP_3 receptor.

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 136 No. 3 283–291
www.jgp.org/cgi/doi/10.1085/jgp.201010453

Ca^{2+} oscillations in cultured portal vein smooth muscle cells (Morel et al., 2003). IP_3R1 activation stimulates propagating intracellular Ca^{2+} waves and causes an increase in global $[Ca^{2+}]_i$ (Zhao et al., 2008). IP_3R1 activation also stimulates a nonselective cation current (I_{Cat}) via an SR Ca^{2+} 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_3R1 to TRPC3 channels, is primarily due to Na^+ influx, and leads to membrane depolarization, voltage-dependent Ca^{2+} channel activation, and a global $[Ca^{2+}]_i$ elevation (Xi et al., 2008; Adebiyi et al., 2010). IP_3R1-mediated SR Ca^{2+} release and TRPC3 channel activation both elevate $[Ca^{2+}]_i$, leading to vasoconstriction (Xi et al., 2008; Zhao et al., 2008).

IP_3R-mediated SR Ca^{2+} release activates BK_{Ca} channels in basilar artery smooth muscle cells (Kim et al., 1998). Given that IP_3Rs directly activate nearby TRPC3 channels in arterial smooth muscle cells (Xi et al., 2008; Zhao et al., 2008; Adebiyi et al., 2010), we studied the mechanisms by which IP_3 and IP_3Rs modulate BK_{Ca} channels. We tested the hypothesis that IP_3 activates BK_{Ca} channels via an SR Ca^{2+} release–independent mechanism. Our data indicate that IP_3 activates BK_{Ca} channels both in intact cells and excised membrane patches where SR Ca^{2+} release cannot occur. IP_3 elevated BK_{Ca} channel apparent Ca^{2+} sensitivity, required IP_3R1 activation, and was absent in IP_3R1-deficient ($IP_3R1^{-/-}$) cells. Our data also indicate that IP_3R1 is located in close spatial proximity to BK_{Ca} channels and coimmunoprecipitates with BK_{Ca} channel α and β1 subunits. This study identifies a novel signaling mechanism whereby IP_3R1 activation stimulates nearby BK_{Ca} channels. Since IP_3Rs and BK_{Ca} 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 $IP_3R1^{-/-}$ mice (3 wk) were euthanized by intraperitoneal injection of 150 mg/kg sodium pentobarbital. Generation of the IP_3R1 knockout mice will be described in a future publication. In brief, exon 5 of IP_3R1 was flanked by two lox P sites, and IP_3R1-floxed mice were generated by homologous recombination. IP_3R1-floxed mice were subsequently crossed with protamine Cre mice (O'Gorman et al., 1997) to generate conventional IP_3R1 knockout mice in which exon 5 is deleted globally and no IP_3R1 protein can be detected (see Fig. 5 A). The phenotype of the global knockout IP_3R1 mice generated is the same as that previously published for global loss of function of IP_3R1 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_2, 5% CO_2), physiological saline solution containing (in mM): 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 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 (Cheranov and Jaggar, 2006). Mouse aorta was used only for Western blotting experiments.

Patch clamp electrophysiology

Single BK_{Ca} 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 $MgCl_2$, 5 EGTA, 1.6 HEDTA, and 10 µM free Ca^{2+}, pH 7.2 with KOH. For inside-out patches, the same pipette and bath solutions were used, except for experiments measuring BK_{Ca} channel Ca^{2+} sensitivity, where free Ca^{2+} concentration was adjusted to between 1 and 300 µM by the addition of $CaCl_2$ and free Mg^{2+} maintained at 1 mM by adjustment of $MgCl_2$. Where indicated, equimolar EGTA was substituted for BAPTA, a fast Ca^{2+} chelator, in both the pipette and bath solutions. Free Ca^{2+} concentration in solutions was measured using a Ca^{2+}-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_{Ca} 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_3R1 (NeuroMab), mouse monoclonal anti–BK_{Ca} α (NeuroMab), or rabbit polyclonal anti–BK_{Ca} 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_3R1 (clone L24/18; NeuroMab) and either rabbit polyclonal anti-BK_{Ca}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_3R1 (Jackson ImmunoResearch Laboratories, Inc.) and Cy2-conjugated goat anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.) for BK_{Ca}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

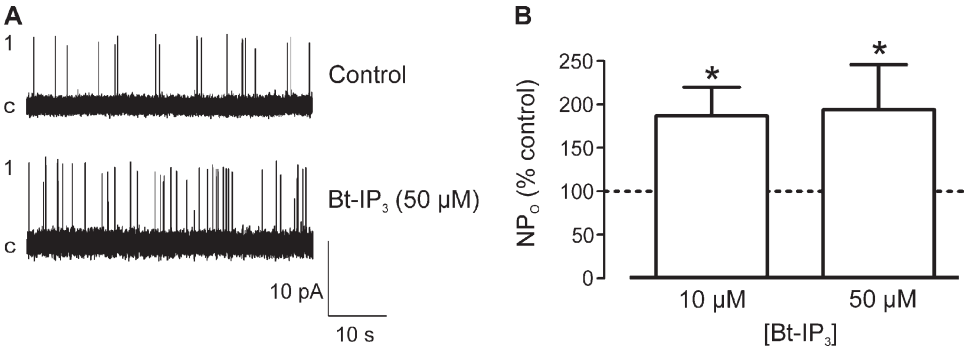

Figure 1. IP_3 activates BK_{Ca} channels in cerebral artery smooth muscle cells. (A) Original recordings obtained from the same cell illustrating activation of BK_{Ca} channel by Bt-IP_3 at +60 mV. (B) Mean data (*n*: control, 5; 10 μM Bt-IP_3, 5; 50 μM Bt-IP_3, 5). *, $P < 0.05$.

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_3R1 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_3R1, mouse monoclonal anti–BK_{Ca} α, or rabbit polyclonal anti–BK_{Ca} β1 primary antibodies, and horseradish peroxidase–conjugated secondary antibodies.

Statistical analysis

BK_{Ca} channel activity (NP_o) was calculated from continuous gap-free data using the following equation: $NP_o = \Sigma\ (t_1 + t_2 ... t_i)$, 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_o by channel number (N). The total number of channels in inside-out patches was determined by introducing 1 mM of free Ca^{2+} into the bath solution at the end of each experiment. BK_{Ca} channel IP_3 sensitivity data and relationships between BK_{Ca} channel open probability (P_o) and free Ca^{2+} concentration were fit with a Boltzmann function: $Y = P_o\text{min} + [(P_o\text{max} - P_o\text{min})/(1+\exp[(K_d\text{-}X)/\text{slope}])]$. 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_{Ca} 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_3 activates BK_{Ca} channels in cerebral artery smooth muscle cells

BK_{Ca} channel regulation by IP_3 was first measured in intact arterial smooth muscle cells using the cell-attached configuration of the patch clamp technique. Bt-IP_3, a membrane-permeant IP_3 analogue, at concentrations of 10 and 50 μM, increased mean BK_{Ca} channel activity

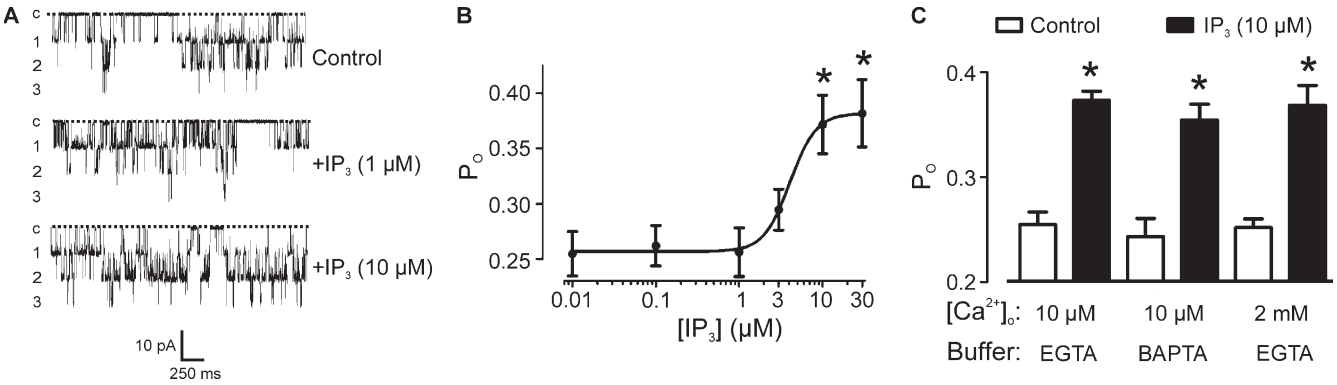

Figure 2. IP_3 activates BK_{Ca} channels in excised inside-out membrane patches. (A) Original recordings from the same inside-out patch illustrating concentration-dependent BK_{Ca} channel activation by IP_3 at −40 mV. (B) Mean data fit with a Boltzmann function. IP_3 increased BK_{Ca} channel P_o with an apparent K_d of 4.1 ± 1.3 μM, a slope of 2.8 ± 1.9, and a maximal P_o of 0.38 ± 0.02. Experimental numbers are ([IP_3]): 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^{2+} with BAPTA or elevating pipette (extracellular) Ca^{2+} from 10 μM to 2 mM does not alter IP_3 (10 μM)-induced BK_{Ca} channel activation (*n*: 10 μM $[Ca^{2+}]_o$/10 μM $[Ca^{2+}]_i$ (EGTA/EGTA); control, 4; IP_3, 4; 10 μM $[Ca^{2+}]_o$/10 μM $[Ca^{2+}]_i$ (BAPTA/BAPTA); control, 4; IP_3, 4; 2 mM $[Ca^{2+}]_o$/10 μM $[Ca^{2+}]_i$ (none/EGTA) control, 3; IP_3, 3). *, $P < 0.05$.

(NP_o) ~1.8- and 1.9-fold, respectively, at +60 mV (Fig. 1, A and B).

Next, we investigated whether IP_3 has effects on BK_{Ca} channels that are independent of plasma membrane Ca^{2+} influx or intracellular Ca^{2+} release. BK_{Ca} 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 $[Ca^{2+}]_i$, 10 µM IP_3 increased mean BK_{Ca} channel open probability (P_o) from ~0.24 to 0.37, or 1.54-fold (Fig. 2, A and B). In contrast, IP_3 did not alter single BK_{Ca} channel amplitude (pA: control, 8.5 ± 0.47; IP_3, 8.0 ± 0.36; $n = 5$; $P > 0.05$; Fig. 2 A). IP_3 also caused concentration-dependent BK_{Ca} channel activation (Fig. 2 B). Fitting an IP_3 concentration–response curve with a Boltzmann function indicated that IP_3 increased BK_{Ca} channel P_o with an apparent K_d of ~4.1 µM, a slope of ~2.8, and a maximal P_o of ~0.38 (Fig. 2 B). IP_3 activated BK_{Ca} channels in excised patches for as long as the seal could be maintained (>30 min), indicating that SR Ca^{2+} 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^{2+} chelator, did not alter IP_3-induced BK_{Ca} channel activation (Fig. 2 C). Elevating pipette free Ca^{2+} from 10 µM to 2 mM also did not alter IP_3-induced BK_{Ca} channel activation, indicating that plasma membrane Ca^{2+} influx was not responsible for channel activation (Fig. 2 C). Collectively, these data indicate that IP_3 activates BK_{Ca} channels via an SR Ca^{2+} release–independent mechanism.

IP_3 elevates BK_{Ca} channel apparent Ca^{2+} sensitivity

IP_3 regulation of BK_{Ca} channel apparent Ca^{2+} sensitivity was examined at a steady membrane voltage of −40 mV. In control, mean apparent K_d for Ca^{2+} was ~20 µM, with a slope of ~1.2, and a maximum P_o of ~0.82 (Fig. 3 A). In the same membrane patches, 10 µM IP_3 decreased the mean K_d for Ca^{2+} to ~12 µM, but did not alter the slope or the maximum P_o (Fig. 3 A). Relative activation by IP_3 increased considerably between 1 and 10 µM Ca^{2+} (Fig. 3 B). For example, with 1 µM of free Ca^{2+}, IP_3 increased mean BK_{Ca} channel P_o 1.14-fold, whereas with 10 µM of free Ca^{2+}, IP_3 increased mean P_o 1.48-fold (Fig. 3 B). At free $[Ca^{2+}]$ >10 µM, relative activation by IP_3 became smaller because channel activity was approaching maximal. These data suggest that IP_3 elevates BK_{Ca} channel apparent Ca^{2+} sensitivity in arterial smooth muscle cells.

IP_3R1 activation mediates IP_3-induced BK_{Ca} channel activation

To identify mechanisms by which IP_3 activates BK_{Ca} channels, we tested the hypothesis that IP_3Rs mediate IP_3-induced BK_{Ca} channel activation. In inside-out patches, 1 mg/ml heparin, an IP_3R blocker, reversed BK_{Ca} channel activation by IP_3 (Fig. 4 A). In contrast, 1 mg/ml heparin alone did not alter mean BK_{Ca} channel P_o (98.9 ± 12.5% of control; $n = 5$; $P > 0.05$). These data indicate that IP_3-induced IP_3R activation stimulates BK_{Ca} channels.

Type 1 IP_3Rs are the principal molecular isoform expressed in cerebral artery and aortic smooth muscle cells (Zhao et al., 2008; Zhou et al., 2008; Adebiyi et al., 2010). Therefore, we studied whether IP_3R1 mediates IP_3-induced BK_{Ca} channel activation. In inside-out patches, a monoclonal IP_3R1 antibody (1:100) reversed IP_3-induced BK_{Ca} channel activation (Fig. 4, B and C). In contrast, the IP_3R1 antibody (1:100) did not alter mean BK_{Ca} 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_3 plus IP_3R1 antibody did not cause any further reduction in BK_{Ca} channel P_o (Fig. 4, B and C). Boiled (95°C for 15 min) IP_3R1 antibody did not alter IP_3-induced BK_{Ca} channel activation (Fig. 4 C). To further examine BK_{Ca} channel regulation by IP_3Rs, we used adenophostin A as an alternate IP_3R agonist. 1 µM adenophostin A increased BK_{Ca} channel activity ~1.60-fold (Fig. 4 D). These data indicate that IP_3R1 activation is necessary for IP_3-mediated BK_{Ca} channel activation in arterial smooth muscle cells.

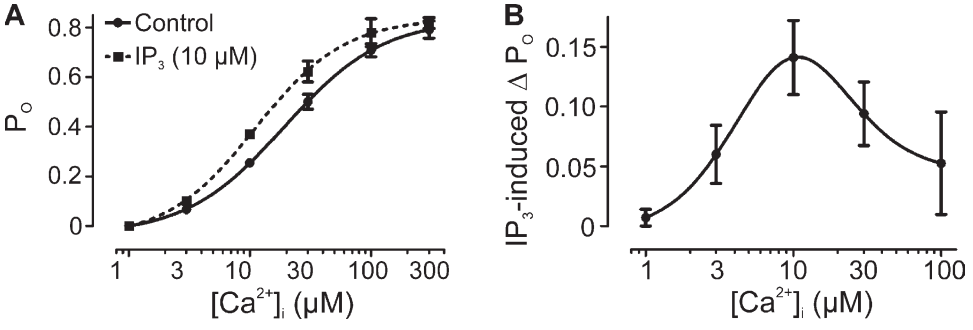

Figure 3. IP_3 elevates BK_{Ca} channel apparent Ca^{2+} sensitivity. (A) Mean data illustrating IP_3-induced BK_{Ca} channel activation over a range of free Ca^{2+} concentrations in inside-out patches at −40 mV. Data are fit with a Boltzmann function constrained to ≥0. Experimental numbers are given after each $[Ca^{2+}]$ (µM): control: 1, 8; 3, 6; 10, 6; 30, 6; 100, 8; 300, 8; 10 µM IP_3: 1, 7; 3, 6; 10, 6; 30, 6; 100, 7; 300, 7. (B) Mean data illustrating IP_3-induced change in BK_{Ca} channel P_o at each $[Ca^{2+}]$. Data are fit with a Gaussian function. In control, the K_d for Ca^{2+} was 20.4 ± 0.9 µM, a slope of 1.2 ± 0.2, and a maximum P_o of 0.82 ± 0.04. 10 µM IP_3 decreased the mean K_d for Ca^{2+} to 12.4 ± 0.8 µM ($P < 0.05$), but did not alter the slope (1.3 ± 0.3) or the maximum P_o (0.83 ± 0.03; $P > 0.05$ for each).

To further investigate the necessity for IP_3R1 and to determine whether IP_3-induced BK_{Ca} channel activation occurs in another species, we studied BK_{Ca} channel regulation in mouse wild-type ($IP_3R1^{+/+}$) and IP_3R1 knockout ($IP_3R1^{-/-}$) cerebral artery smooth muscle cells. Western blotting confirmed that IP_3R1 was present in $IP_3R1^{+/+}$ mouse aorta, but absent in $IP_3R1^{-/-}$ mouse aorta (Fig. 5 A). BK_{Ca} channel P_o ($IP_3R1^{+/+}$, 0.30 ± 0.08; $IP_3R1^{-/-}$, 0.27 ± 0.03; $n = 5$) and amplitude (pA: $IP_3R1^{+/+}$, 10.2 ± 0.8; $IP_3R1^{-/-}$, 11.4 ± 0.8; $n = 5$) were similar in inside-out patches from $IP_3R1^{+/+}$ and $IP_3R1^{-/-}$ cerebral artery smooth muscle cells exposed to 10 μM Ca^{2+} ($P > 0.05$ for each). IP_3 increased mean BK_{Ca} channel P_o to ~142% of control in inside-out patches from mouse $IP_3R1^{+/+}$ cells (Fig. 5 B). Furthermore, in patches from $IP_3R1^{+/+}$ cells, IP_3-induced BK_{Ca} channel activation was reversed by heparin (Fig. 5 B). In contrast, IP_3 or IP_3 plus heparin did not alter BK_{Ca} channel P_o in excised patches from $IP_3R1^{-/-}$ arterial smooth muscle cells (Fig. 5 B). These data indicate that IP_3R1 is essential for IP_3-induced BK_{Ca} channel activation in cerebral artery smooth muscle cells.

BK_{Ca} channel α subunits colocalize with IP_3R1

Our data indicate that IP_3R1 and BK_{Ca} 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_3R1 and BK_{Ca} 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_3R1 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_3R1 and BK_{Ca} channels was observed both at the plasma membrane and intracellularly. Antigenic peptides abolished fluorescent labeling by BK_{Ca} and TRPM4 channel antibodies, respectively (Fig. S1). An antigenic peptide was not available for the monoclonal IP_3R1 antibody, but this antibody detects only IP_3R1 protein in a Western blot, indicating selectivity (Adebiyi et al., 2010). These data indicate that IP_3R1 is located in close proximity to plasma membrane BK_{Ca} channels in arterial smooth muscle cells.

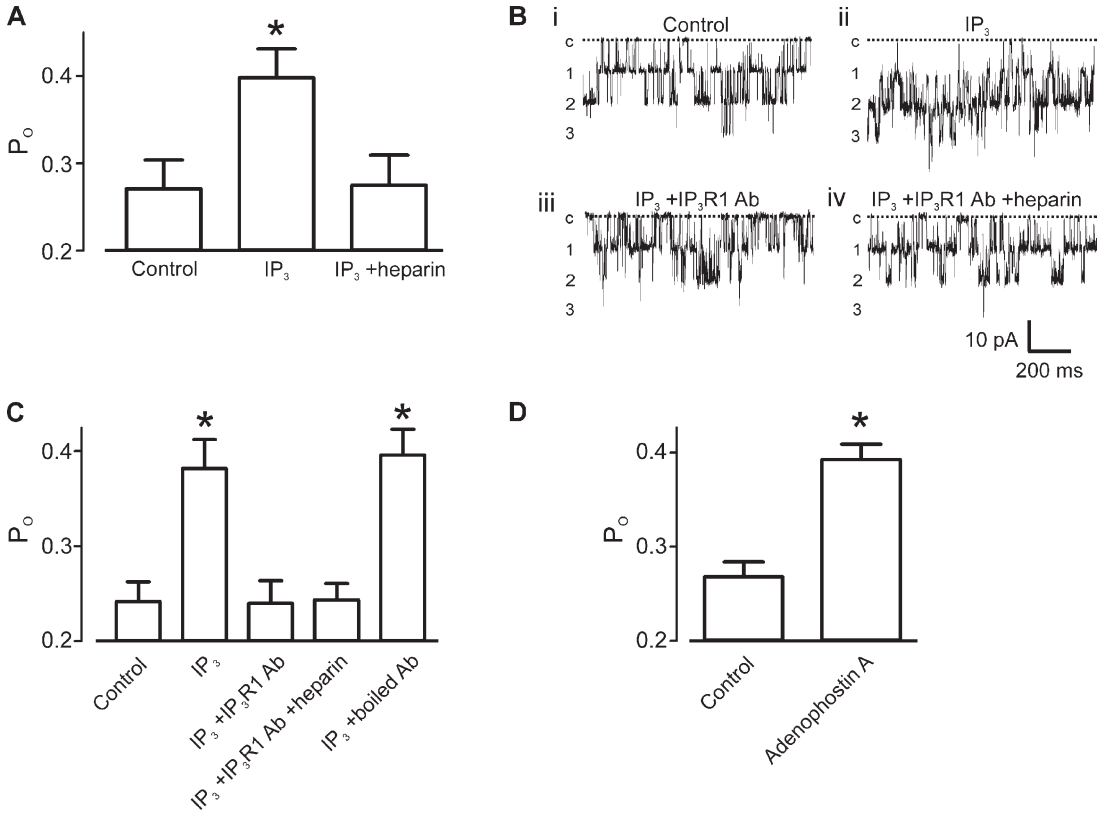

Figure 4. IP_3R1 mediates IP_3-induced BK_{Ca} channel activation. (A) Mean data illustrating that heparin reverses IP_3-induced BK_{Ca} channel activation (n: control (10 μM Ca^{2+}), 5; 30 μM IP_3, 5; 30 μM IP_3 plus 1 mg/ml heparin, 5). (B) Original recording from the same inside-out patch illustrating control BK_{Ca} channel activity (i), IP_3 (30 μM)-induced channel activation (ii), reversal by monoclonal IP_3R1 antibody (iii, 1:100; clone L24/18; NeuroMab), and no further effect of heparin applied in the continued presence of IP_3R1 antibody (iv). (C) Mean data illustrating that monoclonal IP_3R1 antibody inhibits IP_3-induced BK_{Ca} channel activation, and that the addition of heparin leads to no further change in activity (control (10 μM Ca^{2+}), $n = 4$; 30 μM IP_3, $n = 4$; 30 μM IP_3 plus IP_3R1 antibody (1:100), $n = 4$; 30 μM IP_3 plus IP_3R1 antibody (1:100), $n = 4$; 30 μM IP_3 plus IP_3R1 antibody (1:100) plus heparin, $n = 4$; IP_3 plus boiled IP_3R1 antibody, $n = 4$). (D) Mean data illustrating that adenophostin A activates BK_{Ca} channels (n: control (10 μM Ca^{2+}), 4; 1 μM adenophostin A, 4). *, $P < 0.05$.

BK_{Ca} channel α and β1 subunits coimmunoprecipitate with IP_3R1

coIP was performed to test the hypothesis that IP_3R1 and BK_{Ca} channels are contained within the same macromolecular protein complex. Due to the small size of the resistance (100–200-μm diameter), cerebral arteries used in this study, arteries collected from ∼15 rats, were required for each coIP experiment. The monoclonal IP_3R1 antibody coimmunoprecipitated IP_3R1 with BK_{Ca} channel α and β1 subunits from cerebral artery lysate (Fig. 6 B). These data indicate that IP_3R1 and BK_{Ca} channels are located within the same macromolecular complex in arterial smooth muscle cells.

DISCUSSION

Here, we demonstrate that IP_3-induced IP_3R1 activation stimulates BK_{Ca} channels via a local SR Ca^{2+} release–independent coupling mechanism in cerebral artery smooth muscle cells. Novel findings are that: (a) IP_3 activates BK_{Ca} channels in excised membrane patches removed from cytosolic signaling pathways; (b) IP_3 elevates BK_{Ca} channel apparent Ca^{2+} sensitivity; (c) IP_3R1 expression and activation are required for IP_3 to stimulate BK_{Ca} channels; and (d) IP_3R1 and BK_{Ca} channel subunits are located in close spatial proximity and coimmunoprecipitate. These data identify a novel signaling mechanism whereby IP_3R1 channels activate plasma membrane BK_{Ca} channels via an SR Ca^{2+} release–independent local coupling mechanism in arterial smooth muscle cells. Given that both IP_3Rs and K_{Ca} channels are widely expressed, such communication may occur in other cell types.

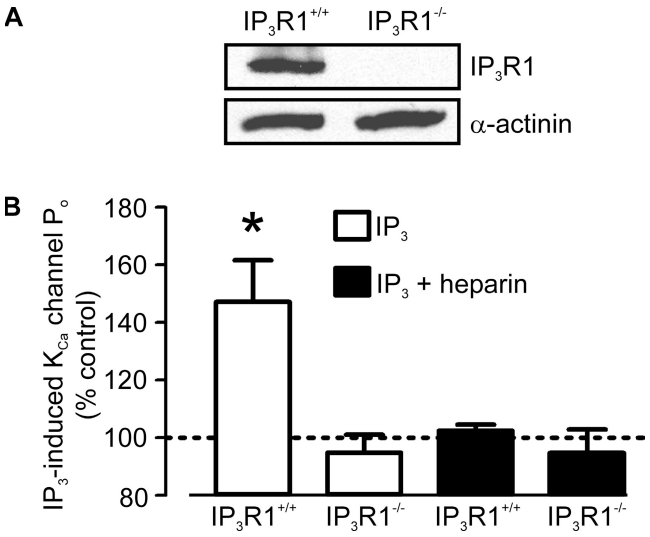

Figure 5. Genetic ablation of IP_3R1 abolishes IP_3-induced BK_{Ca} channel activation in cerebral artery smooth muscle cells. (A) Western blot indicating that IP_3R1 protein (∼270 kD) is present in $IP_3R1^{+/+}$ mouse aorta and absent in $IP_3R1^{-/-}$ aorta. (B) Mean data illustrating that 10 μM IP_3 activates BK_{Ca} channels in inside-out patches from mouse $IP_3R1^{+/+}$ cerebral artery smooth muscle cells, but not in mouse $IP_3R1^{-/-}$ cerebral artery smooth muscle cells. Mean data also indicate that 1 mg/ml heparin reverses IP_3-induced BK_{Ca} channel activation in $IP_3R1^{+/+}$ cells. Membrane voltage was −40 mV. $IP_3R1^{-/-}$: control, $n = 5$; IP_3, $n = 5$; IP_3 plus heparin, $n = 5$. $IP_3R1^{+/+}$: control, $n = 5$; IP_3, $n = 5$; IP_3 plus heparin, $n = 5$. *, $P < 0.05$.

Agonist binding to PLC-coupled receptors leads to phosphatidylinositol 4,5-bisphosphate cleavage and an elevation in both diacyglycerol and IP_3 in smooth muscle cells. Diacyglycerol 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_{Ca} 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 ∼−60 and −20 mV (Knot and Nelson, 1998; Davis and Hill, 1999). Here, BK_{Ca} 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_3 activated BK_{Ca} channels in both intact cells and in excised membrane patches. In excised patches, IP_3 increased BK_{Ca} channel activity with an apparent K_d of ∼4 μM. Previous studies have estimated global intracellular IP_3 concentration ($[IP_3]_i$) to be 0.1–3 μM in unstimulated cells and 1–20 μM 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_3 biosensors suggested that receptor agonists elevate global $[IP_3]_i$ to ∼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_3 gradients higher than global $[IP_3]_i$ should exist, particularly within the immediate vicinity of PLC where IP_3 is generated. The $[IP_3]_i$ nearby a target protein such as an IP_3R will depend on many factors, including PLC activity, proximity of IP_3Rs to PLC, and IP_3 metabolism. K_{Ca} channel IP_3 sensitivity determined here may indicate IP_3 concentrations that would occur nearby IP_3Rs that are located in close proximity to plasma membrane BK_{Ca} channels.

IP_3 acts primarily by relieving Ca^{2+} inhibition of IP_3Rs, thereby permitting Ca^{2+}-induced channel activation (Thrower et al., 2001; Foskett et al., 2007). Physiological micromolar $[Ca^{2+}]_i$ concentrations used here to study K_{Ca} channel activity would be expected to attenuate IP_3-induced IP_3R activation and may provide one explanation for the micromolar IP_3 sensitivity of BK_{Ca} channel activation. IP_3R IP_3 sensitivity can also vary widely from nanomolar to micromolar depending on multiple factors in addition to $[Ca^{2+}]_i$, including IP_3R isoform, splice variation, which can occur in many regions of the

protein including the IP_3-binding domain, and potentially through isoform heterotetramerization (Thrower et al., 2001; Foskett et al., 2007). For example, canine cerebellar IP_3R1 is sensitive to IP_3 over a broad concentration range and exhibits high (nM) and low (10 μM) affinity IP_3-binding sites. The IP_3 and Ca^{2+} sensitivity of arterial smooth muscle cell IP_3Rs has not been determined, nor have these channels been cloned. Therefore, deriving mechanistic details regarding IP_3R communication with K_{Ca} channels is difficult until detailed knowledge of smooth muscle cell IP_3Rs is available, particularly IP_3 and Ca^{2+} sensitivity. Our data indicate that under physiological voltage and local $[Ca^{2+}]_i$, micromolar $[IP_3]_i$ is required for IP_3Rs to activate BK_{Ca} channels.

Data indicate that IP_3 does not directly activate BK_{Ca} channels. Rather, IP_3R1 mediates IP_3-induced BK_{Ca} channel activation. This conclusion is supported by our observation that heparin, an IP_3R1 antibody, and IP_3R1 ablation all blocked IP_3-induced BK_{Ca} channel activation. These data also indicate that functional IP_3R1 protein is excised together with BK_{Ca} 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_3R1 must be located in very close spatial proximity to BK_{Ca} channels. Supporting local interaction between these proteins, coIP data indicated that IP_3R1 and BK_{Ca} channel α and β1 subunits were located in the same macromolecular complex. In contrast to FRET data indicating close spatial proximity of IP_3R1 and BK_{Ca} channels, immuno-FRET data indicated that IP_3R1 and TRPM4 channels are not spatially localized in arterial smooth muscle cells, in agreement with a recent report (Adebiyi et al., 2010). BK_{Ca} channels are also located nearby IP_3Rs in cultured glioma cells, but in contrast to the observations made here, glioma cell IP_3Rs activate BK_{Ca} channels via Ca^{2+} 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_3R1 and BK_{Ca} 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_3R1 and BK_{Ca} channel α subunits was observed both at the plasma membrane and intracellularly. The physiological function of close localization between IP_3R1 and intracellular BK_{Ca} channels is unclear. Conceivably, BK_{Ca} channels may be contained within the SR or Golgi before membrane trafficking, and intracellular FRET may reflect the close proximity of these intracellular BK_{Ca} channels to IP_3R1 located on the SR membrane. Alternatively, IP_3R1 and BK_{Ca} channels may form into a protein complex before membrane trafficking.

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

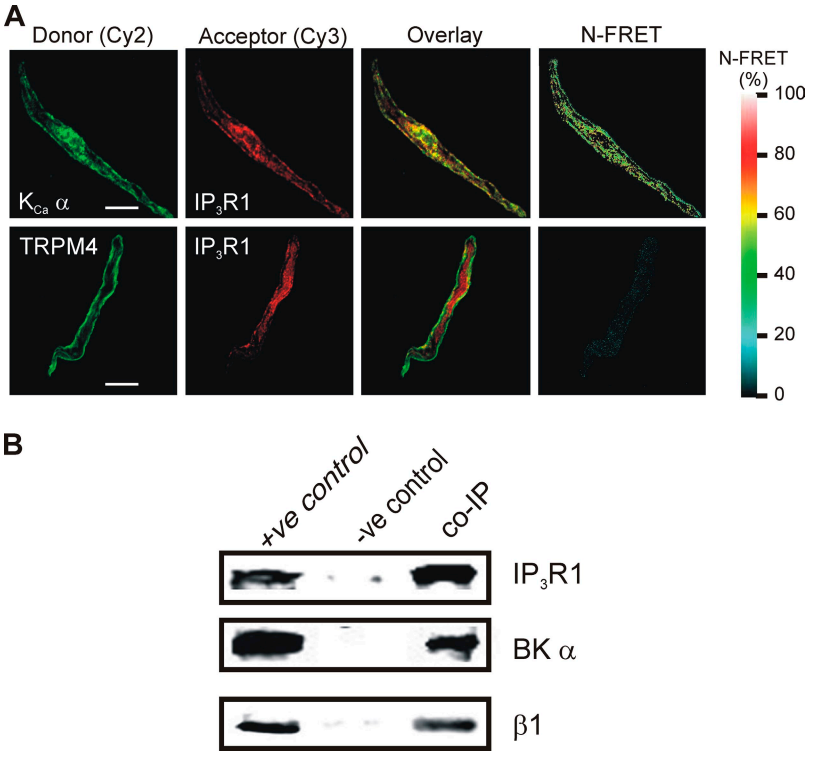

Figure 6. BK_{Ca} channel subunits are located in close spatial proximity to IP_3R1 in cerebral artery smooth muscle cells. (A) Immuno-FRET data illustrating close spatial proximity of IP_3R1 to BK_{Ca} channel α subunits. Cy2- and Cy3-labeled secondary antibodies bound to IP_3R1 and BK_{Ca} channels generate N-FRET. In contrast, secondary antibodies bound to IP_3R1 and TRPM4 do not generate significant N-FRET. Bars, 10 μm. (B) Monoclonal IP_3R1 antibody coimmunoprecipitates IP_3R1 (~270 kD), BK_{Ca} 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_3 activated BK_{Ca} channels in patches that had been excised for >30 min and in solutions that contained BAPTA, which would rapidly buffer any Ca^{2+} released by SR. Therefore, in inside-out patches studied here, IP_3R-mediated SR Ca^{2+} release cannot underlie IP_3-induced BK_{Ca} channel activation. Recently, we demonstrated that IP_3R1 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_3R1, TRPC3, and BK_{Ca} channels may coassemble within a macromolecular complex that regulates Ca^{2+} signaling, membrane potential, and arterial contractility. Conceivably, IP_3R-induced Ca^{2+} influx through plasma membrane TRPC3 channels could elevate submembrane $[Ca^{2+}]_i$ and activate nearby BK_{Ca} channels (Adebiyi et al., 2010). Several observations indicate that such a mechanism does not underlie IP_3-induced BK_{Ca} channel activation reported here: (a) the driving force for Ca^{2+} influx (symmetrical 10 µM Ca^{2+}) is weak and unlikely to sufficiently elevate $[Ca^{2+}]_i$ to activate BK_{Ca} channels; (b) BK_{Ca} channel activation occurred in the presence of BAPTA, which would rapidly buffer any entering Ca^{2+}; and (c) elevating pipette free $[Ca^{2+}]$ to 2 mM did not alter IP_3-induced BK_{Ca} channel activation. In intact cells with physiological Ca^{2+} buffers and cation gradients (i.e., 2 mM $[Ca^{2+}]_o$ and 100–300 nM $[Ca^{2+}]_i$), Ca^{2+} influx through TRPC3 channels may contribute to IP_3R1-induced BK_{Ca} channel activation. However, TRPC3 channels are also Na^+ permeant, and the larger driving force for Na^+ influx would limit Ca^{2+} influx, reducing any potential Ca^{2+} signal to BK_{Ca} channels.

BK_{Ca} channels exhibited a micromolar K_d for Ca^{2+} similar to that previously determined in rat cerebral artery smooth muscle cells at the same voltage (Pérez et al., 2001). IP_3 caused an elevation in BK_{Ca} channel apparent Ca^{2+} sensitivity with activation most prominent between 1 and 10 µM $[Ca^{2+}]_i$. These data indicate that IP_3 will increase BK_{Ca} channel sensitivity to local micromolar Ca^{2+} gradients, but will not shift BK_{Ca} channel Ca^{2+} sensitivity into the global nanomolar $[Ca^{2+}]_i$ range. Data here and in a previous study indicate that IP_3R1 activation both stimulates SR Ca^{2+} release and amplifies the sensitivity of nearby BK_{Ca} channels to micromolar $[Ca^{2+}]_i$ (Zhao et al., 2008). Therefore, the molecular coupling mechanism likely sensitizes BK_{Ca} channels to SR Ca^{2+} released from nearby IP_3Rs.

When considering the physiological function of the local coupling mechanism between IP_3R1 and BK_{Ca} channels, it is important to consider that PLC activation not only elevates IP_3, but also stimulates PKC. PKC inhibits arterial smooth muscle cell BK_{Ca} channels both by reducing Ca^{2+} spark frequency and by direct BK_{Ca} channel inhibition (Jaggar and Nelson, 2000; Jaggar et al., 2000). Thus, PKC reduces BK_{Ca} activation by RYR channels that generate Ca^{2+} sparks (Jaggar et al., 2000). However, vasoconstrictors also activate Ca^{2+} waves and elevate global $[Ca^{2+}]_i$ (Jaggar and Nelson, 2000; Mauban et al., 2001; Zhao et al., 2008; Adebiyi et al., 2010). IP_3R1 activation is essential for vasoconstrictor-induced Ca^{2+} waves and contributes to the global $[Ca^{2+}]_i$ elevation (Zhao et al., 2008). Collectively, these studies suggest that PLC-coupled receptor agonists shift control of BK_{Ca} channel activity from RYR channels to IP_3Rs. IP_3-induced BK_{Ca} channel activation would limit PKC-induced BK_{Ca} channel inhibition and attenuate the membrane depolarization and vasoconstriction.

The signaling mechanism by which IP_3R1 elevates BK_{Ca} channel apparent Ca^{2+} sensitivity was not studied here. Several possibilities exist, including that an IP_3-induced conformational change in IP_3R1 may lead to direct interaction with either the BK_{Ca} channel α and/or β1 subunit. IP_3R1 may also activate BK_{Ca} 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_3Rs stimulate BK_{Ca} channels will require further investigation.

In summary, data indicate that IP_3-induced IP_3R1 activation elevates BK_{Ca} channel apparent Ca^{2+} sensitivity through localized molecular coupling in arterial smooth muscle cells. This negative feedback mechanism would limit IP_3-induced vasoconstriction in cerebral arteries. These data also raise the possibility that local communication between IP_3Rs and BK_{Ca} channels may occur in a wide variety of cell types that express both of these proteins.

We thank Drs. Alejandro M. Dopico and Damodaran Narayanan for helpful comments on the manuscript.

This study was supported by grants from the National Institutes of Health (HL67061, HL077678, and HL094378 to J.H. Jaggar; HL096411 to A. Adebiyi; and HL080101 to J. Chen).

Lawrence G. Palmer served as editor.

Submitted: 16 April 2010
Accepted: 28 July 2010

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