

Type 1 IP₃ receptors activate BK_{Ca} channels via local molecular coupling in arterial smooth muscle cells

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Plasma membrane large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels and sarcoplasmic reticulum inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) are expressed in a wide variety of cell types, including arterial smooth muscle cells. Here, we studied BK_{Ca} channel regulation by IP₃ and IP₃Rs in rat and mouse cerebral artery smooth muscle cells. IP₃ activated BK_{Ca} channels both in intact cells and in excised inside-out membrane patches. IP₃ caused concentration-dependent BK_{Ca} channel activation with an apparent dissociation constant (K_d) of ~4 μ M at physiological voltage (−40 mV) and intracellular Ca²⁺ concentration ([Ca²⁺]_i; 10 μ M). IP₃ also caused a leftward-shift in BK_{Ca} channel apparent Ca²⁺ sensitivity and reduced the K_d for free [Ca²⁺]_i from ~20 to 12 μ M, but did not alter the slope or maximal P_o. BAPTA, a fast Ca²⁺ buffer, or an elevation in extracellular Ca²⁺ concentration did not alter IP₃-induced BK_{Ca} channel activation. Heparin, an IP₃R inhibitor, and a monoclonal type 1 IP₃R (IP₃R1) antibody blocked IP₃-induced BK_{Ca} channel activation. Adenophostin A, an IP₃R agonist, also activated BK_{Ca} channels. IP₃ activated BK_{Ca} channels in inside-out patches from wild-type (IP₃R1^{+/+}) mouse arterial smooth muscle cells, but had no effect on BK_{Ca} channels of IP₃R1-deficient (IP₃R1^{−/−}) mice. Immunofluorescence resonance energy transfer microscopy indicated that IP₃R1 is located in close spatial proximity to BK_{Ca} α subunits. The IP₃R1 monoclonal antibody coimmunoprecipitated IP₃R1 and BK_{Ca} channel α and β 1 subunits from cerebral arteries. In summary, data indicate that IP₃R1 activation elevates BK_{Ca} channel apparent Ca²⁺ sensitivity through local molecular coupling in arterial smooth muscle cells.

INTRODUCTION

Large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channels are expressed in a wide variety of cell types and consist of pore-forming α subunits and auxiliary β 1–4 subunits that modify channel function (Lu et al., 2006). In arterial smooth muscle cells, β 1 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 Ca²⁺ channel activity, a decrease in intracellular Ca²⁺ concentration ([Ca²⁺]_i), and vasodilation (Davis and Hill, 1999; Jaggar et al., 2000). In contrast, BK_{Ca} channel inhibition causes membrane depolarization, which activates voltage-dependent Ca²⁺ channels, leading to an [Ca²⁺]_i elevation and vasoconstriction.

Global cytosolic [Ca²⁺]_i is typically 100–300 nM, whereas BK_{Ca} channels are sensitive to micromolar [Ca²⁺]_i (Jaggar et al., 2000; Pérez et al., 2001). In arterial smooth muscle cells, BK_{Ca} channels are activated by localized micromolar

[Ca²⁺]_i transients termed Ca²⁺ sparks (Nelson et al., 1995; Jaggar et al., 2000). Ca²⁺ sparks are generated by the concerted opening of several SR ryanodine-sensitive Ca²⁺ release (RyR) channels (Nelson et al., 1995; Jaggar et al., 2000). Ca²⁺ spark-induced BK_{Ca} currents induce membrane hyperpolarization and vasodilation.

SR inositol 1,4,5-trisphosphate (IP₃)-gated Ca²⁺ 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, IP₃ receptor (IP₃R) activation stimulates propagating intracellular Ca²⁺ waves and elevates global [Ca²⁺]_i (Lee et al., 2002; Lamont and Wier, 2004; Wilkerson et al., 2006; Zhao et al., 2008). Three different IP₃R 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 IP₃Rs are the principal molecular and functional IP₃R isoform mediating agonist and IP₃-induced intracellular Ca²⁺ signals in aortic and cerebral artery smooth muscle cells (Zhao et al., 2008; Zhou et al., 2008). IP₃R2 also contributes to acetylcholine-induced

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Abbreviations used in this paper: BK_{Ca}, large-conductance Ca²⁺-activated K⁺; [Ca²⁺]_i, intracellular Ca²⁺ concentration; coIP, coimmunoprecipitation; immuno-FRET, immunofluorescence resonance energy transfer; IP₃, 1,4,5-trisphosphate; [IP₃]_i, intracellular IP₃ concentration; IP₃R, IP₃ receptor.

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Ca^{2+} oscillations in cultured portal vein smooth muscle cells (Morel et al., 2003). $\text{IP}_3\text{R1}$ activation stimulates propagating intracellular Ca^{2+} waves and causes an increase in global $[\text{Ca}^{2+}]_i$ (Zhao et al., 2008). $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}$ to TRPC3 channels, is primarily due to Na^+ influx, and leads to membrane depolarization, voltage-dependent Ca^{2+} channel activation, and a global $[\text{Ca}^{2+}]_i$ elevation (Xi et al., 2008; Adebisi et al., 2010). $\text{IP}_3\text{R1}$ -mediated SR Ca^{2+} release and TRPC3 channel activation both elevate $[\text{Ca}^{2+}]_i$, 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; Adebisi 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 $\text{IP}_3\text{R1}$ activation, and was absent in $\text{IP}_3\text{R1}$ -deficient ($\text{IP}_3\text{R1}^{-/-}$) cells. Our data also indicate that $\text{IP}_3\text{R1}$ is located in close spatial proximity to BK_{Ca} channels and coimmunoprecipitates with BK_{Ca} channel α and $\beta 1$ subunits. This study identifies a novel signaling mechanism whereby $\text{IP}_3\text{R1}$ 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 $\text{IP}_3\text{R1}^{-/-}$ mice (3 wk) were euthanized by intraperitoneal injection of 150 mg/kg sodium pentobarbital. Generation of the $\text{IP}_3\text{R1}$ knockout mice will be described in a future publication. In brief, exon 5 of $\text{IP}_3\text{R1}$ was flanked by two lox P sites, and $\text{IP}_3\text{R1}$ -floxed mice were generated by homologous recombination. $\text{IP}_3\text{R1}$ -floxed mice were subsequently crossed with protamine Cre mice (O’Gorman et al., 1997) to generate conventional $\text{IP}_3\text{R1}$ knockout mice in which exon 5 is deleted globally and no $\text{IP}_3\text{R1}$ protein can be detected (see Fig. 5A). The phenotype of the global knockout $\text{IP}_3\text{R1}$ mice generated is the same as that previously published for global loss of function of $\text{IP}_3\text{R1}$ 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_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 – $200\ \mu\text{m}$ 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 (Cherianov 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 $-40\ \text{mV}$, 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- $\text{IP}_3\text{R1}$ (NeuroMab), mouse monoclonal anti- $\text{BK}_{\text{Ca}}\ \alpha$ (NeuroMab), or rabbit polyclonal anti- $\text{BK}_{\text{Ca}}\ \beta 1$ (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- $\text{IP}_3\text{R1}$ (clone L24/18; NeuroMab) and either rabbit polyclonal anti- $\text{BK}_{\text{Ca}}\ \alpha$ (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 $\text{IP}_3\text{R1}$ (Jackson ImmunoResearch Laboratories, Inc.) and Cy2-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) for $\text{BK}_{\text{Ca}}\ \alpha$ 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 $\geq 560\ \text{nm}$, 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

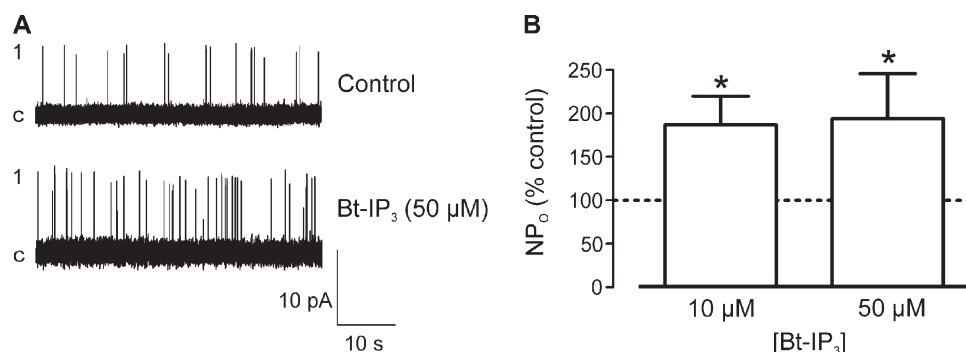


Figure 1. IP₃ activates BK_{Ca} channels in cerebral artery smooth muscle cells. (A) Original recordings obtained from the same cell illustrating activation of BK_{Ca} channel by Bt-IP₃ at +60 mV. (B) Mean data (*n*: control, 5; 10 μM Bt-IP₃, 5; 50 μM Bt-IP₃, 5). *, *P* < 0.05.

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 IP₃R1 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-IP₃R1, 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 (NP₀) was calculated from continuous gap-free data using the following equation: $NP_0 = \sum (t_1 + t_2 \dots t_i)$, where t_i is the relative open time (time open/total time) for each channel level. Open probability (P_o) was calculated by dividing NP₀ by channel number (*N*). The total number of channels in inside-out patches was determined by introducing 1 mM of free Ca²⁺ into the

bath solution at the end of each experiment. BK_{Ca} channel IP₃ sensitivity data and relationships between BK_{Ca} channel open probability (P_o) and free Ca²⁺ concentration were fit with a Boltzmann function: $Y = P_{o,min} + [(P_{o,max} - P_{o,min}) / (1 + \exp[(K_d - X) / \text{slope}])]$. 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

IP₃ activates BK_{Ca} channels in cerebral artery smooth muscle cells

BK_{Ca} channel regulation by IP₃ was first measured in intact arterial smooth muscle cells using the cell-attached configuration of the patch clamp technique. Bt-IP₃, a membrane-permeant IP₃ analogue, at concentrations of 10 and 50 μM, increased mean BK_{Ca} channel activity

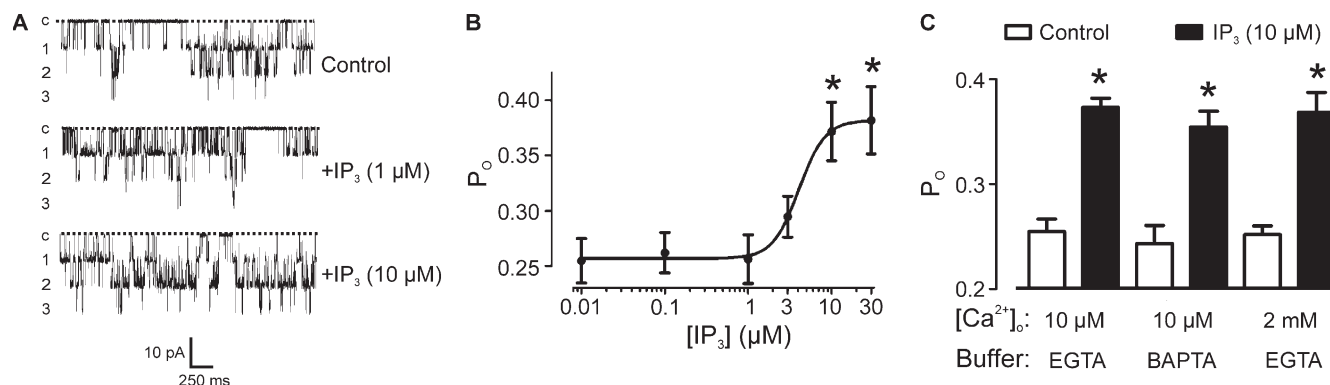


Figure 2. IP₃ activates BK_{Ca} channels in excised inside-out membrane patches. (A) Original recordings from the same inside-out patch illustrating concentration-dependent BK_{Ca} channel activation by IP₃ at -40 mV. (B) Mean data fit with a Boltzmann function. IP₃ increased BK_{Ca} channel P_o with 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 ([IP₃]): 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 Ca²⁺ with BAPTA or elevating pipette (extracellular) Ca²⁺ from 10 μM to 2 mM does not alter IP₃ (10 μM)-induced BK_{Ca} channel activation (*n*: 10 μM [Ca²⁺]_o/10 μM [Ca²⁺]_i (EGTA/EGTA); control, 4; IP₃, 4; 10 μM [Ca²⁺]_o/10 μM [Ca²⁺]_i (BAPTA/BAPTA); control, 4; IP₃, 4; 2 mM [Ca²⁺]_o/10 μM [Ca²⁺]_i (none/EGTA) control, 3; IP₃, 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 IP₃ has effects on BK_{Ca} channels that are independent of plasma membrane Ca²⁺ influx or intracellular Ca²⁺ 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 [Ca²⁺]_i, 10 μM IP₃ 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, IP₃ did not alter single BK_{Ca} channel amplitude (pA: control, 8.5 ± 0.47; IP₃, 8.0 ± 0.36; *n* = 5; *P* > 0.05; Fig. 2 A). IP₃ also caused concentration-dependent BK_{Ca} channel activation (Fig. 2 B). Fitting an IP₃ concentration-response curve with a Boltzmann function indicated that IP₃ 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). IP₃ activated BK_{Ca} channels in excised patches for as long as the seal could be maintained (>30 min), indicating that SR Ca²⁺ 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 Ca²⁺ chelator, did not alter IP₃-induced BK_{Ca} channel activation (Fig. 2 C). Elevating pipette free Ca²⁺ from 10 μM to 2 mM also did not alter IP₃-induced BK_{Ca} channel activation, indicating that plasma membrane Ca²⁺ influx was not responsible for channel activation (Fig. 2 C). Collectively, these data indicate that IP₃ activates BK_{Ca} channels via an SR Ca²⁺ release-independent mechanism.

IP₃ elevates BK_{Ca} channel apparent Ca²⁺ sensitivity

IP₃ regulation of BK_{Ca} channel apparent Ca²⁺ sensitivity was examined at a steady membrane voltage of -40 mV. In control, mean apparent *K*_d for Ca²⁺ 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 IP₃ decreased the mean *K*_d for Ca²⁺ to ~12 μM, but did not alter the slope or the maximum P_o (Fig. 3 A). Relative activation

by IP₃ increased considerably between 1 and 10 μM Ca²⁺ (Fig. 3 B). For example, with 1 μM of free Ca²⁺, IP₃ increased mean BK_{Ca} channel P_o 1.14-fold, whereas with 10 μM of free Ca²⁺, IP₃ increased mean P_o 1.48-fold (Fig. 3 B). At free [Ca²⁺] >10 μM, relative activation by IP₃ became smaller because channel activity was approaching maximal. These data suggest that IP₃ elevates BK_{Ca} channel apparent Ca²⁺ sensitivity in arterial smooth muscle cells.

IP₃R1 activation mediates IP₃-induced BK_{Ca} channel activation

To identify mechanisms by which IP₃ activates BK_{Ca} channels, we tested the hypothesis that IP₃Rs mediate IP₃-induced BK_{Ca} channel activation. In inside-out patches, 1 mg/ml heparin, an IP₃R blocker, reversed BK_{Ca} channel activation by IP₃ (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 IP₃-induced IP₃R activation stimulates BK_{Ca} channels.

Type 1 IP₃Rs 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 IP₃R1 mediates IP₃-induced BK_{Ca} channel activation. In inside-out patches, a monoclonal IP₃R1 antibody (1:100) reversed IP₃-induced BK_{Ca} channel activation (Fig. 4, B and C). In contrast, the IP₃R1 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 IP₃ plus IP₃R1 antibody did not cause any further reduction in BK_{Ca} channel P_o (Fig. 4, B and C). Boiled (95°C for 15 min) IP₃R1 antibody did not alter IP₃-induced BK_{Ca} channel activation (Fig. 4 C). To further examine BK_{Ca} channel regulation by IP₃Rs, we used adenophostin A as an alternate IP₃R agonist. 1 μM adenophostin A increased BK_{Ca} channel activity ~1.60-fold (Fig. 4 D). These data indicate that IP₃R1 activation is necessary for IP₃-mediated BK_{Ca} channel activation in arterial smooth muscle cells.

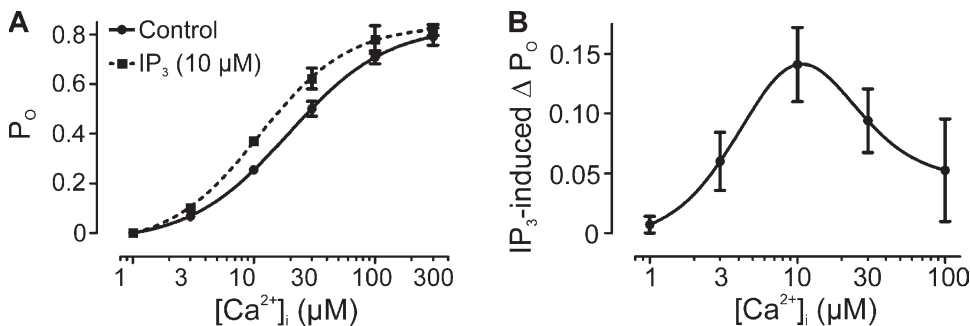


Figure 3. IP₃ elevates BK_{Ca} channel apparent Ca²⁺ sensitivity. (A) Mean data illustrating IP₃-induced BK_{Ca} channel activation over a range of free Ca²⁺ concentrations in inside-out patches at -40 mV. Data are fit with a Boltzmann function constrained to ≥0. Experimental numbers are given for each [Ca²⁺] (μM): control, 1, 8; 3, 6; 10, 6; 30, 6; 100, 8; 300, 8; 10 μM IP₃: 1, 7; 3, 6; 10, 6; 30, 6; 100, 7;

300, 7. (B) Mean data illustrating IP₃-induced change in BK_{Ca} channel P_o at each [Ca²⁺]. Data are fit with a Gaussian function. In control, the *K*_d for Ca²⁺ 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 IP₃ decreased the mean *K*_d for Ca²⁺ 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 IP₃R1 and to determine whether IP₃-induced BK_{Ca} channel activation occurs in another species, we studied BK_{Ca} channel regulation in mouse wild-type (IP₃R1^{+/+}) and IP₃R1 knockout (IP₃R1^{-/-}) cerebral artery smooth muscle cells. Western blotting confirmed that IP₃R1 was present in IP₃R1^{+/+} mouse aorta, but absent in IP₃R1^{-/-} mouse aorta (Fig. 5 A). BK_{Ca} channel P_o (IP₃R1^{+/+}, 0.30 ± 0.08; IP₃R1^{-/-}, 0.27 ± 0.03; *n* = 5) and amplitude (pA: IP₃R1^{+/+}, 10.2 ± 0.8; IP₃R1^{-/-}, 11.4 ± 0.8; *n* = 5) were similar in inside-out patches from IP₃R1^{+/+} and IP₃R1^{-/-} cerebral artery smooth muscle cells exposed to 10 μM Ca²⁺ (*P* > 0.05 for each). IP₃ increased mean BK_{Ca} channel P_o to ~142% of control in inside-out patches from mouse IP₃R1^{+/+} cells (Fig. 5 B). Furthermore, in patches from IP₃R1^{+/+} cells, IP₃-induced BK_{Ca} channel activation was reversed by heparin (Fig. 5 B). In contrast, IP₃ or IP₃ plus heparin did not alter BK_{Ca} channel P_o in excised patches from IP₃R1^{-/-} arterial smooth muscle cells (Fig. 5 B). These data indicate that IP₃R1 is essential for IP₃-induced BK_{Ca} channel activation in cerebral artery smooth muscle cells.

BK_{Ca} channel α subunits colocalize with IP₃R1

Our data indicate that IP₃R1 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 IP₃R1 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 IP₃R1 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 IP₃R1 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 IP₃R1 antibody, but this antibody detects only IP₃R1 protein in a Western blot, indicating selectivity (Adebiyi et al., 2010). These data indicate that IP₃R1 is located in close proximity to plasma membrane BK_{Ca} channels in arterial smooth muscle cells.

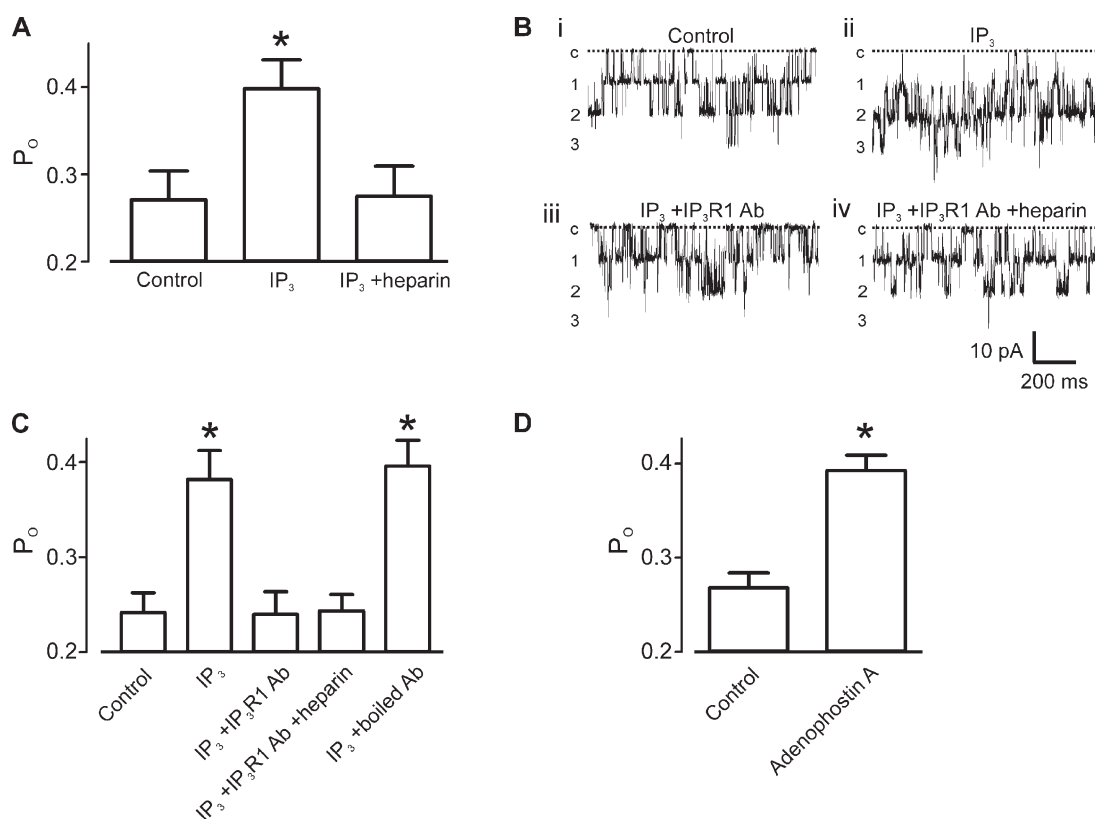


Figure 4. IP₃R1 mediates IP₃-induced BK_{Ca} channel activation. (A) Mean data illustrating that heparin reverses IP₃-induced BK_{Ca} channel activation (*n*: control (10 μM Ca²⁺), 5; 30 μM IP₃, 5; 30 μM IP₃ plus 1 mg/ml heparin, 5). (B) Original recording from the same inside-out patch illustrating control BK_{Ca} channel activity (i), IP₃ (30 μM)-induced channel activation (ii), reversal by monoclonal IP₃R1 antibody (iii, 1:100; clone L24/18; NeuroMab), and no further effect of heparin applied in the continued presence of IP₃R1 antibody (iv). (C) Mean data illustrating that monoclonal IP₃R1 antibody inhibits IP₃-induced BK_{Ca} channel activation, and that the addition of heparin leads to no further change in activity (control (10 μM Ca²⁺), *n* = 4; 30 μM IP₃, *n* = 4; 30 μM IP₃ plus IP₃R1 antibody (1:100), *n* = 4; 30 μM IP₃ plus IP₃R1 antibody (1:100) plus heparin, *n* = 4; IP₃ plus boiled IP₃R1 antibody, *n* = 4). (D) Mean data illustrating that adenophostin A activates BK_{Ca} channels (*n*: control (10 μM Ca²⁺), 4; 1 μM adenophostin A, 4). *, *P* < 0.05.

BK_{Ca} channel α and $\beta 1$ subunits coimmunoprecipitate with IP₃R1

coIP was performed to test the hypothesis that IP₃R1 and BK_{Ca} channels are contained within the same macromolecular protein complex. Due to the small size of the resistance (100–200- μ m diameter), cerebral arteries used in this study, arteries collected from ~ 15 rats, were required for each coIP experiment. The monoclonal IP₃R1 antibody coimmunoprecipitated IP₃R1 with BK_{Ca} channel α and $\beta 1$ subunits from cerebral artery lysate (Fig. 6 B). These data indicate that IP₃R1 and BK_{Ca} channels are located within the same macromolecular complex in arterial smooth muscle cells.

DISCUSSION

Here, we demonstrate that IP₃-induced IP₃R1 activation stimulates BK_{Ca} channels via a local SR Ca²⁺ release-independent coupling mechanism in cerebral artery smooth muscle cells. Novel findings are that: (a) IP₃ activates BK_{Ca} channels in excised membrane patches removed from cytosolic signaling pathways; (b) IP₃ elevates BK_{Ca} channel apparent Ca²⁺ sensitivity; (c) IP₃R1 expression and activation are required for IP₃ to stimulate BK_{Ca} channels; and (d) IP₃R1 and BK_{Ca} channel subunits are located in close spatial proximity and coimmunoprecipitate. These data identify a novel signaling

mechanism whereby IP₃R1 channels activate plasma membrane BK_{Ca} channels via an SR Ca²⁺ release-independent local coupling mechanism in arterial smooth muscle cells. Given that both IP₃Rs and K_{Ca} 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 IP₃ 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_{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). IP₃ activated BK_{Ca} channels in both intact cells and in excised membrane patches. In excised patches, IP₃ increased BK_{Ca} channel activity with an apparent K_d of ~ 4 μ M. Previous studies have estimated global intracellular IP₃ concentration ($[IP_3]_i$) to be 0.1–3 μ M in unstimulated cells and 1–20 μ 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 IP₃ 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 IP₃ gradients higher than global $[IP_3]_i$ should exist, particularly within the immediate vicinity of PLC where IP₃ is generated. The $[IP_3]_i$ nearby a target protein such as an IP₃R will depend on many factors, including PLC activity, proximity of IP₃Rs to PLC, and IP₃ metabolism. K_{Ca} channel IP₃ sensitivity determined here may indicate IP₃ concentrations that would occur nearby IP₃Rs that are located in close proximity to plasma membrane BK_{Ca} channels.

IP₃ acts primarily by relieving Ca²⁺ inhibition of IP₃Rs, thereby permitting Ca²⁺-induced channel activation (Thrower et al., 2001; Foscett et al., 2007). Physiological micromolar $[Ca^{2+}]_i$ concentrations used here to study K_{Ca} channel activity would be expected to attenuate IP₃-induced IP₃R activation and may provide one explanation for the micromolar IP₃ sensitivity of BK_{Ca} channel activation. IP₃R IP₃ sensitivity can also vary widely from nanomolar to micromolar depending on multiple factors in addition to $[Ca^{2+}]_i$, including IP₃R isoform, splice variation, which can occur in many regions of the

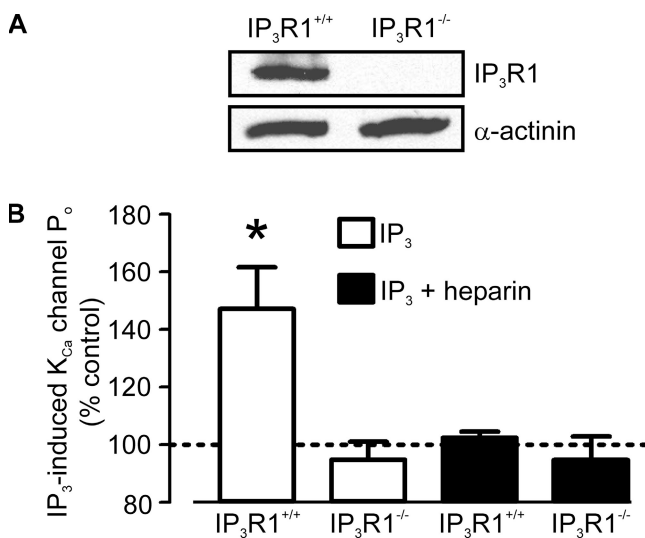


Figure 5. Genetic ablation of IP₃R1 abolishes IP₃-induced BK_{Ca} channel activation in cerebral artery smooth muscle cells. (A) Western blot indicating that IP₃R1 protein (~ 270 kD) is present in IP₃R1^{+/+} mouse aorta and absent in IP₃R1^{-/-} aorta. (B) Mean data illustrating that 10 μ M IP₃ activates BK_{Ca} channels in inside-out patches from mouse IP₃R1^{+/+} cerebral artery smooth muscle cells, but not in mouse IP₃R1^{-/-} cerebral artery smooth muscle cells. Mean data also indicate that 1 mg/ml heparin reverses IP₃-induced BK_{Ca} channel activation in IP₃R1^{+/+} cells. Membrane voltage was -40 mV. IP₃R1^{-/-}: control, $n = 5$; IP₃, $n = 5$; IP₃ plus heparin, $n = 5$. IP₃R1^{+/+}: control, $n = 5$; IP₃, $n = 5$; IP₃ plus heparin, $n = 5$. *, $P < 0.05$.

protein including the IP₃-binding domain, and potentially through isoform heterotetramerization (Thrower et al., 2001; Foskett et al., 2007). For example, canine cerebellar IP₃R1 is sensitive to IP₃ over a broad concentration range and exhibits high (nM) and low (10 μ M) affinity IP₃-binding sites. The IP₃ and Ca²⁺ sensitivity of arterial smooth muscle cell IP₃Rs has not been determined, nor have these channels been cloned. Therefore, deriving mechanistic details regarding IP₃R communication with K_{Ca} channels is difficult until detailed knowledge of smooth muscle cell IP₃Rs is available, particularly IP₃ and Ca²⁺ sensitivity. Our data indicate that under physiological voltage and local [Ca²⁺]_i, micromolar [IP₃]_i is required for IP₃Rs to activate BK_{Ca} channels.

Data indicate that IP₃ does not directly activate BK_{Ca} channels. Rather, IP₃R1 mediates IP₃-induced BK_{Ca} channel activation. This conclusion is supported by our observation that heparin, an IP₃R1 antibody, and IP₃R1 ablation all blocked IP₃-induced BK_{Ca} channel activation. These data also indicate that functional IP₃R1 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, IP₃R1 must be located in very close spatial proximity to BK_{Ca} channels. Supporting local interaction between these proteins, coIP data indicated that IP₃R1 and BK_{Ca} channel α and β 1 subunits were located in the same

macromolecular complex. In contrast to FRET data indicating close spatial proximity of IP₃R1 and BK_{Ca} channels, immuno-FRET data indicated that IP₃R1 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 IP₃Rs in cultured glioma cells, but in contrast to the observations made here, glioma cell IP₃Rs activate BK_{Ca} channels via Ca²⁺ 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 (\sim 20 nm) in arterial smooth muscle cells (Devine et al., 1972). Conceivably, IP₃R1 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 IP₃R1 and BK_{Ca} channel α subunits was observed both at the plasma membrane and intracellularly. The physiological function of close localization between IP₃R1 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 IP₃R1 located on the SR membrane. Alternatively, IP₃R1 and BK_{Ca} channels may form into a protein complex before membrane trafficking.

Intact SR may have been excised within membrane patches, although SR Ca²⁺ would be depleted due to the recording conditions used (no ATP in bath solution).

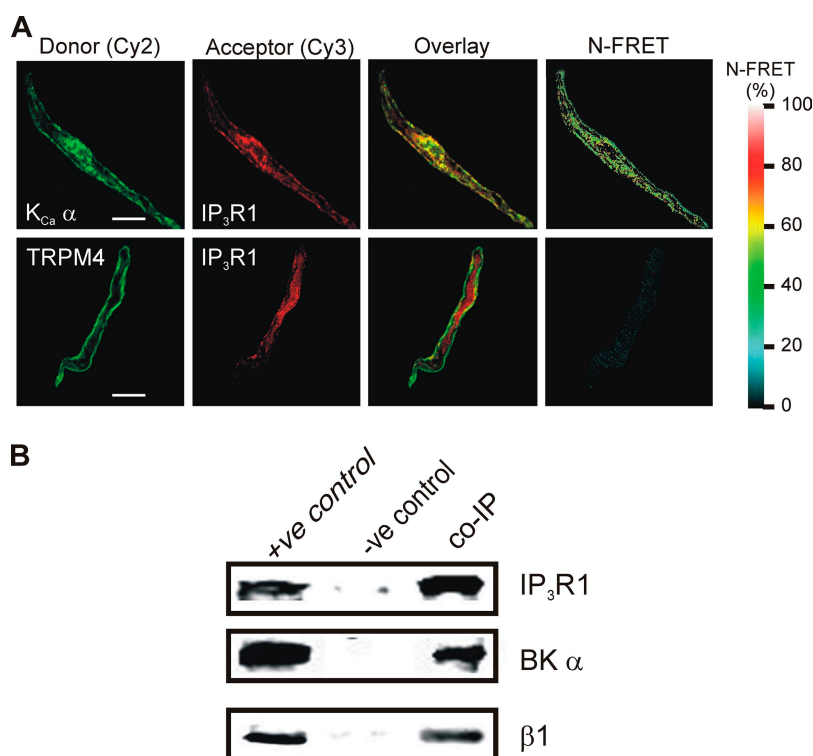


Figure 6. BK_{Ca} channel subunits are located in close spatial proximity to IP₃R1 in cerebral artery smooth muscle cells. (A) Immuno-FRET data illustrating close spatial proximity of IP₃R1 to BK_{Ca} channel α subunits. Cy2- and Cy3-labeled secondary antibodies bound to IP₃R1 and BK_{Ca} channels generate N-FRET. In contrast, secondary antibodies bound to IP₃R1 and TRPM4 do not generate significant N-FRET. Bars, 10 μ m. (B) Monoclonal IP₃R1 antibody coimmunoprecipitates IP₃R1 (\sim 270 kD), BK_{Ca} channel α (\sim 125 kD), and β 1 (\sim 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, IP₃ activated BK_{Ca} channels in patches that had been excised for >30 min and in solutions that contained BAPTA, which would rapidly buffer any Ca²⁺ released by SR. Therefore, in inside-out patches studied here, IP₃R-mediated SR Ca²⁺ release cannot underlie IP₃-induced BK_{Ca} channel activation. Recently, we demonstrated that IP₃R1 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, IP₃R1, TRPC3, and BK_{Ca} channels may coassemble within a macromolecular complex that regulates Ca²⁺ signaling, membrane potential, and arterial contractility. Conceivably, IP₃R-induced Ca²⁺ influx through plasma membrane TRPC3 channels could elevate submembrane [Ca²⁺]_i and activate nearby BK_{Ca} channels (Adebiyi et al., 2010). Several observations indicate that such a mechanism does not underlie IP₃-induced BK_{Ca} channel activation reported here: (a) the driving force for Ca²⁺ influx (symmetrical 10 μM Ca²⁺) is weak and unlikely to sufficiently elevate [Ca²⁺]_i to activate BK_{Ca} channels; (b) BK_{Ca} channel activation occurred in the presence of BAPTA, which would rapidly buffer any entering Ca²⁺; and (c) elevating pipette free [Ca²⁺] to 2 mM did not alter IP₃-induced BK_{Ca} channel activation. In intact cells with physiological Ca²⁺ buffers and cation gradients (i.e., 2 mM [Ca²⁺]_o and 100–300 nM [Ca²⁺]_i), Ca²⁺ influx through TRPC3 channels may contribute to IP₃R1-induced BK_{Ca} channel activation. However, TRPC3 channels are also Na⁺ permeant, and the larger driving force for Na⁺ influx would limit Ca²⁺ influx, reducing any potential Ca²⁺ signal to BK_{Ca} channels.

BK_{Ca} channels exhibited a micromolar K_d for Ca²⁺ similar to that previously determined in rat cerebral artery smooth muscle cells at the same voltage (Pérez et al., 2001). IP₃ caused an elevation in BK_{Ca} channel apparent Ca²⁺ sensitivity with activation most prominent between 1 and 10 μM [Ca²⁺]_i. These data indicate that IP₃ will increase BK_{Ca} channel sensitivity to local micromolar Ca²⁺ gradients, but will not shift BK_{Ca} channel Ca²⁺ sensitivity into the global nanomolar [Ca²⁺]_i range. Data here and in a previous study indicate that IP₃R1 activation both stimulates SR Ca²⁺ release and amplifies the sensitivity of nearby BK_{Ca} channels to micromolar [Ca²⁺]_i (Zhao et al., 2008). Therefore, the molecular coupling mechanism likely sensitizes BK_{Ca} channels to SR Ca²⁺ released from nearby IP₃Rs.

When considering the physiological function of the local coupling mechanism between IP₃R1 and BK_{Ca} channels, it is important to consider that PLC activation not only elevates IP₃, but also stimulates PKC. PKC inhibits arterial smooth muscle cell BK_{Ca} channels both by reducing Ca²⁺ 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 Ca²⁺ sparks (Jaggar et al., 2000). However,

vasoconstrictors also activate Ca²⁺ waves and elevate global [Ca²⁺]_i (Jaggar and Nelson, 2000; Mauban et al., 2001; Zhao et al., 2008; Adebiyi et al., 2010). IP₃R1 activation is essential for vasoconstrictor-induced Ca²⁺ waves and contributes to the global [Ca²⁺]_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 IP₃Rs. IP₃-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 IP₃R1 elevates BK_{Ca} channel apparent Ca²⁺ sensitivity was not studied here. Several possibilities exist, including that an IP₃-induced conformational change in IP₃R1 may lead to direct interaction with either the BK_{Ca} channel α and/or β1 subunit. IP₃R1 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 IP₃Rs stimulate BK_{Ca} channels will require further investigation.

In summary, data indicate that IP₃-induced IP₃R1 activation elevates BK_{Ca} channel apparent Ca²⁺ sensitivity through localized molecular coupling in arterial smooth muscle cells. This negative feedback mechanism would limit IP₃-induced vasoconstriction in cerebral arteries. These data also raise the possibility that local communication between IP₃Rs and BK_{Ca} channels may occur in a wide variety of cell types that express both of these proteins.

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