Type 1 inositol 1,4,5-trisphosphate receptors mediate UTP-induced cation currents, Ca\(^{2+}\) signals, and vasoconstriction in cerebral arteries

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Zhao G, Adebiyi A, Blaskova E, Xi Q, Jaggar JH. Type 1 inositol 1,4,5-trisphosphate receptors mediate UTP-activated cation currents, Ca\(^{2+}\) signals, and vasocostriction in cerebral arteries. Am J Physiol Cell Physiol 295: C1376–C1384, 2008. First published September 17, 2008; doi:10.1152/ajpcell.00362.2008. —Inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) regulate diverse physiological functions, including contraction and proliferation. There are three IP\(_3\)R isoforms, but their functional significance in arterial smooth muscle cells is unclear. Here, we investigated relative expression and physiological functions of IP\(_3\)R isoforms in cerebral artery smooth muscle cells. We show that 2-aminoethoxydiphenyl borate and xestospongin C, membrane-permeant IP\(_3\)R blockers, reduced Ca\(^{2+}\) wave activation and global intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) elevation stimulated by UTP, a phospholipase C-coupled purinergic receptor agonist. Quantitative PCR, Western blotting, and immunofluorescence indicated that all three IP\(_3\)R isoforms were expressed in acutely isolated cerebral artery smooth muscle cells, with IP\(_3\)R1 being the most abundant isoform at 82% of total IP\(_3\)R message. IP\(_3\)R1 knockdown with short hairpin RNA (shRNA) did not alter baseline Ca\(^{2+}\) wave frequency and global [Ca\(^{2+}\)]\(_i\), but abolished UTP-induced Ca\(^{2+}\) wave activation and reduced the UTP-induced global [Ca\(^{2+}\)]\(_i\), elevation by \(\sim 61\%\). Antibodies targeting IP\(_3\)R1 and IP\(_3\)R1 knockdown reduced UTP-induced nonselective cation current (\(I_{\text{cat}}\)) activation. IP\(_3\)R1 knockdown also reduced UTP-induced vasoconstriction in pressurized arteries with both intact and depleted sarcoplasmic reticulum (SR) Ca\(^{2+}\) \(\sim 45\%\). These data indicate that IP\(_3\)R1 is the predominant IP\(_3\)R isoform expressed in rat cerebral artery smooth muscle cells. IP\(_3\)R1 stimulation contributes to UTP-induced \(I_{\text{cat}}\) activation. Ca\(^{2+}\) wave generation, global [Ca\(^{2+}\)]\(_i\), elevation, and vasocostriction. In addition, IP\(_3\)R1 activation constrains cerebral arteries in the absence of SR Ca\(^{2+}\) release by stimulating plasma membrane \(I_{\text{cat}}\). Cerebral artery smooth muscle cells; calcium wave; short hairpin RNA

Inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)Rs) are expressed in many cell types and regulate several physiological functions, including development, muscle contraction, cell proliferation, and differentiation (1, 45, 47, 56). In vascular smooth muscle cells, IP\(_3\)Rs modulate vasocostriction and proliferation by altering spatial and temporal properties of local and global Ca\(^{2+}\) signals (51, 53). Vasocostricitors, including norepinephrine, phenylephrine, UTP, and vasopressin, stimulate Ca\(^{2+}\) waves and Ca\(^{2+}\) oscillations in smooth muscle cells of portal vein, rat tail, mesenteric and cerebral arteries, and cultured A7r5 smooth muscle cells (5, 7, 21, 23, 29). Vasocostricitors also elevate arterial smooth muscle cell global intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and induce vasocostriction by stimulating IP\(_3\)Rs (7, 43, 54). IP\(_3\) also activates cation channels in vascular smooth muscle cells through mechanisms that do not require the stimulation of sarcoplasmic reticulum (SR) Ca\(^{2+}\) release (27, 54). In cerebral artery smooth muscle cells, IP\(_3\)R activation stimulates TRPC3 channels, leading to Na\(^+\) influx, membrane depolarization, voltage-dependent Ca\(^{2+}\) channel activation, and vasocostriction (54). Thus IP\(_3\)Rs regulate ion channel activity, Ca\(^{2+}\) signals, and physiological functions in the vasculature. However, despite the functional importance of IP\(_3\)Rs, specific IP\(_3\)R isoforms that are expressed in this cell type and their functional significance are poorly understood.

Three IP\(_3\)R isoforms, designated IP\(_3\)R1, IP\(_3\)R2, and IP\(_3\)R3, are encoded by distinct genes (6, 37). IP\(_3\)R isoform expression varies widely between different cell types. Cerebellar Purkinje neurons express predominantly IP\(_3\)R1, pancreatic β-cells primarily express IP\(_3\)R3, and cardiac myocytes express IP\(_3\)R2 (14, 48). All three IP\(_3\)R isoforms are expressed in whole aorta, mesenteric and basilar arteries, and cultured aortic smooth muscle cells (17, 56). In primary cultured portal vein smooth muscle cells, IP\(_3\)R1 and IP\(_3\)R2 were detected by immunofluorescence, whereas IP\(_3\)R3 was absent (32). In contrast, in primary cultured rat ureter smooth muscle cells and cultured A7r5 cells, IP\(_3\)R1 and IP\(_3\)R3 were expressed and IP\(_3\)R2 was absent (32, 51).

IP\(_3\)R isoforms may also regulate vascular smooth muscle Ca\(^{2+}\) signaling in a cell-specific manner. IP\(_3\)R2 activation contributes to ACh-induced Ca\(^{2+}\) oscillations in cultured portal vein smooth muscle cells (32). In contrast, IP\(_3\)R1 was required for ACh-induced Ca\(^{2+}\) elevations in cultured portal vein smooth muscle cells (32), ATP-induced Ca\(^{2+}\) transients in cultured aortic smooth muscle cells (56), and vasopressin-evoked Ca\(^{2+}\) release and capacitative Ca\(^{2+}\) entry in cultured A7r5 smooth muscle cells (51). These studies suggest that IP\(_3\)R isoform expression may differ between smooth muscle cell types, leading to tissue-specific regulation of intracellular Ca\(^{2+}\) signaling.

The major goal of this study was to determine the functional significance of IP\(_3\)R isoforms that are expressed in smooth muscle cells of resistance-size cerebral arteries. We demonstrate that all three IP\(_3\)R isoforms are expressed in rat cerebral artery smooth muscle cells and that IP\(_3\)R1 is, far the most abundant. We also show that IP\(_3\)R1 suppression using antibodies and short hairpin RNA (shRNA) reduces UTP-induced elevations in Ca\(^{2+}\) wave frequency and global [Ca\(^{2+}\)]\(_i\), cation current (\(I_{\text{cat}}\)) activation, and vasocostriction. These data identify IP\(_3\)R1 as the principal molecular and functional IP\(_3\)R isoform in cerebral artery smooth muscle cells.

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MATERIALS AND METHODS

Tissue preparation. Animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Sprague-Dawley rats (200–250 g body wt) were euthanized with pentobarbital sodium (150 mg/kg). The brain was removed and placed into ice-cold (4°C) oxygenated (21% O₂, 5% CO₂, 74% N₂) physiological saline solution (PSS) containing (in mM) 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, and 11 glucose (with pH adjusted to 7.4 with NaOH). Posterior cerebral, middle cerebral, and cerebellar arteries (~50–200 μm diameter) were removed and cleaned of connective tissue. When appropriate, endothelium was removed by introducing an air bubble into the arterial lumen for 2 min, followed by a 30-s wash with PSS. Cerebral artery smooth muscle cells were isolated as previously described (10).

RT-PCR and real-time PCR. IP₃R fragments were amplified by nested PCR using the Tag DNA polymerase kit (Invitrogen). Reactions were run at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s for 35 cycles each, with the following primers: CACCCAAAGAA-GAGGCTGGTCC (forward, R1), CTGGTCCATCGTCTTTGACCTG (forward, R2), and GTCAGGAACTGGCAGATGGCAGGA (reverse) for IP₃R1; CCTGAGATGAGCCTGGCTGAGGTTCAA (forward) and TGTTGCAGGGCAGTAATCTC (reverse) for IP₃R3; and ATCCTGTGGCATTCCATGAAACTAC (forward) and GCCACAGATGAAGCAGGTTGTC (R2) for IP₃R2; and ATCCTGGAGAGCTTGAC (forward) and GTCAGGAACTGGCAGATGCGAG (reverse) for IP₃R3. PCR products were separated on 2% agarose gels, and bands were visualized by staining with ethidium bromide.

For real-time PCR, IP₃R isoform-specific primers were as follows: GAGATGAGGCTGGTGGTCA (forward) and TTGTGCCCTCTCCCTGACATGGCA (reverse) for IP₃R1, CAACAGACGCTGGAGAAGCTTGAC (forward) and GTCCAGAGACTGGCAGATGGCAGGT (reverse) for IP₃R2, AGACGCGCTGGCTACTATGGAGAA (forward), GTCAGAGACTGGCAGTGCCGACGT (reverse) for IP₃R3, and ATCCCTGTGGCATTCCCTGATAGAAC (forward) and AGAGGACGGGAGCAGTAAATTC (reverse) for β-actin. Each PCR mixture (25 μl) contained 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 400 nM primer, and an equal volume of cDNA template. Reactions were performed in triplicate on a sequence detection system (Prism 7700, Applied Biosystems) at 95°C for 10 min and 40 cycles of 20 s at 95°C, 20 s at 65°C, and 45 s at 72°C. A negative control, with smooth muscle cell RNA, instead of cDNA, was carried out in each experiment. The integrity of each PCR was verified by using dissociation curve analysis. Relative IP₃R isoform mRNA expression was calculated from the difference between fluorescence threshold (Ct) values (ΔCt) of each IP₃R isoform sample using the ΔCt method.

Immunoblotting. Rat whole brain or cerebral arteries were homogenized in Laemmli sample buffer (2.5% SDS, 10% glycerol, 0.01% β-mercaptoethanol in 100 mM Tris-HCl, pH 6.8) and centrifuged at 10,000 × g for 10 min. Proteins (30 μg/lane) were separated by 4–15% gradient SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA). Membranes were then blocked with 10% goat serum, smooth muscle cells were imaged using a Zeiss LSM Pascal scanning confocal microscope. Negative controls were prepared by omission of primary antibodies.

IP₃R1 knockdown. Two different IP₃R1 silencing vectors were constructed by GenScript (Piscataway, NJ) using pRNA-U6.1/Neo as a backbone plasmid to encode shRNA targeting IP₃R1. The vectors encode CGGTGCAAAGATTCAGGATA (IP₃R1shV1) or GCAG-CAACGTGATGAGATGTAC (IP₃R1shV2). A pRNA-U6.1/Neo vector that encodes a scrambled sequence (TGAAATCATATGGCTAC-GTTC) was used as a control (IP₃R1scrn). To deliver plasmids into arterial wall cells, reverse permeabilization was used (54). IP₃R1shV1 (10 μg/ml) was used for Western blotting, patch clamp, and diameter measurements, and IP₃R1shV1 (5 μg/ml) + IP₃R1shV2 (5 μg/ml) was used for Western blotting and Ca²⁺ imaging experiments, with IP₃R1scrn (10 μg/ml) as control for each.

Patch-clamp electrophysiology. Iₛ₄ was measured in the presence or absence of UTP (30 μM) using the conventional whole cell patch-clamp configuration (Axopatch 200B, Clampex 8.2). The pipette solution contained (in mM) 140 CsCl, 10 HEPES, 10 glucose, 5 MgATP, and 5 EGTA (with pH adjusted to 7.2 with CsOH), with free Ca²⁺ adjusted to 100 nM using Ca²⁺-sensitive (catalog no. 476601, Corning) and reference (catalog no. 476370, Corning) electrodes. Bath solution contained (in mM) 140 NaCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose (with pH adjusted to 7.4 with NaOH). For experiments studying Iₛ₄ regulation in myocytes treated with IP₃R1 scrambled and suppression vectors, thapsigargin (100 nM) was included in the pipette solution to induce SR Ca²⁺ depletion. Where appropriate, IP₃R1 antibody (Alomone Labs) or a denatured IP₃R1 antibody control (95°C, 10 min) was included in the pipette solution (1:100 dilution). Whole cell currents were measured by applying 940-ms voltage ramps between −120 and +20 mV with a holding potential of −40 mV. Current amplitude was analyzed offline using Clampfit 9.2.

Laser-scanning confocal Ca²⁺ imaging. Cerebral artery segments were cannulated and incubated in the dark with fluo-4 AM (10 μM) and 0.05% Pluronic F-127 for 1 h. Intracellular Ca²⁺ signals in cerebral artery smooth muscle cells were imaged using a Noran Oz laser-scanning confocal microscope, as described previously (11). Fluo-4 AM was excited with 488-nm light, and emitted light >510 nm was collected. Images (112.6 × 105.6 μm) were acquired at a rate of 30 s⁻¹. Under each condition at least two different representative areas of the same arterial segment were each scanned for 10 s. The same area of artery was scanned only once to avoid any laser-induced changes in Ca²⁺, where appropriate, IP₃R1 antibody (Alomone Labs) or a denatured IP₃R1 antibody control (95°C, 10 min) was included in the pipette solution (1:100 dilution). Whole cell currents were measured by applying 940-ms voltage ramps between −120 and +20 mV with a holding potential of −40 mV. Current amplitude was analyzed offline using Clampfit 9.2.

antibody (1:10,000 dilution; Chemicon International) and then with horseradish peroxidase-conjugated anti-mouse IgG. Band intensities were quantified by digital densitometry using Quantity One version 4.4.1 software. IP₃R isoform band intensity was normalized to actin.

Immunofluorescence. Freshly isolated smooth muscle cells were fixed with 3.7% paraformaldehyde in PBS (Invitrogen, Carlsbad, CA) for 15 min and permeabilized with 0.1% Triton X-100 for 1 min at room temperature. After 1 h of incubation in PBS containing 5% BSA, smooth muscle cells were treated overnight at 4°C with antibodies to IP₃R1 (UC Davis/NINDS/NIMH NeuroMab Facility), IP₃R2 (Affinity Bio), or IP₃R3 (BD Transduction Laboratories), each at a dilution of 1:100 in PBS containing 5% BSA. After they were washed and blocked with 10% goat serum, smooth muscle cells were incubated with secondary antibodies (1:100 dilution; Invitrogen-Molecular Probes) as follows: goat anti-mouse IgG with Alexa Fluor 546 for IP₃R1, goat anti-rabbit IgG with Alexa Fluor 488 for IP₃R2, or anti-mouse IgG2a with Alexa Fluor 555 for IP₃R3. The cells were washed and mounted, and fluorescence images were obtained using a Zeiss LSM Pascal scanning confocal microscope. Negative controls were prepared by omission of primary antibodies.
fractional fluorescence increase (F/F₀), and [Ca$$^{2+}$$]_{test} is [Ca$$^{2+}$$]_{rest} at F₀ (193 nM) (10).

**Fura-2 imaging.** IP3R1scrm- or IP3R1shV-treated cerebral artery segments were incubated in HEPES-buffered solution (in mM: 134 NaCl, 6 KCl, 2 CaCl$$₂$$, 1 MgCl$$₂$$, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with NaOH) containing fura-2 AM (5 μM) and 0.05% Pluronic F-127 for 30 min and then washed for 15 min. Fura-2 was alternately excited at 340 or 380 nm using a personal computer-driven hyperswitch (Ionoptix, Milton, MA). Background-corrected ratios were collected every 1 s at 510 nm using a photomultiplier tube. SR Ca$$^{2+}$$ load was estimated by measuring the amplitude of caffeine (10 mM)-induced [Ca$$^{2+}$$] transients, as we described previously (11).

**Pressurized artery diameter measurement.** Endothelium-denuded cerebral artery segments were cannulated at each end in a perfusion chamber. The chamber was continuously perfused with PSS and maintained at 37°C. Intravascular pressure was altered using an attached reservoir and monitored using a pressure transducer. Wall diameter was measured at 1 Hz using a charge-coupled device camera and the edge-detection function of IonWizard (Ionoptix). Pharmacological agents were applied via chamber perfusion. Passive diameter was determined by applying Ca$$^{2+}$$-free PSS supplemented with 5 mM EGTA. The magnitude of myogenic tone was calculated using the following equation: myogenic tone (%) = (1 - active diameter/passive diameter) × 100.

**Reagents.** Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM, fura-2 AM, and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR), and papain was obtained from Worthington Biochemical (Lakewood, NJ).

**Statistical analysis.** Values are means ± SE. Student’s t-test and Student-Newman-Keuls test were used for comparison of paired or unpaired data and multiple data sets, respectively. P < 0.05 was considered significant.

**RESULTS**

**IP$$₃$$R stimulation contributes to UTP-induced Ca$$^{2+}$$ wave activation and global [Ca$$^{2+}$$] elevation in rat cerebral artery smooth muscle cells.** Ca$$^{2+}$$ signals in smooth muscle cells of intact cerebral arteries were acquired by imaging Fluo-4 AM fluorescence at a rate of 30 images per second. UTP (30 μM) elevated mean Ca$$^{2+}$$ wave frequency in smooth muscle cells from 0.07 to 0.70 Hz, or ~10-fold (Fig. 1, A and B). UTP also elevated mean global [Ca$$^{2+}$$] from 193 nM (10) to ~318 nM (Fig. 1C). 2-Aminoethoxydiphenyl borate (2-APB, 2-APB) and xestospongin C (XeC), IP$$₃$$R blockers both prevented UTP-induced Ca$$^{2+}$$ wave activation (Fig. 1, A and B). The mean UTP-induced global [Ca$$^{2+}$$] elevation was reduced by ~54% by XeC and blocked by 2-APB (Fig. 1C). XeC did not significantly change spontaneous Ca$$^{2+}$$ wave frequency (0.07 ± 0.05 Hz, n = 4, P > 0.05), whereas 2-APB abolished these events (0 ± 0 Hz, n = 4, P < 0.05). These data indicate that IP$$₃$$R activation contributes to UTP-induced Ca$$^{2+}$$ waves and global [Ca$$^{2+}$$], elevation in cerebral artery smooth muscle cells.

**IP$$₃$$R isoform expression in rat cerebral artery smooth muscle cells.** We sought to examine the physiological function of individual IP$$₃$$R isoforms in mediating UTP regulation of arterial smooth muscle cell Ca$$^{2+}$$ signals. RT-PCR was performed on lysate isolated from small groups (~100) of individually collected cerebral artery smooth muscle cells, as we have done previously (9). Transcripts for all three IP$$₃$$R isoforms were amplified using conventional RT-PCR (Fig. 2A). Quantitative real-time PCR indicated that IP$$₃$$R1 mRNA was the most abundant, at ~82% of total IP$$₃$$R message (Fig. 2B).

**Fig. 1.** UTP activates Ca$$^{2+}$$ waves and elevates global intracellular Ca$$^{2+}$$ concentration ([Ca$$^{2+}$$]$$₉$$) as a result of inositol 1,4,5-trisphosphate (IP$$₃$$) receptor (IP$$₃$$R) activation in cerebral artery smooth muscle cells. A: Ca$$^{2+}$$ signals recorded in smooth muscle cells of an intact cerebral artery under control conditions and in the presence of UTP (30 μM) and UTP + 2-aminooxydiphenyl borate (2-APB, 100 μM). Top: colored boxes (2.2 × 2.2 μm, 10 × 10 pixels) indicate locations of changes in fluorescence ratio (F/F₀) measured over 10 s in arterial smooth muscle cells. Bottom: colored traces showing changes in F/F₀ for respective colored boxes over 10 s. Images were acquired at 30 Hz. B: mean data illustrating inhibition of UTP-induced Ca$$^{2+}$$ waves in smooth muscle cells of cerebral artery segments by 2-APB (100 μM) and xestospongin C (XeC, 20 μM). C: UTP-induced global [Ca$$^{2+}$$]$$₉$$ elevations are abolished by 2-APB and attenuated by XeC. Changes in global [Ca$$^{2+}$$]$$₉$$ were calculated from a control value previously determined using fura-2 (10). Values are means ± SE; n = 27, 13, 4, and 9 for control, UTP, UTP + 2-APB, and UTP + XeC, respectively. *P < 0.05 vs. control. #P < 0.05 vs. UTP.

Western blotting was performed using IP$$₃$$R isoform-specific antibodies to detect proteins in rat cerebral artery lysate. Consistent with data obtained from RT-PCR experiments, protein bands for IP$$₃$$R1, IP$$₃$$R2, and IP$$₃$$R3 were detected in whole brain and rat cerebral artery lysates, with the IP$$₃$$R1 band the most prominent and IP$$₃$$R2 the least intense (Fig. 2C). Immunofluorescence also identified IP$$₃$$R1, IP$$₃$$R2, and IP$$₃$$R3 protein in freshly isolated cerebral artery smooth muscle cells (Fig. 2D). Collectively, these data indicate that IP$$₃$$R1 is the most abundant IP$$₃$$R isoform expressed in rat cerebral artery smooth muscle cells.

**IP$$₃$$R1 knockdown using shRNA in intact cerebral arteries.** Two different silencing vectors (IP$$₃$$R1shV1 and IP$$₃$$R1shV2) were constructed to express IP$$₃$$R1-specific shRNA. IP$$₃$$R1shV1 and IP$$₃$$R1shV2 target exons 32 and 24, respectively, in IP$$₃$$R1.
A vector that encodes scrambled shRNA (IP3R1scrm) was used as a control. Reversible permeabilization was used to introduce IP3R1scrm, IP3R1shV1, IP3R1shV2, and IP3R1shV1 + IP3R1shV2 into intact cerebral arteries. Western blot analysis indicated that IP3R1shV1 and IP3R1shV2 reduced mean IP3R1 protein to ~67 and 53%, respectively, of that in arteries treated with IP3R1scrm (Fig. 3). Insertion of both IP3R1shV1 + IP3R1shV2 decreased mean IP3R1 expression to ~48% of that in arteries treated with IP3R1scrm (Fig. 3). IP3R1 knockdown did not alter IP3R3 protein, as determined by reprobing membranes with an IP3R3-selective antibody (Fig. 3B).

IP3R1 knockdown attenuates UTP-induced \( \text{Ca}^2+ \) wave activation and global \( \text{[Ca}^2+]_i \) elevation. To study the function of IP3R1 in mediating UTP-induced \( \text{Ca}^2+ \) wave and global \( \text{[Ca}^2+]_i \) elevations, we studied arteries in which IP3R1 expression was reduced with IP3R1shV1 + IP3R1shV2. IP3R1 knockdown did not alter spontaneous \( \text{Ca}^2+ \) wave frequency but prevented UTP-induced \( \text{Ca}^2+ \) wave activation and reduced the UTP-induced global \( \text{[Ca}^2+]_i \) elevation by ~61% (Fig. 4, A and B). IP3R1 knockdown did not alter resting \( \text{[Ca}^2+]_i \) [fura-2 ratio at 340-nm emission to 380-nm emission = 1.03 ± 0.05 (\( n = 16 \)) for IP3R1scrm and 1.08 ± 0.08 (\( n = 14 \), \( P > 0.05 \)) for IP3R1shV1] or SR Ca\(^{2+}\) load, as determined by measuring caffeine (10 mM)-induced \( \text{[Ca}^{2+}]_i \) transients (Fig. 4, C and D). These data indicate that IP3R1 activation is essential for UTP-induced \( \text{Ca}^2+ \) signal modification in arterial smooth muscle cells.

IP3R1 knockdown and an antibody targeting IP3R1 attenuate UTP-induced \( I_{\text{cat}} \) activation. We have previously shown that IP3R1 knockdown attenuates \( I_{\text{cat}} \) in cerebral artery smooth muscle cells (54). Therefore, we studied the importance of IP3R1 for this response. Inclusion of an anti-IP3R1 antibody in the pipette solution reduced mean baseline \( I_{\text{cat}} \) density by ~70% and attenuated the UTP-induced \( I_{\text{cat}} \) elevation by ~85% compared with a denatured antibody control (Fig. 5). \( I_{\text{cat}} \) regulation was also studied in smooth muscle cells isolated from arteries in which IP3R1 expression was reduced using shRNA. Baseline current density in IP3R1shV1 and IP3R1shV2 were similar (~3.8 ± 0.7 pA/pF (\( n = 11 \)) and ~3.9 ± 1.1 pA/pF (\( n = 4 \)), respectively, \( P > 0.05 \); Fig. 6). However, UTP-induced \( I_{\text{cat}} \) activation was reduced by ~87% in IP3R1shV-treated smooth muscle cells compared with cells

Fig. 2. PCR, Western analysis, and immunofluorescence identify IP3R1, IP3R2, and IP3R3 expression in cerebral artery smooth muscle cells. A: RT-PCR detection of IP3R1 (285 bp), IP3R2 (241 bp), IP3R3 (319 bp), and \( \beta \)-actin (257 bp) in isolated smooth muscle cells. NC, negative control using smooth muscle cell RNA, instead of cDNA. Right: 50-bp-increment ladders are shown. B: real-time PCR data indicating percent message for IP3R1, IP3R2, and IP3R3 in isolated smooth muscle cells. Values are means ± SE; \( n = 3 \) for each. C: Western blot detection of IP3R1, IP3R2, and IP3R3 in cerebral arteries and brain. D: immunofluorescence results showing IP3R1, IP3R2, and IP3R3 expression and localization in isolated smooth muscle cells.

Fig. 3. IP3R1 short hairpin RNA (shRNA) suppression vectors attenuate IP3R1 protein in cerebral arteries. A: Western blot illustrating that IP3R1shV1, IP3R1shV2, and IP3R1shV1 + IP3R1shV2 reduce IP3R1 protein in cerebral arteries. Western blot analysis indicated that IP3R1shV1 and IP3R1shV2 reduced mean IP3R1 protein to ~67 and 53%, respectively, of that in arteries treated with IP3R1scrm (Fig. 3). Insertion of both IP3R1shV1 + IP3R1shV2 decreased mean IP3R1 expression to ~48% of that in arteries treated with IP3R1scrm (Fig. 3). IP3R1 knockdown did not alter IP3R3 protein, as determined by reprobing membranes with an IP3R3-selective antibody (Fig. 3B).
IP3R isoforms are expressed in cultured aortic smooth muscle in the vasculature by performing RT-PCR and immunofluorescence (H11005/UTP-induced vasoconstriction in cerebral arteries. Physiological functions of IP3R1, diameter regulation of presynaptic terminals were similar in IP3R1scrm- and IP3R1shV-treated cerebral arteries, D: mean change in fura-2 ratio (∆340/380) in cerebral arteries treated with IP3R1scrm (n = 16) and IP3R1shV (n = 14). Values are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. IP3R1scrm.

DISCUSSION

We have investigated the relative expression and physiological functions of IP3R isoforms in cerebral artery smooth muscle cells. IP3R1 knockdown attenuates UTP-induced vasconstriction in arteries with intact and depleted SR Ca2+. To investigate physiological functions of IP3R1, diameter regulation of pressurized (20 mmHg) endothelium-denuded arteries was studied. IP3R1 knockdown did not alter baseline myogenic tone (23 ± 1.8% (n = 6) vs. 19 ± 0.7% for scrambled control (n = 6), P > 0.05) but reduced mean UTP (1 μM)-induced constriction by ∼52% compared with scrambled control (Fig. 7). Thapsigargin (100 nM–1 μM), at concentrations that deplete SR Ca2+ in cerebral artery smooth muscle cells (54), did not alter arterial diameter (Fig. 7). In SR Ca2+-depleted arteries, IP3R1 knockdown also reduced mean UTP-induced vasconstriction by ∼57% (100 nM thapsigargin) and 62% (1 μM thapsigargin). These data indicate that IP3R1 activation is essential for UTP-induced vasconstriction in cerebral arteries.

Treated IP3R1scrm (Fig. 6). These data indicate that IP3R1 is required for UTP-induced Icat activation in cerebral artery smooth muscle cells.

IP3R1 knockdown attenuates UTP-induced vasconstriction in arteries with intact and depleted SR Ca2+. To investigate physiological functions of IP3R1, diameter regulation of pressurized (20 mmHg) endothelium-denuded arteries was studied. IP3R1 knockdown did not alter baseline myogenic tone (23 ± 1.8% (n = 6) vs. 19 ± 0.7% for scrambled control (n = 6), P > 0.05) but reduced mean UTP (1 μM)-induced constriction by ∼52% compared with scrambled control (Fig. 7). Thapsigargin (100 nM–1 μM), at concentrations that deplete SR Ca2+ in cerebral artery smooth muscle cells (54), did not alter arterial diameter (Fig. 7). In SR Ca2+-depleted arteries, IP3R1 knockdown also reduced mean UTP-induced vasconstriction by ∼57% (100 nM thapsigargin) and 62% (1 μM thapsigargin). These data indicate that IP3R1 activation is essential for UTP-induced vasconstriction in cerebral arteries.

UTP-induced phospholipase C activation elevates diacylglycerol (DAG), leading to PKC activation, and increases IP3, leading to IP3R activation (35, 44). Vasoconstrictors, including UTP, block Ca2+ sparks, activate Ca2+ waves and oscillations, and elevate global [Ca2+]i in arterial smooth muscle cells (23, 31, 52). UTP-induced Ca2+ spark inhibition occurs as a result of PKC activation (23). Mechanisms that generate Ca2+ waves and the physiological functions of these Ca2+ signals in vascular smooth muscle cells are unclear. Vasoconstrictors elevate Ca2+ wave frequency, and depletion of SR Ca2+ abolishes these events (23, 30). Different proposals have been developed to explain mechanisms that generate and propagate Ca2+ waves (7, 15, 16, 18). One model suggests that ryanodine receptor (RyR) channel and IP3R activation is required for Ca2+ wave generation and propagation (7, 15, 16, 26). In this scenario, IP3 acts as a trigger to prime Ca2+ release through SR IP3Rs. Released Ca2+ then stimulates further Ca2+ release by activating RyR channels, and waves proceed independently of IP3Rs. Another proposal suggests that IP3R activation alone is sufficient to initiate and propagate waves through Ca2+-induced Ca2+ release (18, 26). Both concepts indicate that IP3Rs are required for Ca2+ wave generation. In cultured human coronary artery smooth muscle cells, IP3 generation and subsequent IP3R activation are required for UTP-induced SR Ca2+ release (44). Our data obtained using pharmacological...
blockers, antibodies, and protein knockdown indicate that IP3R1 is required for UTP-induced Ca2+ wave generation and propagation. IP3R1 knockdown did not change SR Ca2+ load, which would have indirectly affected Ca2+ wave frequency (23, 30). Thus data suggest that IP3R1 knockdown inhibits Ca2+ waves by reducing the number of IP3R1 channels that contribute to these signaling events. Intracellular IP3 concentration should be low in the absence of exogenous receptor agonists, although UTP may be released from endothelial cells under resting conditions, which may generate additional IP3 (24, 25, 38). Since IP3R1 knockdown and XeC did not alter baseline Ca2+ wave frequency in the absence of UTP, our data suggest that the contribution of IP3 to Ca2+ waves in the absence of agonists is small. These different findings raise the possibility that IP3Rs exhibit variable expression profiles and physiological functions in smooth muscle cells of anatomically different vessels and in cultured versus noncultured smooth muscle cells.

Collectively, data presented here and in our previous study indicate that IP3R1 activation contributes to agonist-induced I_cat activation in cerebral artery smooth muscle cells (54). TRPC channels, including TRPC1, TRPC3, TRPC5, TRPC6, and TRPC7, are expressed in vascular smooth muscle cells and may contribute to I_cat (2, 12, 28, 36, 39, 40, 54). In cerebral artery smooth muscle cells, TRPC3 channel knockdown attenuated endothelin-1- and IP3-induced [Ca2+]i elevation and constriction, indicating that this isoform is a major contributor to the IP3R-induced I_cat in this cell type (54). The mechanism by which IP3R activation stimulates I_cat in arterial smooth muscle cells is unclear. Studies using protein overexpression have demonstrated that a consensus sequence present on the COOH terminus of all TRPC channel isoforms recognizes an NH2-terminal binding domain found in all IP3R isoforms (46).

It has been proposed that physical coupling of these two domains removes inhibitory calmodulin from the TRPC channel COOH terminus, leading to channel activation (46, 55). Whether a similar coupling mechanism underlies IP3R regulation of I_cat and TRPC3 channels in arterial smooth muscle cells remains to be determined but is one possibility.

Our data indicate that IP3R1 suppression with antibodies or shRNA attenuated UTP-induced I_cat activation by ~85%. This level of inhibition is large, given that UTP-induced phospholipase C activation also elevates DAG, and DAG and 1-oleoyl-

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**Fig. 5.** Anti-IP3R1 antibody inhibits UTP-induced cation current (I_cat) in rat cerebral artery smooth muscle cells. A: exemplar traces obtained using whole cell patch-clamp configuration with anti-IP3R1 (1:100 dilution) or its heat-denatured control in the pipette before or after UTP (30 μM) application. B: mean data showing the effect of anti-IP3R1 antibody on I_cat. Values are means ± SE; n = 11 (denatured control Ab) and 5 (all others). *P < 0.05 vs. control. #P < 0.05 vs. denatured Ab.

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**Fig. 6.** IP3R1 knockdown inhibits I_cat activation by UTP in cerebral artery smooth muscle cells. A: exemplar traces illustrating UTP-induced I_cat activation in IP3R1scrm- and IP3R1shV-treated smooth muscle cells. B: mean current density data for IP3R1scrm- and IP3R1shV-treated cells. *P < 0.05 vs. control. #P < 0.05 vs. IP3R1scrm.
2-acetyl-sn-glycerol (OAG), a DAG analog, activated \( I_{\text{cat}} \) in rat cerebral and rabbit coronary artery smooth muscle cells, portal vein smooth muscle cells, and cells overexpressing TRPC channels (3, 20, 34, 42). In cerebral artery smooth muscle cells, PKC activation stimulates an \( I_{\text{cat}} \) by increasing the apparent micromolar \( \text{Ca}^{2+} \) sensitivity of TRPM4 channels (13, 42). In our patch-clamp experiments, EGTA was used to clamp free \( \text{Ca}^{2+} \) at a physiological concentration of 100 nM. Therefore, UTP-induced PKC activation would not be expected to activate TRPM4 channels, and the strong inhibitory effect of IP\(_3\)R1 inhibition on UTP-induced \( I_{\text{cat}} \) may be due to an effect on TRPC channels alone. However, PKC regulation of TRPC channels is complex, with studies reporting activation, inhibition, or no effect on TRP channels, including those using smooth muscle cells from a variety of blood vessels (4, 34, 41, 49, 50). In rabbit coronary artery smooth muscle cells, IP\(_3\) potentiated DAG-induced \( I_{\text{cat}} \) activation, and heparin blocked this activation, indicating that IP\(_3\)Rs mediated this response (34). In cerebral artery smooth muscle cell inside-out patches, IP\(_3\) and OAG did not activate cation channels, and OAG did not facilitate IP\(_3\)-induced cation channel activation (54). Whether interactions occur between IP\(_3\)Rs and PKC in \( I_{\text{cat}} \) activation and what the mechanisms are in cerebral artery smooth muscle cells remain to be determined. Nevertheless, our data indicate that IP\(_3\)R activation is a key mechanism mediating agonist-induced \( I_{\text{cat}} \) activation in these cells.

An elevation in global \( [\text{Ca}^{2+}]_i \), stimulates vasoconstriction, whereas a reduction in global \( [\text{Ca}^{2+}]_i \) leads to vasodilation. UTP elevated global \( [\text{Ca}^{2+}]_i \), and this effect was blocked by 2-APB and attenuated by XeC and IP\(_3\)R1 knockdown. To study physiological functions of IP\(_3\)R1 that occur through SR \( \text{Ca}^{2+} \) release-dependent and -independent mechanisms, diameter measurements were obtained at an intravascular pressure of 20 mmHg to reduce the influence of SR \( \text{Ca}^{2+} \) depletion regulating diameter through \( \text{Ca}^{2+} \) spark inhibition, as we have done previously (54). UTP-induced vasoconstriction was inhibited by IP\(_3\)R1 knockdown, consistent with effects on \( \text{Ca}^{2+} \) signals. More importantly, UTP-induced vasoconstriction was inhibited by IP\(_3\)R1 knockdown, even though SR \( \text{Ca}^{2+} \) depletion did not alter UTP-induced vasoconstriction. In addition, SR \( \text{Ca}^{2+} \) depletion did not alter arterial diameter at 20 mmHg, consistent with previous data (54). Taken together, data indicate that 1) IP\(_3\)R1 activation contributes to UTP-induced vasoconstriction, 2) at 20 mmHg, SR \( \text{Ca}^{2+} \) release does not contribute to UTP-induced vasoconstriction, 3) SR \( \text{Ca}^{2+} \) load does not regulate arterial diameter at low pressure, and 4) UTP-induced IP\(_3\)R1 activation constricts cerebral arteries through an SR \( \text{Ca}^{2+} \) release-independent mechanism. We propose that, in cerebral artery smooth muscle cells, UTP stimulates IP\(_3\)R1, leading to \( I_{\text{cat}} \) activation, membrane depolarization, voltage-dependent \( \text{Ca}^{2+} \) channel activation, a global \( [\text{Ca}^{2+}]_i \), elevation, and vasoconstriction. We also show that UTP-induced \( \text{Ca}^{2+} \) waves require IP\(_3\)R1 activation and SR \( \text{Ca}^{2+} \) release (22). Since SR \( \text{Ca}^{2+} \) depletion did not alter UTP-induced vasoconstriction, our data suggest that SR \( \text{Ca}^{2+} \) release and, thus, \( \text{Ca}^{2+} \) waves do not contribute to UTP-induced vasoconstriction. These data support our previous observation that SR \( \text{Ca}^{2+} \) release does not contribute to IP\(_3\)-induced vasoconstriction at low pressure (54). We have also shown that elevating pressure to 60 mmHg increases the contribution of SR \( \text{Ca}^{2+} \) release to IP\(_3\)-induced vasoconstriction (54). Conceivably, vasoconstrictor-induced \( \text{Ca}^{2+} \) waves may directly contribute to contraction at higher pressures.

Physiological functions of IP\(_3\)R2 and IP\(_3\)R3 were not directly investigated in the present study. By comparing effects of XeC, a blocker of all IP\(_3\)R isoforms, and those of IP\(_3\)R1 knockdown and antibodies, data suggest that IP\(_3\)R1 is the principal molecular isoform mediating UTP-induced \( I_{\text{cat}} \) activation, \( \text{Ca}^{2+} \) wave stimulation, and contraction in cerebral artery smooth muscle cells. Partial IP\(_3\)R1 knockdown almost completely blocked UTP-induced \( I_{\text{cat}} \) and \( \text{Ca}^{2+} \) wave activation and partially attenuated vasoconstriction. IP\(_3\)R2 and IP\(_3\)R3 are expressed and therefore, these proteins are highly likely to perform physiological functions in arterial myocytes. We do not rule out physiological functions for IP\(_3\)R2 and IP\(_3\)R3. However, our data indicate a principal function for IP\(_3\)R1 in mediating these responses.

In summary, the present study provides evidence that all three IP\(_3\)R isoforms are expressed in rat cerebral artery smooth muscle cells and that IP\(_3\)R1 is the most abundant subtype. We also show that IP\(_3\)R1 activation contributes to UTP-induced \( I_{\text{cat}} \) activation, \( \text{Ca}^{2+} \) wave stimulation, global \( [\text{Ca}^{2+}]_i \), elevation, and vasoconstriction. These data indicate that IP\(_3\)R1 activation is necessary for UTP-induced vasoconstriction.

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