Isoform-Selective Physical Coupling of TRPC3 Channels to IP3 Receptors in Smooth Muscle Cells Regulates Arterial Contractility

Adebowale Adebiyi, Guiling Zhao, Damodaran Narayanan, Candice M. Thomas, John P. Bannister, Jonathan H. Jaggar

Rationale: Inositol 1,4,5-trisphosphate (IP3)-induced vasoconstriction can occur independently of intracellular Ca2+ release and via IP3 receptor (IP3R) and canonical transient receptor potential (TRPC) channel activation, but functional signaling mechanisms mediating this effect are unclear.

Objectives: Study mechanisms by which IP3Rs stimulate TRPC channels in myocytes of resistance-size cerebral arteries.

Methods and Results: Immunofluorescence resonance energy transfer (immuno-FRET) microscopy using isoform-selective antibodies indicated that endogenous type 1 IP3Rs (IP3R1) are in close spatial proximity to TRPC3, but distant from TRPC6 or TRPM4 channels in arterial myocytes. Endothelin-1 (ET-1), a phospholipase C-coupled receptor agonist, elevated immuno-FRET between IP3R1 and TRPC3, but not between IP3R1 and TRPC6 or TRPM4. TRPC3, but not TRPC6, communoprecipitated with IP3R1. TRPC3 and TRPC6 antibodies selectively inhibited recombinant channels, but only the TRPC3 antibody blocked IP3-induced nonselective cation current (Icat) in myocytes. TRPC3 knockdown attenuated immuno-FRET between IP3R1 and TRPC3, IP3-induced Icat activation, and ET-1 and IP3-induced vasoconstriction, whereas TRPC6 channel knockdown had no effect. ET-1 did not alter total or plasma membrane-localized TRPC3, as determined using surface biotinylation. RT-PCR demonstrated that C-terminal calmodulin and IP3R binding (CIRB) domains are present in myocyte TRPC3 and TRPC6 channels. A peptide corresponding to the IP3R N-terminal region that can interact with TRPC channels demonstrated that CIRB domains are present in myocyte TRPC3 and TRPC6 channels. A peptide corresponding to the IP3R N-terminal region that can interact with TRPC channels and inhibit recombinant channels, but only the TRPC3 antibody blocked IP3-induced nonselective cation current (Icat). A TRPC3 CIRB domain peptide attenuated IP3- and ET-1-induced Icat activation and vasoconstriction.

Conclusions: IP3 stimulates direct coupling between IP3R1 and membrane-resident TRPC3 channels in arterial myocytes, leading to Icat activation and vasoconstriction. Close spatial proximity between IP3R1 and TRPC3 establishes this isoform-selective functional interaction. (Circ Res. 2010;106:00-00.)

Key Words: inositol 1,4,5-trisphosphate ▪ canonical transient receptor potential channel ▪ coupling ▪ vasoconstriction

Activation of plasma membrane phospholipase (PLC)-coupled receptors by vasoconstrictor agonists leads to phosphatidylinositol 4,5-biphosphate (PIP2) hydrolysis and the generation of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. In vascular myocytes, diacylglycerol (DAG) activates protein kinase (PKC), leading to the phosphorylation of a wide variety of proteins, including ion channels. IP3 binds to sarcoplasmic reticulum (SR) IP3 receptors (IP3Rs), resulting in SR Ca2+ release, an elevation in intracellular Ca2+ concentration ([Ca2+]i), and vasoconstriction. Recent evidence also indicates that IP3-induced vasoconstriction can occur independently of SR Ca2+ release and via the activation of type 1 IP3 receptors (IP3R1) and type 3 canonical transient receptor potential (TRPC) channels. However, the functional signaling mechanisms by which IP3Rs and TRPC channels communicate in arterial myocytes are unclear.

The mammalian TRP channel superfamily is encoded by at least 28 different genes that are subdivided into 7 families. These families encode ion channels with diverse ion selectivity, modes of regulation, and physiological functions. Vascular myocytes express at least 4 TRP families, including TRPC, TRPM, TRPV, and TRPP. These channels regulate arterial myocyte membrane potential, [Ca2+]i, contractility, and proliferation, and are implicated in the etiology of vascular diseases. Given the diversity of vascular myocyte TRP channels, it has become important to identify signaling pathways that specifically regulate individual chan-
TRPC Channel Knockdown
Silencing vectors that express TRPC3- (TRPC3shV), TRPC6- (TRPC6shV), or scrambled control (scrm) short hairpin RNA were inserted into cerebral arteries using reverse permeabilization, as previously described.6

Coimmunoprecipitation
Arterial lysate was incubated with control mouse IgG or IP$_3$R1 monoclonal antibody and then incubated with protein A sepharose beads. Protein samples were then analyzed by Western blotting using mouse monoclonal anti-IP$_3$R1, mouse polyclonal anti-TRPC3, rabbit polyclonal anti-TRPC6, and horseradish peroxidase–conjugated secondary antibodies.

Surface Biotinylation
Membrane expression of TRPC3 channels was measured using surface biotinylation of intact arteries, as previously described.17

Cell Culture and Transfection
HEK293 cells were transfected with vectors encoding recombinant TRPC3 or TRPC6, kindly provided by Dr James Putney (National Institute of Environmental Health Sciences) and Dr Jochen Reiser (University of Miami, Fla), respectively. Electrophysiology and Western blotting experiments were performed 36 to 72 hour after transfection.

Polymerase Chain Reaction
RT-PCR was performed on pure populations of $\approx$100 selected cerebral artery myocytes, as described previously.18

Patch-Clamp Electrophysiology
Membrane cation currents were measured in arterial myocytes and HEK293 cells using the conventional whole cell patch-clamp configuration, as performed previously.4,5

Pressurized Artery Diameter Measurement
Arterial diameter was measured using pressurized artery myography, as described previously.18

Statistical Analysis
Data are expressed as mean±standard error of the mean. Statistical significance was calculated by using Student’s t-tests for paired or unpaired data or ANOVA followed by Student–Newman–Keuls test for multiple data sets. P<0.05 was considered significant.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results
IP$_3$R1 Is in Close Proximity to TRPC3 Channels in Cerebral Artery Myocytes
IP$_3$R1 is the principal molecular and functional IP$_3$R isoform that is expressed in myocytes of cerebral arteries and aorta.5,19

To explore spatial proximity between IP$_3$R1 and 3 different TRP channel isoforms in cerebral artery myocytes, we used immunofluorescence resonance energy transfer. Specifically, spatial localization between IP$_3$R1 and TRPC3, TRPC6, or TRPM4 channels was examined. Cy3- and Cy2-labeled secondary antibodies bound to primary antibodies targeting IP$_3$R1 (monoclonal) and TRPC3 (polyclonal), respectively, produced mean N-FRET of $\approx$25% (Figure 1A and 1B). In contrast, fluorescent antibodies bound to IP$_3$R1 and TRPM4 isoforms and to determine whether individual TRP channel isoforms perform distinct physiological functions. For instance, arterial myocytes express multiple TRPC isoforms, including 1, 3, and 6, but whether signaling pathways specifically regulate individual members and what the mechanisms are that mediate such effects are poorly understood.9,10 In cerebral artery myocytes, vasoconstrictors activate TRPC3,9,10 whereas intravascular pressure stimulates TRPC6.9,10 Thus, TRPC3 and TRPC6 channels perform distinct physiological functions, but signaling pathways that mediate this differential regulation are unclear.

Here, we studied mechanisms by which IP$_3$R1, the principal molecular and functional arterial myocyte IP$_3$R isoform,5 stimulates TRPC currents in resistance-size cerebral arteries. Data suggest that IP$_3$R1 is in close spatial proximity to, and associates with, TRPC3, but not TRPC6 or TRPM4 channels. Endothelin (ET)-1, a PLC-coupled receptor agonist, and IP$_3$ associate with, TRPC3, but not TRPC6 or TRPM4 channels. Therefore, TRPC3 and TRPC6 channels perform distinct physiological functions, but signaling pathways that mediate this differential regulation are unclear.

Non-standard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>CIRB</td>
<td>calmodulin and inositol 1,4,5-trisphosphate receptor binding</td>
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<td>ET</td>
<td>endothelin</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<tr>
<td>I$_{cat}$</td>
<td>nonselective cation current</td>
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<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
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<td>IP$_3$R</td>
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<td>IP$_3$RNTP</td>
<td>inositol 1,4,5-trisphosphate receptor N-terminal sequence peptide</td>
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<tr>
<td>N-FRET</td>
<td>normalized fluorescence resonance energy transfer</td>
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Tissue Preparation
Animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Miami. Sprague–Dawley rat (≈250 g) resistance-size cerebral arteries and myocytes from these arteries were isolated as previously described.4

Immunofluorescence Resonance Energy Transfer
Paraformaldehyde-fixed myocytes were incubated with primary antibodies: mouse monoclonal anti-IP$_3$R1 and rabbit polyclonal anti-TRPC3, rabbit polyclonal anti-TRPC6, or rabbit polyclonal anti-TRPM4. Cells were then labeled with secondary antibodies: Cy3-conjugated donkey antimouse for IP$_3$R1 and Cy2-conjugated goat anti-rabbit for TRPC3, TRPM4, or TRPC6. Fluorescent images, acquired using a Zeiss LSM 5 Pascal confocal microscope, were background-subtracted and normalized fluorescence resonance energy transfer (N-FRET)–calculated using the method of Xia and Liu.15

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(polyclonal), or IP$_R$1 and TRPC6 (polyclonal), generated mean N-FRET of $\approx 11\%$ and $\approx 12\%$, respectively (Figure 1A and 1B). ET-1 increased mean N-FRET between IP$_R$1 and TRPC3-bound antibodies to $\approx 34\%$. In contrast, N-FRET between IP$_R$1 and TRPC3-bound antibodies was not altered by membrane depolarization with 30 mmol/L K$^+$ (Figure 1B). ET-1 did not change N-FRET between antibodies bound to IP$_R$1 and TRPC6 or IP$_R$1 and TRPM4 (Figure 1A and 1B). Antigenic peptides for TRPC3, TRPC6, and TRPM4 specifically blocked immunofluorescence produced by each antibody, but did not alter immunofluorescence detection by the other 2 antibodies (Online Figure I, A).

Next, N-FRET was measured in myocytes in which TRPC3 or TRPC6 expression was reduced using short hairpin RNA and quantified using Western blotting. The selectivity of antibodies for Western blotting experiments was examined. Whole Western blots for IP$_R$1, TRPC3 and TRPC6 antibodies on arterial lysate are illustrated in Online Figure I (B). The monoclonal IP$_R$1 antibody detected only 1 band corresponding to IP$_R$1 (Online Figure I, B), and this band is reduced by IP$_R$1 knockdown, indicating specificity.5 Antigenic peptides abolished appropriate size bands for each TRPC isoform but did not alter detection of the other TRPC protein (Online Figure I, C). The TRPC3 antibody detected recombinant TRPC3 expressed in HEK293 cells, but did not detect recombinant TRPC6 (Online Figure II, A). Similarly, the TRPC6 antibody detected recombinant TRPC6, but did not detect recombinant TRPC3 (Online Figure II, A). Endogenous TRPC3 and TRPC6 proteins were detected in HEK293 cells, consistent with a previous report.20 Taken together, these data further indicate that the TRPC3 and TRPC6 antibodies are selective for their respective TRPC channel isoforms.

Western blotting indicated that TRPC3shV and TRPC6shV reduced mean TRPC3 and TRPC6 protein by $\approx 40$ and 43%, respectively, of that in arteries treated with scrm (Figure 1C and 1D). TRPC3shV did not alter TRPC6 and IP$_R$1 expression and TRPC6shV did not alter TRPC3 and IP$_R$1 expression (Figure 1C and 1D). TRPC3 knockdown reduced control N-FRET and the ET-1–induced elevation in N-FRET between IP$_R$1 and TRPC3 (Figure 1E). In contrast, TRPC6 knockdown had no effect on N-FRET between IP$_R$1 and TRPC6 in control or ET-1 (Figure 1E), indicating that this signal represents background N-FRET.

Coimmunoprecipitation (co-IP) was used to examine whether IP$_R$1 physically associates with TRPC3 and TRPC6
channels in small cerebral arteries. Because of the small size of the resistance-size vessels used in this study, arteries collected from \( \sim 20 \) rats were required for each experiment. The IP\( _{\alpha} \)R1 monoclonal antibody coimmunoprecipitated both IP\( _{\alpha} \)R1 and TRPC3 channels from lysate (Figure 2). In contrast, TRPC6 was not detected in the same IP\( _{\alpha} \)R1 immunoprecipitate (Figure 2).

In summary, data indicate that TRPC3, but not TRPM4 or TRPC6, is in close spatial proximity to, and associates with, IP\( _{\alpha} \)R1 channels in myocytes of resistance-size cerebral arteries. Data also demonstrate that ET-1 modifies the molecular relationship between IP\( _{\alpha} \)R1 and TRPC3.

TRPC3 Mediates IP\( _{3} \)-Induced \( I_{\text{Cat}} \) Activation in Cerebral Artery Myocytes

The contribution of TRPC3 and TRPC6 channels to IP\( _{3} \)-induced \( I_{\text{Cat}} \) was examined. The TRPC3 antibody blocked whole-cell currents generated by recombinant TRPC3 expressed in HEK293 cells (Figure 3A and 3B; Online Figure II, B). In contrast, the TRPC3 antibody did not alter currents produced by recombinant TRPC6 channels. Similarly, the TRPC6 antibody blocked recombinant TRPC6 channel currents, but did not alter TRPC3 currents (Figure 3A and 3B; Online Figure II, B). Therefore, the antibodies selectively inhibit their respective TRPC currents. The TRPC3 antibody reduced mean IP\( _{3} \)-induced \( I_{\text{Cat}} \) density by \( \sim 57\% \) in arterial myocytes (Figure 3C and 3D). In contrast, the TRPC6 antibody had no effect on IP\( _{3} \)-induced \( I_{\text{Cat}} \) density in arterial myocytes (Figure 3C and 3D).

IP\( _{3} \)-induced \( I_{\text{Cat}} \) activation and vasoconstriction were studied in myocytes and arteries in which TRPC3 or TRPC6 expression was reduced using short hairpin RNA. TRPC3 knockdown reduced IP\( _{3} \)-induced \( I_{\text{Cat}} \) density by \( \sim 41\% \). In contrast, TRPC6 knockdown did not alter IP\( _{3} \)-induced \( I_{\text{Cat}} \) density (Figure 4A and 4B). TRPC6 knockdown reduces myogenic tone at intravascular pressures \( >40 \) mm Hg. Therefore, diameter regulation was measured in arteries pressurized to \( 20 \) mm Hg to avoid differences in baseline tone between arteries in which TRPC3 and TRPC6 were knocked down. TRPC3 knockdown attenuated IP\( _{3} \)- and ET-1–induced vasoconstriction by \( \sim 43 \) and \( 49\% \), respectively (Figure 4C through 4E). These data indicate that TRPC3, but not TRPC6, channels mediate IP\( _{3} \)-induced \( I_{\text{Cat}} \) activation and contraction in cerebral artery myocytes.

ET-1 Does Not Increase Surface Expression of TRPC3 Channels in Myocytes

An increase in ion channel activity (NP\( _{o} \)) can occur because of both an elevation in the number of channels (N) and an increase in channel open probability (P\( _{o} \)). To investigate whether ET-1 alters plasma membrane TRPC3 channel protein, we used surface biotinylation. Confirmation that biotin specifically labels surface proteins was obtained using biotin-treated arterial segments exposed to Texas Red-conjugated streptavidin (Online Figure III). In addition, heat shock protein 90, an intracellular protein, was detected only in the nonbiotinylated (cytosolic) fraction of arterial myocytes, suggesting that biotin specifically labels surface proteins.
Biotinylation indicated that in control, ≈30% of total TRPC3 protein was present in the arterial plasma membrane (Figure 5B and 5C). ET-1 did not alter the percentage of plasma membrane-localized TRPC3 (Figure 5B and 5C). These data indicate that a significant proportion of arterial TRPC3 is cytosolic and that ET-1 elevates myocyte \( I_{\text{cat}} \) by stimulating plasma membrane-resident TRPC3 channels.

Calmodulin and IP3R Binding Domains Are Present in Myocyte TRPC3 and TRPC6 Channels

Recombinant IP3R N-termini contain a conserved amino acid sequence that can interact with a conserved C-terminal CIRB (calmodulin and IP3R binding) domain found on all TRPC isoforms.11,22 TRPM4 does not contain a CIRB domain (GenBank accession no. XM_574447). Our data indicate that isoform-selective association of TRPC3 channels to IP3R1 occurs in arterial myocytes (Figures 1 through 4). Therefore, we used RT-PCR to investigate whether myocyte TRPC channels contain CIRB domains. Primers used were selective for TRPC3 and TRPC6 CIRB domains (Online Figure IV). RT-PCR was performed on manually selected arterial myocytes to prevent message contamination from other vascular wall cell types.18 Data indicated that the C-termini of arterial myocyte TRPC3 and TRPC6 channels contain CIRB domains (Figure 6A).

TRPC3-IP3R1 Physical Coupling Is Required for ET-1- and IP3-Induced \( I_{\text{cat}} \) Activation

We tested the hypothesis that IP3-induced \( I_{\text{cat}} \) activation in arterial myocytes occurs through an interaction between the conserved IP3R interaction domain and the TRPC CIRB domain. Synthetic peptides corresponding to a region in the TRPC3 CIRB domain (QIMKRLIKRYVLKAQVDKEND, CIRBP)21–24 and a scrambled control peptide (RDLKKAQNLVEIQKKYDRMVI, CIRBPscrm) were generated. CIRBP and CIRBPscrm alone did not alter mean baseline \( I_{\text{cat}} \) density (pA/pF: no peptide, 3.5±0.5, n=5; CIRBPscrm, -3.7±0.9, n=10; CIRBP, -2.9±0.6, n=11; \( P>0.05 \) for each) in arterial myocytes. In contrast, CIRBP reduced IP3 and ET-1–induced \( I_{\text{cat}} \) activation by 63% and 60%, respectively, when compared with CIRBPscrm (Figure 6B through 6D).

To study physiological functions of the IP3R N terminus, a peptide was constructed corresponding to the IP3R N-terminal sequence containing the conserved TRPC-binding region (EEVWLFWRD, IP3RntP). IP3RntP increased mean \( I_{\text{cat}} \) density by ≈260% (Figure 7A and 7B). Gadolinium (Gd3+), a nonselective cation channel blocker, had no significant effect on baseline currents, but reduced mean IP3RntP-induced \( I_{\text{cat}} \) density by ≈70% (Figure 7A and 7B). These data indicate that physical association between the IP3R1 N terminus and the TRPC C terminus CIRB domain leads to \( I_{\text{cat}} \) activation in arterial myocytes.
CIRBP Attenuates ET-1– and IP₃-Induced Vasoconstriction

The physiological function of molecular coupling between IP₃R and TRPC3 channels was investigated by measuring diameter regulation of pressurized (60 mm Hg) arteries. Membrane-permeant CIRBP (CIRBP-TAT) and scrambled control (CIRBPscrm-TAT) peptides were each generated by conjugation to a HIV-1 TAT sequence (RRRQRRKKRGY). CIRBP-TAT reduced vasoconstriction caused by Bt-IP3, a membrane permeant IP₃ analog, and ET-1 by 78% and 65%, respectively, when compared with CIRBPscrm-TAT (Figure 8A through 8D). These data indicate that interaction between the IP₃R1 N terminus and the TRPC3 channel CIRB domain contributes to IP₃ and ET-1–induced vasoconstriction.

Discussion

Here, we identify a novel vasoregulatory signaling mechanism whereby IP₃Rs couple to plasma membrane TRPC3 channels in arterial myocytes (Online Figure V). Novel findings of this study are that in cerebral artery myocytes: (1) IP₃R1 is located in close proximity to TRPC3, but not TRPC6 or TRPM4 channels; (2) ET-1 elevates FRET between fluorescent antibodies bound to IP₃R1 and TRPC3 channels but does not alter FRET between IP₃R1 and TRPC6 or TRPM4 channels; (3) TRPC3 channels co-IP with IP₃R1, whereas TRPC6 channels do not; (4) TRPC3, but not TRPC6, channels underlie IP₃-induced \( I_{\text{Cat}} \); (5) ET-1 does not alter total or plasma membrane-localized TRPC3 channel protein; (6) TRPC3 and TRPC6 both contain CIRB domains; (7) a TRPC3 CIRB domain peptide reduces IP₃ and ET-1–induced \( I_{\text{Cat}} \) activation and a peptide corresponding to the IP₃R1 N-terminal sequence that interacts with the TRPC CIRB domain activates \( I_{\text{Cat}} \); and (8) CIRBP attenuates IP₃ and ET-1–induced vasoconstriction. Taken together, these data indicate that interaction between the IP₃R1 N terminus and the TRPC3 channel CIRB domain contributes to \( I_{\text{Cat}} \) and vasoconstriction. Physical coupling between all IP₃ and TRPC channel isoforms occurs with recombinant channels. To our knowledge, this is the
first time that molecular coupling between IP3R1 and TRPC channels has been demonstrated to occur in a native cell type, to perform a physiological function, and to exhibit TRPC channel isoform selectivity. Our data also suggest that close spatial proximity of IP3R1 to TRPC3 channels establishes selective molecular coupling of these proteins. In contrast, although TRPC6 contains a CIRB domain, spatial separation appears to prevent this channel from coupling to IP3R1 in arterial myocytes. Our study demonstrates that arterial myocyte IP3R channels, previously considered to function solely by releasing SR Ca2+/H11001, directly activate TRPC3 channels, thereby regulating arterial contractility. This study also illustrates that manipulating physical coupling between IP3R1 and TRPC3 channels is a novel mechanism that could be exploited to regulate arterial contractility.

Rat cerebral artery myocytes express several TRP isoforms, including TRPC1, -3, -6, TRPM4, and TRPV4.7,9–11 Mammalian TRPC channel isoforms share >30% amino acid sequence identity,27 with all TRPC channel isoforms containing a C-terminal CIRB domain.21,22 Cerebral artery myocytes express all 3 IP3R isoforms, with IP3R1 being the principal molecular and functional isoform.5 All recombinant IP3R isoforms contain an N-terminal TRPC channel interaction domain.21,22 Coupling of the IP3R TRPC binding domain to the TRPC channel CIRB domain displaces inhibitory calmodulin, leading to channel activation.22 The CIRB domain may also be a TRPC channel trafficking sequence.24 These previous studies were performed primarily by studying recombinant channels. It has been proposed that the physical interaction between IP3Rs and TRPC channels may have occurred in response to TRPC channel overexpression.21–23,26 Therefore, it was unclear whether physical coupling between IP3Rs and TRPC channels would occur in native cell types. Here, we demonstrate that this coupling mechanism does occur in a native cell type, selectively regulates TRPC3 currents in arterial myocytes, and leads to vasoconstriction.

In cerebral artery myocytes, IP3R stimulation leads to the activation of a ICat to which TRPC3 channels contribute.4,9 IP3R-mediated ICat activation leads primarily to Na+/H11001 influx, resulting in membrane depolarization, voltage-dependent Ca2+ channel activation, an [Ca2+]i elevation, and vasoconstriction.4 The mechanism by which IP3Rs stimulate TRPC3 channels occurs independently of SR Ca2+ release and appears to be a major mechanism by which PLC-coupled receptor agonists cause vasoconstriction.4 IP3R1 and TRPC3 channels do not contribute to the cerebral artery myogenic response.4,9 In contrast, TRPC6 channels do not contribute to ET-1 or UTP-induced cerebral artery constriction, but are essential for the myogenic response.4,9,10 The different mechanisms by which vasoconstrictors and IP3 activate TRPC3, whereas intravascular pressure stimulates TRPC6 were unclear. Fluorescent antibodies bound to endogenous IP3R1 and TRPC3 generated FRET that was elevated by ET-1 and higher than for TRPC6 or TRPM4. TRPC3 knockdown reduced basal and ET-1–induced IP3R1/TRPC3 FRET. The calculated Förster distance between Cy2 and Cy3 is 5 to 6 nm, supporting other evidence in this study that IP3R1 and
TRPC3 are close enough to physically interact. In contrast, the FRET signal between IP₃R1 and TRPC6 and TRPM4 was consistent with background,²⁸ was not elevated by ET-1, and was not altered by TRPC6 knockdown. These data indicate that that TRPC6 and TRPM4 are not in close proximity to IP₃R1. Mechanisms that position TRPC3 nearby IP₃R1, but TRPC6 and TRPM4 away from IP₃R1, are unclear. Several possibilities exist, including differential localization by local lipid environments, including caveolae. It is also possible that IP₃R1 and TRPC3 are maintained by scaffolding proteins in a macromolecular complex. Scaffolding proteins, including Homer, establish close proximity of TRPC channels to other proteins, including IP₃Rs.²⁹ The ET-1–induced elevation in immunofluorescence resonance energy transfer may occur because of an IP₃-induced conformational change in IP₃R1 that locates the Cy3-tagged antibody closer to Cy2-bound TRPC3. Alternatively, ET-1 may cause IP₃R clustering,³⁰ thereby locating more Cy3-tagged IP₃R1 channels in the vicinity of Cy2-bound TRPC3. The FRET method cannot differentiate between these potential mechanisms, but these possibilities deserve future investigation.

Co-IP data indicated that IP₃R1 and TRPC3 are located in the same arterial macromolecular complex that does not contain TRPC6. The small arteries used for co-IP experiments inevitably contained endothelium. Complementary data, including those obtained using FRET, patch-clamp electrophysiology, RNA interference, antibodies and peptides, indicate that close spatial and functional interaction occurs between IP₃R1 and TRPC3 in isolated myocytes. Arterial myocyte TRPC3 and TRPC6 channels both contained CIRB domains. Thus, although both TRPC3 and TRPC6 could interact molecularly with IP₃R1, spatial proximity determines their ability to communicate. The CIRBP sequence used here as a competitive antagonist is identical to the sequence in TRPC3, and between 11% (TRPC1) and 89% (TRPC7) homologous to sequences in other TRPC channel isoforms.²⁴ The CIRBP peptide did not alter Iₐᵥ or diameter in the absence of ET-1 or IP₃, but reduced ET-1 and IP₃-induced Iₐᵥ activation and vasoconstriction. Additional support that the CIRB domain is functional in arterial myocytes was data indicating that IP₃RntP stimulated a Gd³⁺-sensitive Iₐᵥ. The IP₃RntP should activate all TRP channels that contain a CIRB domain, including TRPC3, regardless of their proximity to IP₃Rs. However, TRPC isoforms also have variable affinities for the IP₃R N terminus.²¹ Taken together our data indicate that: (1) IP₃R1 and TRPC3 proteins are maintained in close proximity in the absence of receptor agonists; (2) physical interaction between the IP₃R1 N terminus and TRPC3 channel C terminus is weak in the absence of IP₃, indicating that the interaction domains do not maintain the close proximity; and 3) IP₃ enhances physical interaction between IP₃R1 and TRPC3. Although IP₃R1 did not activate TRPC6, IP₃R1 may activate TRPC isoforms other than TRPC3 in arterial myocytes. Given that there are seven TRPC isoforms and 3 IP₃R isoforms, it was beyond the scope of the current study to determine which of the potential twenty-one interactions may occur molecularly and functionally in arterial myocytes. TRPC channel heteromultimers have been reported in cultured vascular myocytes and A7r5 cells,³¹ but it is unclear whether TRPC channels that couple to IP₃R1 in arterial myocytes are homomultimers or heteromultimers. TRPC3 knockdown reduced TRPC3 protein by ≈40% and attenuated IP₃-induced Iₐᵥ by ≈41%. A TRPC3-selective antibody also similarly inhibited recombinant TRPC3 currents and IP₃-induced Iₐᵥ in arterial myocytes. Therefore, any IP₃-induced current mediated by channels other than TRPC3 would be small in arterial myocytes. In other native cell types, IP₃Rs and TRPC channels may exhibit different isoform-dependent coupling patterns, including promiscuous coupling similar to that with recombinant channels.

Several stimuli, including receptor agonists, increase plasma membrane trafficking of recombinant TRPC3 channels.³² Biotinylation indicated that over the same time course that ET-1 activated Iₐᵥ in arterial myocytes, total TRPC3 or plasma membrane-localized TRPC3 did not change. These data indicate that ET-1 elevates Iₐᵥ by increasing the Pᵥ of membrane-resident TRPC3 channels. Biotinylation also indicated that a large proportion of TRPC3 protein is intracellular. One explanation for this data are that TRPC3 channels may be retained, for example by RNF24,³³ and that signaling pathways other than those activated by acute ET-1 application may stimulate plasma membrane insertion. TRPC3 channels are also present on intracellular organelle membranes.²⁹ Given that FRET was detected at the plasma membrane and intracellularly, our data indicate that intracellular TRPC3 channels are also in close proximity to IP₃R1. Protein complexes containing both IP₃R1 and TRPC3 may also form before plasma membrane insertion of TRPC3.²⁹ PLC not only generates IP₃, but also elevates DAG, which activates PKC. CIRBP reduced ET-1– and IP₃-induced Iₐᵥ activation by >60%. These data indicate that ET-1 and IP₃ stimulate a Iₐᵥ primarily through molecular coupling of IP₃Rs to TRPC channels, rather than through DAG and PKC activation. This conclusion is consistent with previous evidence that IP₃R1 antibodies and IP₃R1 knockdown also inhibit UTP-induced Iₐᵥ activation in arterial myocytes.⁵ DAG and PKC can regulate TRPC channels, but in vascular myocytes isolated from a variety of different blood vessels, regulation is complex with studies reporting PKC-mediated activation, inhibition, or no effect.¹⁴,³⁴–³⁶ In cerebral artery myocytes, PKC stimulates a Iₐᵥ by elevating TRPM4 channel apparent micromolar Ca²⁺ sensitivity.³⁷,³⁸ Here, in patch-clamp experiments, free intracellular Ca²⁺ was strongly buffered using EGTA and ET-1–induced PKC activation would not stimulate TRPM4 channels.³⁷,³⁸ Conceivably, a proportion of the CIRBP-insensitive ET-1–induced vasoconstriction may occur because of PKC activation of other TRP channels, including TRPM4. Agonists also stimulate vasoconstriction via additional mechanisms, including activation of Ca²⁺ waves and voltage-dependent Ca²⁺ channels, and elevation of myofilament Ca²⁺ sensitivity.²,³
In summary, we describe a novel mechanism by which IP$_3$Rs regulate arterial diameter. Our data indicate that ET-1 and IP$_3$ activate membrane-resident TRPC3 channels in arterial myocytes by causing an interaction between the IP$_3$R1 N-terminal TRPC channel interaction sequence and the C-terminal TRPC3 channel CIRB domain. We also provide a mechanism by which IP$_3$R1 activation selectively stimulates TRPC3, but not TRPC6, channels and indicate that this occurs because of the close spatial proximity of these proteins which allows physical interaction. Physical coupling between IP$_3$R1 and TRPC3 channels could be manipulated therapeutically to regulate arterial contractility and, thus, brain blood flow.

Acknowledgments
We thank Dr Lidia A. Gardner for technical advice with FRET measurements.

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Disclosures
None.

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Disclosures
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References

**Novelty and Significance**

**What Is Known?**
- Phospholipase C–coupled receptor agonists elevate inositol 1,4,5-trisphosphate (IP₃) in arterial smooth muscle cells, leading to the activation of sarcoplasmic reticulum (SR) type 1 IP₃ receptors (IP₃Rs), an increase in intracellular calcium ([Ca²⁺]ᵢ) concentration, and vasoconstriction.
- Canonical transient receptor potential (TRPC) channels are molecularly and functionally diverse proteins that regulate smooth muscle cell plasma membrane cation influx and vascular contractility.
- The conventional view has been that IP₃Rs stimulate vasoconstriction by releasing SR Ca²⁺, although recent evidence indicates that IP₃Rs also induce vasoconstriction via an SR Ca²⁺ release-independent mechanism that involves TRPC channel activation; the mechanism is unidentified.

**What New Information Does This Article Contribute?**
- Type 1 IP₃ receptors are located in very close spatial proximity to plasma membrane TRPC3 channels in arterial smooth muscle cells.
- Vasoconstrictor agonists and IP₃ induce physical interaction between the IP₃R1 N terminus and the TRPC3 channel C-terminus calmodulin and IP₃/R binding domain, leading to TRPC3 channel activation and vasoconstriction.

Many vasoconstrictors elevate intracellular IP₃, but the mechanisms by which this second messenger stimulates vasoconstriction are poorly understood. We used a combination of molecular, cellular, and physiological approaches to examine IP₃R regulation of TRPC channels in cerebral artery smooth muscle cells. We found that IP₃R1, the predominant functional IP₃R isoform in arterial smooth muscle cells, physically interacts with and directly activates plasma membrane TRPC3, but not TRPC6, channels. To our knowledge, this is the first demonstration that:
1. native IP₃Rs physically interact with and activate native TRPC channels, and
2. IP₃Rs directly regulate the activity of a plasma membrane ion channel in arterial smooth muscle cells. Physical coupling of IP₃R1 to TRPC3 channels occurs due close spatial proximity of these proteins. In contrast, TRPC6 channels are not located nearby IP₃R1 and do not couple. Our findings identify a novel mechanism of arterial contractility regulation whereby agonist-induced IP₃ stimulates coupling of IP₃Rs to TRPC3 channels, resulting in plasma membrane cation influx, and vasoconstriction. We also show that manipulating the interaction between IP₃R1 and TRPC3 channels is a novel mechanism that could be exploited to regulate arterial contractility.
Isocform-Selective Physical Coupling of TRPC3 Channels to IP$_3$ Receptors in Smooth Muscle Cells Regulates Arterial Contractility
Adebowale Adebiyi, Guiling Zhao, Damodaran Narayanan, Candice M. Thomas, John P. Bannister and Jonathan H. Jaggar

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Materials and Methods

Tissue Preparation and Smooth Muscle Cell Isolation
Posterior cerebral, cerebellar, and middle cerebral arteries (100–200 µm) were harvested from rat brain maintained in ice-cold (4°C) oxygenated (21% O2-5% CO2-74% N2) physiological saline solution (PSS) containing: (in mmol/L) 119 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.6 CaCl2, 1.2 MgSO4, and 11 glucose (pH 7.4). Individual smooth muscle cells were dissociated from the cerebral arteries using a HEPES-buffered solution containing: (in mmol/L) 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl2, 10 HEPES, and 10 glucose (pH 7.3), as previously described. Briefly, cerebral arteries were placed into isolation solution containing 0.7 mg/ml papain, 1 mg/ml dithioerythreitol and 1 mg/ml bovine serum albumin (BSA) for 12 minutes at 37 °C. Arteries were then immediately transferred to isolation solution containing 1 mg/ml collagenase F and H (2:1), 100 μmol/L CaCl2 and 1 mg/ml BSA for 10 minutes at 37 °C. Arteries were subsequently washed in isolation solution and triturated using a fire-polished glass pipette to yield single smooth muscle cells. Cells were maintained at 4°C, and used for experimentation within 8 hours.

Immunofluorescence resonance energy transfer (immuno-FRET)
Freshly isolated cerebral artery smooth muscle cells were allowed to adhere to poly-L-lysine coated coverslips. Cells were fixed with 3.7% paraformaldehyde in Phosphate-Buffered Saline (PBS) for 15 min. Paraformaldehyde-fixed cells were then washed three times with PBS and permeabilized with 0.1% triton X-100 for 1 min at room temperature. Following a 1 h incubation in PBS containing 5% bovine serum albumin (BSA), smooth muscle cells were treated for 1 h at 37°C with primary antibodies: mouse monoclonal anti-IP3R1 (NeuroMab) and either rabbit polyclonal anti-TRPC3 (Alomone), rabbit polyclonal anti-TRPC6 (Alomone), or rabbit polyclonal anti-TRPM4 (Affinity BioReagents, Golden CO), each at a dilution of 1:100 in PBS containing 5% BSA. After a wash and block with PBS containing 5% BSA, smooth muscle cells were incubated for 1 h at 37°C with secondary antibodies: Cy3-conjugated Donkey Anti-Mouse for IP3R1 (Jackson ImmunoResearch) and Cy2-conjugated Goat Anti-Rabbit (Jackson ImmunoResearch) for TRPC3, TRPM4, or TRPC6. Following wash and mount, fluorescence images were acquired using a Zeiss LSM Pascal laser-scanning confocal microscope. Cy2 and Cy3 were excited at 488 and 543 nm and emission 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 Xia method and Zeiss LSM FRET Macro tool version 2.5.

Reverse transcription polymerase chain reaction (RT-PCR)
Briefly, total RNA was prepared from isolated cerebral artery smooth muscle cells using Absolutely RNA nanoprep kit (Stratagene, La Jolla, CA). cDNA was synthesized from DNase-treated RNA samples using AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene). cDNA products were amplified by nested-PCR using gene-specific oligonucleotide primer pairs. The oligonucleotide primer sequences used for RT-PCR were as follows: TRPC3 channel CIRB domain, first-round PCR: 5’-ATCATGAGGATCACTAACTTTTCCA-3’ (forward) and 5’-TATCGTGTGCTGATGAGA-3’ (reverse); nested-PCR: 5’-GAAGTAGGCGATGGTAAAC-3’ (forward) and 5’-TGCTGATTGAGAATGCTGTTA-3’ (reverse). TRPC6 channel CIRB domain, first-round PCR: 5’-TGACAGAATCGCTGTCGACA-3’ (forward) and 5’-TGGGCCTGTCAGTACCT-3’ (reverse); nested-PCR: 5’-GAAATCGCTGCGCAGACACAA-3’ (forward) and 5’-CATGATTTCCTGATCGCTCTTGA-3’ (reverse). GeneBank accession numbers used to design TRPC3 and TRPC6 CIRB domains were NM_021771.1 and NM_053559.1, respectively. PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY) with the
following reaction conditions: an initial denaturation at 94 °C for 2 min, followed by 40 cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s), with a final extension at 72 °C for 10 min. PCR products were separated by agarose gel (1.5%) electrophoresis and sequenced.

**Short Hairpin RNA Silencing Vector Construction**

Using pRNA-U6.1/Neo as a template, silencing vectors were constructed to express short hairpin (sh)RNA (GenScript Corp, Piscataway, NJ) as previously described.3 Expressed DNA sequences were as follows: for TRPC3shV, GTTCATACTTTACTCCTACTA; and for TRPC6shV, AGCTCAGAAGATTTCCATTTA.

**Reverse Permeabilization**

Silencing vectors were inserted intracellularly into cerebral artery segments using a reverse permeabilization procedure, as previously described.4 Arteries were placed into serum-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin and incubated at 37°C (95%O2, 5% CO2) for 4 days.

**Western Immunoblotting**

Western Immunoblotting was performed as previously described.3, 5 Briefly, rat cerebral artery lysate protein concentrations were determined spectrophotometrically with amido black solution. Proteins were separated by 4-15% gradient SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes using a Mini Trans Blot Cell (Bio-Rad, Hercules, CA). Membranes were then incubated in respective antibodies and developed using enhanced chemiluminescence (Pierce, Rockford IL).

**Co-immunoprecipitation**

For each experiment, lysate was harvested from cerebral arteries pooled from ~20 rats using ice-cold lysis buffer, giving ~1 mg total protein. The composition of lysis buffer was (in mmol/L): 140 NaCl, 8 sodium phosphate, 2 potassium phosphate, 10 KCl, and 0.2% Triton X-100, plus 1% protease inhibitor cocktail (Sigma). Arterial lysate was incubated with control mouse IgG or IP3R1 mouse monoclonal antibody (5 µg) at 4 °C overnight. Samples were then incubated with 50 µL protein A Sepharose beads (Pierce, Rockford, IL) for 2 h at room temperature. Beads were subsequently collected by centrifugation at 5000 g for 5 min at 4 °C and washed three times with lysis buffer. Laemmli sample buffer (2x, 25 µL) was added to protein beads and incubated for ~ 10 min at room temperature, followed by heating in boiling water for 5 min. Protein samples were then analyzed by Western blotting using mouse monoclonal anti-IP3R1 (NeuroMab), mouse polyclonal anti-TRPC3 (Abnova), rabbit polyclonal anti-TRPC6 (Alomone), and horseradish peroxidase-conjugated secondary antibodies, as previously described.3, 5

**Surface Biotinylation**

For each experiment, intact cerebral arteries from ~ 2 rats were incubated for 1 h at room temperature in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Pierce) in PBS pH 7.4. Unbound biotin was removed by quenching with PBS supplemented with 100 mmol/L glycine and excess removed by washing with PBS. Arteries were then homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. The total protein concentration of the supernatant was determined and used to calculate the amount of lysate to be used for Avidin (Monomeric Avidin, Pierce) pull-down of biotinylated surface proteins. This ensured that the same amount of total protein was present in all samples. Following pull-down, biotinylated proteins remained bound to the Avidin beads, whereas the supernatant contained non-biotinylated cytosolic proteins. Biotinylated proteins were eluted from Avidin beads by boiling in 1x SDS-buffer containing 2% 2-mercaptoethanol. Western blotting was used to quantify surface and cytosolic TRPC3 protein using rabbit polyclonal anti-TRPC3 (Abnova) and horseradish peroxidase-conjugated secondary antibody, as previously described.3, 5 Mouse monoclonal anti-Hsp90 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
Band intensity was determined using Quantity One software (BioRad). The percentage of total protein at the surface was calculated as 100 × [biotinylated/(biotinylated+non-biotinylated)].

**Cell culture and Transfection**

Human Embryonic Kidney 293 (HEK293) cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard tissue culture conditions (21% O₂-5% CO₂; 37°C). HEK293 cells were transfected with vectors encoding GFP (control), GFP-tagged TRPC3, or GFP-tagged TRPC6 (2 µg of each). Transfection was done using the Ca²⁺ phosphate method. TRPC3-GFP and TRPC6-GFP constructs were kindly provided by Dr. James Putney (NIEHS, Research Triangle Park) and Dr. Jochen Reiser (University of Miami Miller School of Medicine), respectively. The vectors used for transfection were confirmed by PCR analysis (Online Figure IV). Transfected cells grown on sterile glass coverslips were used for electrophysiological experiments. Transfected cells grown on 35 mm culture dishes were used for Western blotting experiments. Cells were used between 36 and 72 h after transfection.

**Patch-Clamp Electrophysiology**

Patch-clamp electrophysiology was performed on isolated arterial smooth muscle cells or HEK293 cells. Isolated smooth muscle cells were allowed to attach to a glass coverslip in the bottom of a chamber for 15 min prior to experimentation. Transfected HEK293 cells expressing GFP, GFP-tagged TRPC3, or GFP-tagged TRPC6 channels were grown on glass coverslips, which were then placed in the bottom of a patch-clamp chamber. GFP-positive HEK293 cells were visualized using a Nikon TS100 microscope using an epi-fluorescence attachment. Fluorescent HEK cells that were not attached to neighboring cells were used to obtain whole cell patch-clamp recordings. Membrane currents were measured using the patch-clamp technique (Axopatch 200B, Clampex 8.2). I_cat was measured using the conventional whole cell patch-clamp configuration, as we have done previously.³,⁵ Whole cell currents were measured by applying 940-ms voltage ramps between -120 and + 20 mV with a holding potential of -40 mV. Bath solution for conventional whole-cell experiments contained (in mmol/L): 140 NaCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 Hepes, and 10 glucose (pH 7.4). The pipette solution contained (in mmol/L): 140 CsCl, 10 HEPES, 10 glucose, 5 Mg-ATP, and 5 EGTA (with pH adjusted to 7.2 with CsOH), and 100 nmol/L free Ca²⁺. Where applicable, anti-TRPC3 and -TRPC6 antibodies were included in the pipette solution. Antibodies were denatured by incubation at 95 °C for 20 minutes. Cells were dialyzed with antibodies or peptides for 10 minutes prior to current recordings. Effects of Gd³⁺ were measured in paired experiments. Current amplitude at -120 mV was analyzed offline using pClamp 9 (Axon Instruments).

**Pressurized Artery Diameter Measurement**

An arterial segment 1–2 mm in length was cannulated at each end in a perfusion chamber (Living Systems Instrumentation; Burlington, VT). The chamber was continuously perfused with PSS equilibrated with a mixture of 21 % O₂-5 % CO₂-74 % N₂, and maintained at 37°C. Intravascular pressure was altered using an attached reservoir and monitored using a pressure transducer. Pressurized arteries were observed with a charge-coupled device camera attached to an inverted microscope (Nikon TE 200). Arterial diameter was measured at 1 Hz using the automatic edge-detection function of IonWizard software (Ionoptix; Milton, MA). Pharmacological compounds were applied via chamber perfusion.

**Chemicals**

Unless otherwise stated, all reagents were purchased from Sigma Chemical (St. Louis, MO). Papain was purchased from Worthington Biochemical (Lakewood, NJ) and 2,3,6-Tri-O-Butyryl-myo-Inositol 1,4,5-Trisphosphate-Hexakis(propionoxymethyl) Ester (Bt-IP₃) was purchased from SiChem (Bremen, Germany). CIRB and IP3RnTP peptides were synthesized by Sigma-Genosys (Woodlands, TX) while CIRBPscrm, CIRBP-TAT, and CIRBPscrm-TAT were synthesized by Molecular Resource Center, UTHSC (Memphis, TN).
Statistical Analysis
GraphPad Instat software (GraphPad Prism, San Diego, CA) was used for statistical analysis.
References

Online Figure Legends

**Online Figure I.** A. Antigenic peptides for each TRP channel primary antibody specifically block immunofluorescence produced by that antibody, but do not alter immunofluorescence detection by the other two primary antibodies. B. Whole western blot illustrating detection of IP$_3$R1 (~270 kDa), TRPC3 (~90 kDa), and TRPC6 (~110 kDa) proteins by their respective antibodies in cerebral arteries. C. TRPC3 antigenic peptide blocked TRPC3 detection, but had no effect on TRPC6 recognition. Similarly, a TRPC6 antigenic peptide blocked TRPC6 detection, but had no effect on TRPC3 recognition.

**Online Figure II.** A. TRPC3 (left panel) and TRPC6 (right panel) antibodies selectively detect overexpressed recombinant TRPC3 and TRPC6 in HEK293 cells. Each lane represents corresponding construct transfected into the cells. Faint TRPC3 and TRPC6 bands indicate endogenous TRPC3 and TRPC6 in HEK293 cells, as previously demonstrated. B. Exemplary recordings of $I_{\text{cat}}$ measured in HEK293 cells transfected with vectors encoding GFP (control), GFP-TRPC3, or GFP-TRPC6 and effects of TRPC3 and TRPC6 antibodies and heat-denatured antibodies (2 µg/mL each).

**Online Figure III.** Surface labeling of myocytes using Texas red-conjugated streptavidin in non-biotinylated (left) and biotinylated (right) intact cerebral arteries. Scale bar = 20 µm.

**Online Figure IV.** First-round PCR analysis of TRPC3 and TRPC6 vector constructs using specific primers.

**Online Figure V.** Proposed mechanisms by which IP$_3$R coupling to TRPC3 channels regulate IP$_3$-induced arterial smooth muscle cell contractility.
Online Fig. I

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- IP₃R1
- TRPC3
- TRPC6

C

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Online Fig. III
Online Fig. IV

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ET-1

Receptor

$G_{i}$

PLC

$IP_3$

$Na^+ Ca^{2+}$

Depolarization

$IP_3R1$

$Ca_{v}1.2$

$Ca^{2+}$

$Ca^{2+}$ waves

global $[Ca^{2+}]_i$

Vasoconstriction

Online Fig. V