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Isoform-selective physical coupling of TRPC3 channels to IP_3 receptors in smooth muscle cells regulates arterial contractility

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Abstract

Rationale—Inositol 1,4,5-trisphosphate (IP_3)-induced vasoconstriction can occur independently of intracellular Ca^{2+} release and via IP_3 receptor (IP_3R) and canonical transient receptor potential (TRPC) channel activation, but functional signaling mechanisms mediating this effect are unclear.

Objectives—Study mechanisms by which IP_3Rs 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 IP_3Rs (IP_3R1) 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 IP_3R1 and TRPC3, but not between IP_3R1 and TRPC6 or TRPM4. TRPC3, but not TRPC6, co-immunoprecipitated with IP_3R1. TRPC3 and TRPC6 antibodies selectively inhibited recombinant channels, but only the TRPC3 antibody blocked IP_3-induced non-selective cation current (I_{Cat}) in myocytes. TRPC3 knockdown attenuated immuno-FRET between IP_3R1 and TRPC3, IP_3-induced I_{Cat} activation, and ET-1 and IP_3-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 IP_3R binding (CIRB) domains are present in myocyte TRPC3 and TRPC6 channels. A peptide corresponding to the IP_3R N-terminal region that can interact with TRPC channels activated I_{Cat}. A TRPC3 CIRB domain peptide attenuated IP_3- and ET-1-induced I_{Cat} activation and vasoconstriction.

Conclusions—IP_3 stimulates direct coupling between IP_3R1 and membrane-resident TRPC3 channels in arterial myocytes, leading to I_{Cat} activation and vasoconstriction. Close spatial proximity between IP_3R1 and TRPC3 establishes this isoform-selective functional interaction.

Keywords

Inositol 1,4,5-trisphosphate; canonical transient receptor potential channel; coupling; vasoconstriction

Introduction

Activation of plasma membrane phospholipase C (PLC)-coupled receptors by vasoconstrictor agonists leads to phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis

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Disclosures
None.

and the generation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol.[1] In vascular myocytes, diacylglyceroI (DAG) activates protein kinase C (PKC), leading to the phosphorylation of a wide variety of proteins, including ion channels.[2] IP_3 binds to sarcoplasmic reticulum (SR) IP_3 receptors (IP_3Rs), resulting in SR Ca^{2+} release, an elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and vasoconstriction.[3] Recent evidence also indicates that IP_3-induced vasoconstriction can occur independently of SR Ca^{2+} release and via the activation of type 1 IP_3 receptors (IP_3R1) and type 3 canonical transient receptor potential (TRPC) channels.[4, 5] However, the functional signaling mechanisms by which IP_3Rs 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.[6] These families encode ion channels with diverse ion selectivity, modes of regulation, and physiological functions.[6] Vascular myocytes express at least four TRP families, including TRPC, TRPM, TRPV, and TRPP.[7–10] These channels regulate arterial myocyte membrane potential, $[Ca^{2+}]_i$, contractility, and proliferation, and are implicated in the etiology of vascular diseases.[4, 8–12] Given the diversity of vascular myocyte TRP channels, it has become important to identify signaling pathways that specifically regulate individual channel 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,[4, 9, 13, 14] 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_3R1, the principal molecular and functional arterial myocyte IP_3R isoform,[5] stimulates TRPC currents in resistance-size cerebral arteries. Data suggest that IP_3R1 is in close spatial proximity to, and associates with, TRPC3, but not TRPC6 or TRPM4 channels. Endothelin-1 (ET-1), a PLC-coupled receptor agonist, and IP_3 alter the interaction between the IP_3R N-terminus and the TRPC3 channel C-terminus, leading to channel activation and vasoconstriction. Data indicate that IP_3R1 selectively couples to TRPC3 channels due to the close spatial proximity of these proteins and that this mechanism is essential for mediating ET-1 and IP_3-induced vasoconstriction.

Materials and Methods

Tissue Preparation

Animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Sprague–Dawley rat (~250 g) resistance-size cerebral arteries and myocytes from these arteries were isolated as previously described.[4]

Immunofluorescence resonance energy transfer (immuno-FRET)

Paraformaldehyde-fixed myocytes were incubated with primary antibodies: mouse monoclonal anti-IP_3R1 and rabbit polyclonal anti-TRPC3, rabbit polyclonal anti-TRPC6, or rabbit polyclonal anti-TRPM4. Cells were then labeled with secondary antibodies: Cy3-conjugated donkey anti-mouse for IP_3R1 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 N-FRET calculated using the Xia method.[15]

TRPC channel knockdown

Silencing vectors that express TRPC3- (TRPC3shV), TRPC6- (TRPC6shV), or scrambled control (scrm) short hairpin RNA (shRNA) were inserted into cerebral arteries using reverse permeabilization, as previously described.[16]

Co-immunoprecipitation (co-IP)

Arterial lysate was incubated with control mouse IgG or IP_3R1 monoclonal antibody and then incubated with protein A sepharose beads. Protein samples were then analyzed by Western blotting using mouse monoclonal anti-IP_3R1, 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 (NIEHS) and Dr. Jochen Reiser (University of Miami), respectively. Electrophysiology and Western blotting experiments were performed 36–72 h after transfection.

Polymerase chain reaction (PCR)

Reverse transcription PCR (RT-PCR) was performed on pure populations of ~100 selected cerebral artery myocytes, as previously described.[18]

Patch-Clamp Electrophysiology

Membrane cation currents were measured in arterial myocytes and HEK293 cells using the conventional whole cell patch-clamp configuration, as done previously.[4, 5]

Pressurized Artery Diameter Measurement

Arterial diameter was measured using pressurized artery myography, as previously described.[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.

Expanded Materials and Methods are available as supplement documentation.

Results

IP_3R1 is in close proximity to TRPC3 channels in cerebral artery myocytes

IP_3R1 is the principal molecular and functional IP_3R isoform that is expressed in myocytes of cerebral arteries and aorta.[5, 19] To explore spatial proximity between IP_3R1 and three different TRP channel isoforms in cerebral artery myocytes, we used immuno-FRET. Specifically, spatial localization between IP_3R1 and TRPC3, TRPC6, or TRPM4 channels was examined. Cy3- and Cy2-labeled secondary antibodies bound to primary antibodies targeting IP_3R1 (monoclonal) and TRPC3 (polyclonal), respectively, produced mean N-FRET of ~25 % (Fig. 1A,B). In contrast, fluorescent antibodies bound to IP_3R1 and TRPM4

(polyclonal), or IP_3R1 and TRPC6 (polyclonal), generated mean N-FRET of ~11 % and ~12 %, respectively (Fig. 1A,B). ET-1 increased mean N-FRET between IP_3R1 and TRPC3-bound antibodies to ~34 %. In contrast, N-FRET between IP_3R1 and TRPC3-bound antibodies was not altered by membrane depolarization with 30 mmol/L K^+ (Fig. 1B). ET-1 did not change N-FRET between antibodies bound to IP_3R1 and TRPC6 or IP_3R1 and TRPM4 (Fig. 1A, B). Antigenic peptides for TRPC3, TRPC6, and TRPM4 specifically blocked immunofluorescence produced by each antibody, but did not alter immunofluorescence detection by the other two antibodies (Online Fig. IA).

Next, N-FRET was measured in myocytes in which TRPC3 or TRPC6 expression was reduced using shRNA and quantified using Western blotting. The selectivity of antibodies for Western blotting experiments was examined. Whole Western blots for IP_3R1, TRPC3 and TRPC6 antibodies on arterial lysate are illustrated in Online Fig. IB. The monoclonal IP_3R1 antibody detected only one band corresponding to IP_3R1 (Online Fig. IB), and this band is reduced by IP_3R1 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 Fig. IC). The TRPC3 antibody detected recombinant TRPC3 expressed in HEK293 cells, but did not detect recombinant TRPC6 (Online Fig. IIA). Similarly, the TRPC6 antibody detected recombinant TRPC6, but did not detect recombinant TRPC3 (Online Fig. IIA). 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 ~40 and 43%, respectively, of that in arteries treated with scrm (Fig. 1C,D). TRPC3shV did not alter TRPC6 and IP_3R1 expression and TRPC6shV did not alter TRPC3 and IP_3R1 expression (Fig. 1C,D). TRPC3 knockdown reduced control N-FRET and the ET-1-induced elevation in N-FRET between IP_3R1 and TRPC3 (Fig. 1E). In contrast, TRPC6 knockdown had no effect on N-FRET between IP_3R1 and TRPC6 in control or ET-1 (Fig. 1E), indicating that this signal represents background N-FRET.

Co-IP was used to examine whether IP_3R1 physically associates with TRPC3 and TRPC6 channels in small cerebral arteries. Due to the small size of the resistance-size vessels used in this study, arteries collected from ~20 rats were required for each experiment. The IP_3R1 monoclonal antibody co-immunoprecipitated both IP_3R1 and TRPC3 channels from lysate (Fig. 2). In contrast, TRPC6 was not detected in the same IP_3R1 immunoprecipitate (Fig. 2).

In summary, data indicate that TRPC3, but not TRPM4 or TRPC6, is in close spatial proximity to, and associates with, IP_3R1 channels in myocytes of resistance-size cerebral arteries. Data also demonstrate that ET-1 modifies the molecular relationship between IP_3R1 and TRPC3.

TRPC3 mediates IP_3-induced I_{Cat} activation in cerebral artery myocytes

The contribution of TRPC3 and TRPC6 channels to IP_3-induced I_{Cat} was examined. The TRPC3 antibody blocked whole-cell currents generated by recombinant TRPC3 expressed in HEK293 cells (Fig. 3A,B, Online Fig. IIB). 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 (Fig. 3A,B, Online Fig. IIB). Therefore, the antibodies selectively inhibit their respective TRPC currents. The TRPC3 antibody reduced mean IP_3-induced I_{Cat} density by ~57 % in arterial myocytes (Fig. 3C,D). In contrast, the TRPC6 antibody had no effect on IP_3-induced I_{Cat} density in arterial myocytes (Fig. 3C,D).

IP_3-induced I_{Cat} activation and vasoconstriction were studied in myocytes and arteries in which TRPC3 or TRPC6 expression was reduced using shRNA. TRPC3 knockdown reduced IP_3-induced I_{Cat} density by ~41 %. In contrast, TRPC6 knockdown did not alter IP_3-induced I_{Cat} density (Fig. 4A,B). TRPC6 knockdown reduces myogenic tone at intravascular pressures >40 mmHg.[10] Therefore, diameter regulation was measured in arteries pressurized to 20 mmHg 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 ~43 and 49 %, respectively (Fig. 4C,D,E). In contrast, TRPC6 knockdown had no effect on IP_3- and ET-1-induced vasoconstriction (Fig. 4C,D,E). These data indicate that TRPC3, but not TRPC6, channels mediate IP_3-induced I_{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 due to 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.[17]

Confirmation that biotin specifically labels surface proteins was obtained using biotin-treated arterial segments exposed to Texas Red-conjugated streptavidin (Online Fig. III). In addition, heat shock protein 90 (HSP90), an intracellular protein, was detected only in the non-biotinylated (cytosolic) fraction (Fig. 5A). Biotinylation indicated that in control, ~30 % of total TRPC3 protein was present in the arterial plasma membrane (Fig. 5B,C). ET-1 did not alter the percentage of plasma membrane-localized TRPC3 (Fig. 5B,C). These data indicate that a significant proportion of arterial TRPC3 is cytosolic and that ET-1 elevates myocyte I_{Cat} by stimulating plasma membrane-resident TRPC3 channels.

Calmodulin and IP_3R binding domain (CIRB) domains are present in myocyte TRPC3 and TRPC6 channels

Recombinant IP_3R N-termini contain a conserved amino acid sequence that can interact with a conserved C-terminal CIRB domain found on all on TRPC isoforms.[21, 22] TRPM4 does not contain a CIRB domain (Genbank Accession number: XM_574447). Our data indicate that isoform-selective association of TRPC3 channels to IP_3R1 occurs in arterial myocytes (Figs. 1⁻4). 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 Fig. 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 (Fig. 6A).

TRPC3-IP_3R1 physical coupling is required for ET-1- and IP_3-induced I_{Cat} activation

We tested the hypothesis that IP_3-induced I_{Cat} activation in arterial myocytes occurs through an interaction between the conserved IP_3R 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_{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 IP_3 and ET-1-induced I_{Cat} activation by ~63 % and 60 %, respectively, when compared with CIRBPscrm (Fig. 6B,C,D).

To study physiological functions of the IP_3R N-terminus, a peptide was constructed corresponding to the IP_3R N-terminal sequence containing the conserved TRPC-binding region (EEVWLFWRD, IP_3RntP). IP_3RntP increased mean I_{Cat} density by ~260 % (Fig.

7A,B). Gadolinium (Gd^{3+}), a non-selective cation channel blocker, had no significant effect on baseline currents, but reduced mean IP_3RntP-induced I_{Cat} densityby ~70% (Fig. 7A,B). These data indicate that physical association between the IP_3R1 N-terminus and the TRPC C-terminus CIRB domain leads to I_{Cat} activation in arterial myocytes.

CIRBP attenuates ET-1 and IP_3-induced vasoconstriction

The physiological function of molecular coupling between IP_3R and TRPC3 channels was investigated by measuring diameter regulation of pressurized (60 mmHg) arteries. Membrane-permeant CIRBP (CIRBP-TAT) and scrambled control (CIRBPscrm-TAT) peptides were each generated by conjugation to a HIV-1 TAT sequence (RRRQRRKKRGY).[25] CIRBP-TAT reduced vasoconstriction caused by Bt-IP_3, a membrane permeant IP_3 analog, and ET-1 by ~78 % and ~65 %, respectively, when compared with CIRBPscrm-TAT (Figs. 8A, B, C, and D). These data indicate that interaction between the IP_3R1 N-terminus and the TRPC3 channel CIRB domain contributes to IP_3 and ET-1-induced vasoconstriction.

Discussion

Here, we identify a novel vasoregulatory signaling mechanism whereby IP_3Rs couple to plasma membrane TRPC3 channels in arterial myocytes (Online Fig. V). Novel findings of this study are that in cerebral artery myocytes: 1) IP_3R1 is located in close proximity to TRPC3, but not TRPC6 or TRPM4 channels, 2) ET-1 elevates FRET between fluorescent antibodies bound to IP_3R1 and TRPC3 channels, but does not alter FRET between IP_3R1 and TRPC6 or TRPM4 channels, 3) TRPC3 channels co-IP with IP_3R1, whereas TRPC6 channels do not, 4) TRPC3, but not TRPC6, channels underlie IP_3-induced I_{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 IP_3- and ET-1-induced I_{Cat} activation and a peptide corresponding to the IP_3R1 N-terminal sequence that interacts with the TRPC CIRB domain activates I_{Cat}, 8) CIRBP attenuates IP_3-and ET-1-induced vasoconstriction. Taken together, these data indicate that IP_3R1 activation causes physical coupling of the IP_3R1 N-terminus to the TRPC3 channel C-terminus, leading to I_{Cat} activation and vasoconstriction. Physical coupling between all IP_3R and TRPC channel isoforms occurs with recombinant channels,[21–23, 26] To our knowledge, this is the first time that molecular coupling between IP_3R1 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 IP_3R1 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 IP_3R1 in arterial myocytes. Our study demonstrates that arterial myocyte IP_3R channels, previously considered to function solely by releasing SR Ca^{2+}, directly activate TRPC3 channels, thereby regulating arterial contractility. This study also illustrates that manipulating physical coupling between IP_3R1 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 three IP_3R isoforms, with IP_3R1 being the principal molecular and functional isoform.[5] All recombinant IP_3R isoforms contain an N-terminal TRPC channel interaction domain.[21, 22] Coupling of the IP_3R 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 IP_3Rs and TRPC channels may have occurred in response to TRPC channel overexpression.[21–23, 26] Therefore, it was unclear whether physical coupling between IP_3Rs 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, IP_3R stimulation leads to the activation of a I_{Cat} to which TRPC3 channels contribute.[4, 9] IP_3R-mediated I_{Cat} activation leads primarily to Na^+ influx, resulting in membrane depolarization, voltage-dependent Ca^{2+} channel activation, an $[Ca^{2+}]_i$ elevation, and vasoconstriction.[4] The mechanism by which IP_3Rs stimulate TRPC3 channels occurs independently of SR Ca^{2+} release and appears to be a major mechanism by which PLC-coupled receptor agonists cause vasoconstriction.[4] IP_3R1 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 IP_3 activate TRPC3, whereas intravascular pressure stimulates TRPC6 were unclear. Fluorescent antibodies bound to endogenous IP_3R1 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 IP_3R1/TRPC3 FRET. The calculated Forster distance between Cy2 and Cy3 is 5–6 nm, supporting other evidence in this study that IP_3R1 and TRPC3 are close enough to physically interact. In contrast, the FRET signal between IP_3R1 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 IP_3R1. Mechanisms that position TRPC3 nearby IP_3R1, but TRPC6 and TRPM4 away from IP_3R1 are unclear. Several possibilities exist, including differential localization by local lipid environments, including caveolae. It is also possible that IP_3R1 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 IP_3Rs.[29] The ET-1-induced elevation in immuno-FRET may occur due to an IP_3-induced conformational change in IP_3R1 that locates the Cy3-tagged antibody closer to Cy2-bound TRPC3. Alternatively, ET-1 may cause IP_3R clustering,[30] thereby locating more Cy3-tagged IP_3R1 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 IP_3R1 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 IP_3R1 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 IP_3R1, 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 I_{Cat} or diameter in the absence of ET-1 or IP_3, but reduced ET-1 and IP_3-induced I_{Cat} activation and vasoconstriction. Additional support that the CIRB domain is functional in arterial myocytes was data indicating that IP_3RntP stimulated a Gd^{3+}-sensitive I_{Cat}. The IP_3RntP should activate all TRP channels that contain a CIRB domain, including TRPC3, regardless of their proximity to IP_3Rs. However, TRPC isoforms also have variable affinities for the IP_3R N-terminus.[21] Taken together our data indicate that: 1) IP_3R1 and TRPC3 proteins are maintained in close proximity in the absence of receptor agonists; 2) physical interaction between the IP_3R1 N-terminus and TRPC3 channel C-terminus is weak

in the absence of IP_3, indicating that the interaction domains do not maintain the close proximity; and 3) IP_3 enhances physical interaction between IP_3R1 and TRPC3. Although IP_3R1 did not activate TRPC6, IP_3R1 may activate TRPC isoforms other than TRPC3 in arterial myocytes. Given that there are seven TRPC isoforms and three IP_3R 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 IP_3R1 in arterial myocytes are homomultimers or heteromultimers. TRPC3 knockdown reduced TRPC3 protein by ~40% and attenuated IP_3-induced I_{Cat} by ~41%. A TRPC3-selective antibody also similarly inhibited recombinant TRPC3 currents and IP_3-induced I_{Cat} in arterial myocytes. Therefore, any IP_3-induced current mediated by channels other than TRPC3 would be small in arterial myocytes. In other native cell types, IP_3Rs 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 I_{Cat} in arterial myocytes, total TRPC3 or plasma membrane-localized TRPC3 did not change. These data indicate that ET-1 elevates I_{Cat} by increasing the P_o of membrane-resident TRPC3 channels. Biotinylation also indicated that a large proportion of TRPC3 protein is intracellular. One explanation for this data is 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 IP_3R1. Protein complexes containing both IP_3R1 and TRPC3 may also form prior to plasma membrane insertion of TRPC3. [29]

Phospholipase C not only generates IP_3, but also elevates DAG, which activates PKC. CIRBP reduced ET-1- and IP_3-induced I_{Cat} activation by >60 %. These data indicate that ET-1 and IP_3 stimulate a I_{Cat} primarily through molecular coupling of IP_3Rs to TRPC channels, rather than through DAG and PKC activation. This conclusion is consistent with previous evidence that IP_3R1 antibodies and IP_3R1 knockdown also inhibit UTP-induced I_{Cat} 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 I_{Cat} by elevating TRPM4 channel apparent micromolar Ca^{2+} sensitivity.[37, 38] Here, in patch clamp experiments, free intracellular Ca^{2+} 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 due to PKC activation of other TRP channels, including TRPM4. Agonists also stimulate vasoconstriction via additional mechanisms, including activation of Ca^{2+} waves and voltage-dependent Ca^{2+} channels, and elevation of myofilament Ca^{2+} sensitivity.[2,3]

In summary, we describe a novel mechanism by which IP_3Rs 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_3R1 N-terminal TRPC channel interaction sequence and the C-terminal TRPC3 channel CIRB domain. We also provide a mechanism by which IP_3R1 activation selectively stimulates TRPC3, but not TRPC6, channels and indicate that this occurs due to the close spatial proximity of these proteins which allows

physical interaction. Physical coupling between IP_3R1 and TRPC3 channels could be manipulated therapeutically to regulate arterial contractility, and thus, brain blood flow.

Novelty and Significance

What Is Known?

- Phospholipase C-coupled receptor agonists elevate inositol 1,4,5-trisphosphate (IP_3) in arterial smooth muscle cells, leading to the activation of sarcoplasmic reticulum (SR) type 1 IP_3 receptors (IP_3Rs), an increase in intracellular calcium ($[Ca^{2+}]_i$) 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 IP_3Rs stimulate vasoconstriction by releasing SR Ca^{2+}, although recent evidence indicates that IP_3Rs also induce vasoconstriction via an SR Ca^{2+} release-independent mechanism that involves TRPC channel activation; the mechanism is unidentified.

What New Information Does This Article Contribute?

- Type 1 IP_3 receptors are located in very close spatial proximity to plasma membrane TRPC3 channels in arterial smooth muscle cells.
- Vasoconstrictor agonists and IP_3 induce physical interaction between the IP_3R1 N-terminus and the TRPC3 channel C-terminal calmodulin and IP_3R binding domain, leading to TRPC3 channel activation and vasoconstriction.
- In arterial smooth muscle cells, IP_3R1 coupling to TRPC3 channels is isoform-selective since IP_3R1 does not activate spatially separated TRPC6 channels.

Many vasoconstrictors elevate intracellular IP_3, but the mechanisms by which this second messenger stimulates vasoconstriction are poorly understood. We utilized a combination of molecular, cellular, and physiological approaches to examine IP_3R regulation of TRPC channels in cerebral artery smooth muscle cells. We found that IP_3R1, the predominant functional IP_3R 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 IP_3Rs physically interact with and activate native TRPC channels, and 2) IP_3Rs directly regulate the activity of a plasma membrane ion channel in arterial smooth muscle cells. Physical coupling of IP_3R1 to TRPC3 channels occurs due close spatial proximity of these proteins. In contrast, TRPC6 channels are not located nearby IP_3R1 and do not couple. Our findings identify a novel mechanism of arterial contractility regulation whereby agonist-induced IP_3 stimulates coupling of IP_3Rs to TRPC3 channels, resulting in plasma membrane cation influx, and vasoconstriction. We also show that manipulating the interaction between IP_3R1 and TRPC3 channels is a novel mechanism that could be exploited to regulate arterial contractility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Scrm	vectors encoding scrambled short hairpin RNA
TRPC3shV	vectors encoding TRPC3 short hairpin RNA
TRPC6shV	vectors encoding TRPC6 short hairpin RNA
PLC	phospholipase C
IP_3R	IP_3 receptors
CIRB	calmodulin and IP_3R binding
Immuno-FRET	Immunofluorescence resonance energy transfer
ET-1	endothelin-1
IP_3RntP	IP_3R1 N-terminal sequence peptide
I_{Cat}	non-selective cation current

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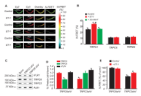

Figure 1. TRPC3, but not TRPM4 or TRPC6, is in close spatial proximity to IP_3R1 channels in arterial myocytes

A, Fluorescent images of individual Cy2 and Cy3 labels, pixel overlay, and N-FRET for indicated TRP channel primary antibody combinations. Scale bar=10 μm; **B,** Mean data illustrating control and ET-1 (100 nmol/L)-induced N-FRET generated by Cy3 bound IP_3R1 and Cy2-bound TRPC3, TRPM4, or TRPC6 antibodies, and effects of ET-1 and 30 mmol/L K^+. * $P<0.05$ compared with TRPC6 or TRPM4; # $P<0.05$ compared with IP_3R1-TRPC3 in control. n for columns from left to right are=27, 11, 15, 20, 28, 17, and 13, respectively. **C**, Representative Western blot illustrating that TRPC3shV causes selective knockdown of TRPC3 and TRPC6shV induces selective knockdown of TRPC6. **D,** Mean data illustrating effects of TRPC3shV and TRPC6shV on TRPC3 (n=4 and 6), TRPC6 (n=4 and 6), and IP_3R1 (n=4 each), respectively (* $P<0.05$). **E,** TRPC6 knockdown did not reduce the N-FRET signal between IP_3R1 and TRPC6 in control (n=15) or ET-1 (n=10), whereas TRPC3 knockdown reduced the N-FRET signal between IP_3R1 and TRPC3 in control (n=10) and ET-1 (n=15). * $P<0.05$ compared with scrm.

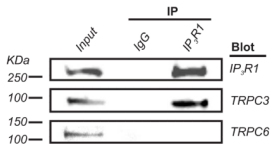

Figure 2. IP_3R1 interacts with TRPC3 but not TRPC6 channels in cerebral arteries

IP_3R1 monoclonal antibody co-immunoprecipitates IP_3R1 (~270 kDa) and TRPC3 (~90 kDa), but not TRPC6 (~110 kDa) in cerebral arteries. Lysate supernatant (20 μg protein) was used as the input control. Mouse IgG was used instead of the IP_3R1 monoclonal antibody as a negative co-IP control.

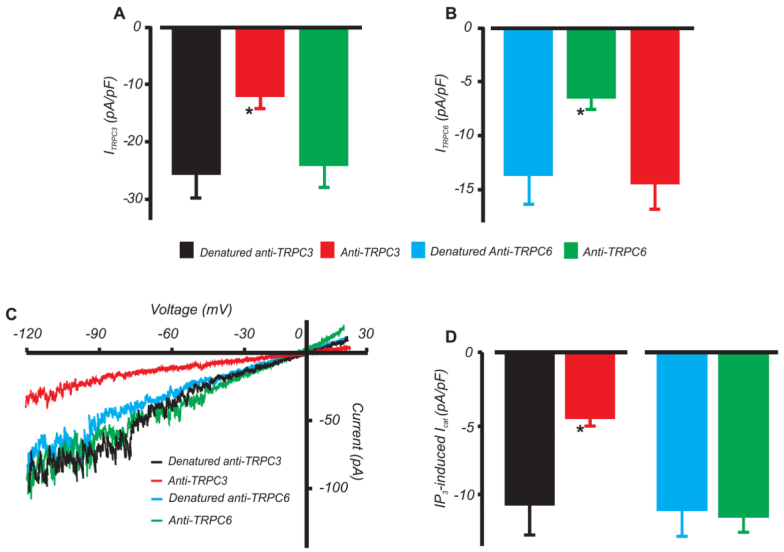

Figure 3. TRPC3 mediates IP_3-induced I_{Cat} in arterial myocytes

A,B, Mean data for current density generated in HEK293 cells in response to transfection with recombinant TRPC3 or TRPC6 channels, and selective inhibition by TRPC3 and TRPC6 antibodies (2 μg/mL each). Whole cell currents were essentially absent in cells transfected with only GFP. n for columns from left to right are = 7, 7, 6, 6, 6, and 4, respectively. **C,** Exemplary recordings illustrating inhibition of IP_3 (10 μmol/L in pipette solution)-induced I_{Cat} by TRPC3 antibody (2 μg/mL), but no effect of TRPC6 antibody (2 μg/mL), in arterial myocytes. **D,** Mean data for active and denatured TRPC3 (n=8 each) and TRPC6 antibody (n=7 each) in arterial myocytes. *P<0.05 vs. denatured control.

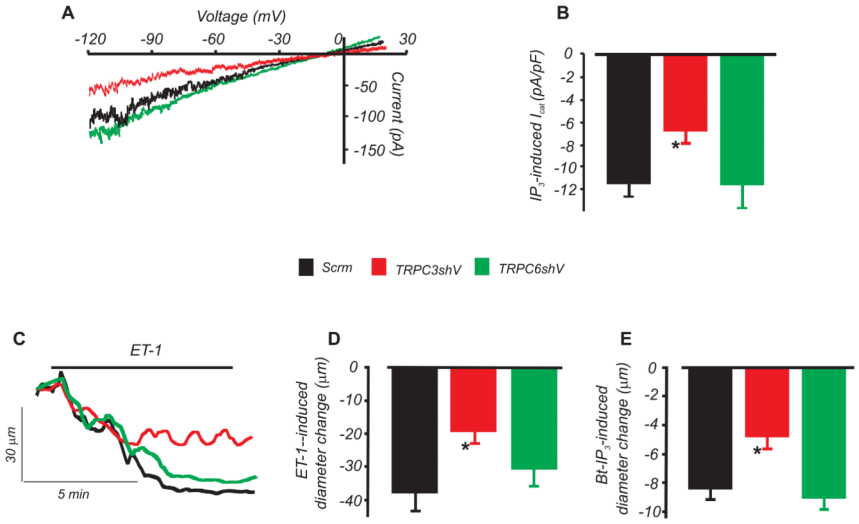

Figure 4. TRPC3 knockdown attenuates IP_3-induced I_{Cat} activation and vasoconstriction whereas TRPC6 knockdown has no effect

A, Exemplary traces of IP_3 (10 μmol/L)-induced I_{Cat} in arterial myocytes treated with scrm, TRPC3shV, or TRPC6shV. **B,** Mean I_{Cat} density (n: scrm, 6; TRPC3shV; 10, TRPC6shV, 8). **C,** Exemplary recordings of ET-1-induced vasoconstriction in pressurized (20 mmHg) arteries treated with scrm, TRPC3shV, or TRPC6shV. **D and E,** Mean data for ET-1- (300 pmol/L) and Bt-IP_3 (10 nmol/L)-induced vasoconstriction (n=5, 4, and 4 for scrm, TRPC3shV, and TRPC6shV, respectively). * P<0.05 versus scrm). Baseline tone was similar in scrm- (12 ± 4%, n=5), TRPC3shV- (10 ± 1%, n=4), and TRPC6shV- (11 ± 2%, n=4) treated arteries (P>0.05 for each).

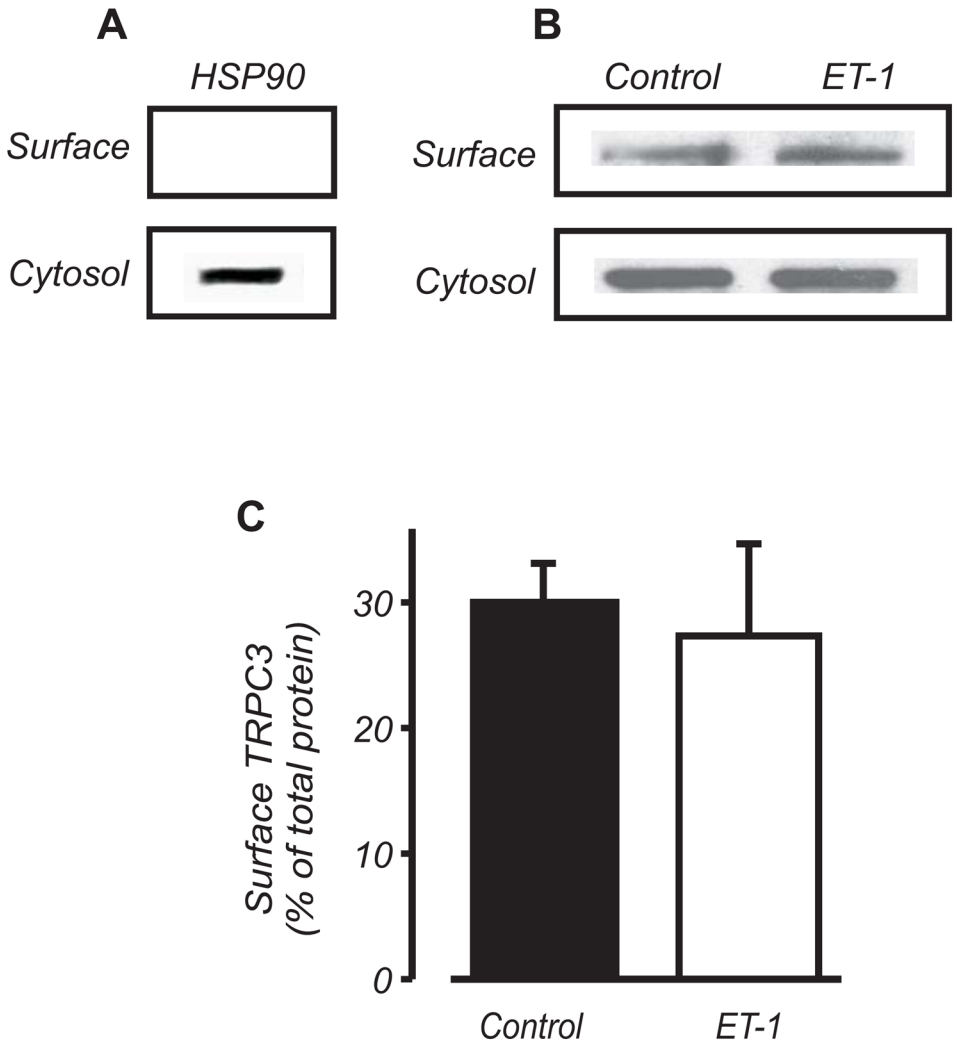

Figure 5. ET-1 does not increase surface expression of TRPC3 channels in cerebral arteries

A, Western blot illustrating that HSP90 is detected only in the non-biotinylated (cytosolic) fraction in cerebral arteries. **B,C,** Western blot and mean data (n=3) indicating that ET-1 (10 nmol/L) does not increase plasma membrane-localized TRPC3.

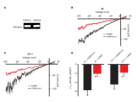

Figure 6. IP_3R to TRPC physical coupling is essential for ET-1- and IP_3-induced I_{Cat} activation in arterial myocytes

A, Cerebral artery myocyte TRPC3 and TRPC6 channels both contain CIRB domains, as determined using nested RT-PCR. **B and C,** Exemplary recordings illustrating attenuation of IP_3 (10 μmol/L) and ET-1 (100 nmol/L)-induced I_{Cat} activation by CIRBP (1 μmol/L via pipette) in arterial myocytes. **D,** Mean IP_3 and ET-1-induced change in I_{Cat} density in CIRBP or CIRBPscrm (1 μmol/L each) -treated myocytes (*P<0.05 vs. CIRBPscrm; n=9, 9, 10, and 8 for ET-1+CIRBPscrm, ET-1+CIRBP, IP_3+CIRBPscrm, and IP_3+CIRBP, respectively).

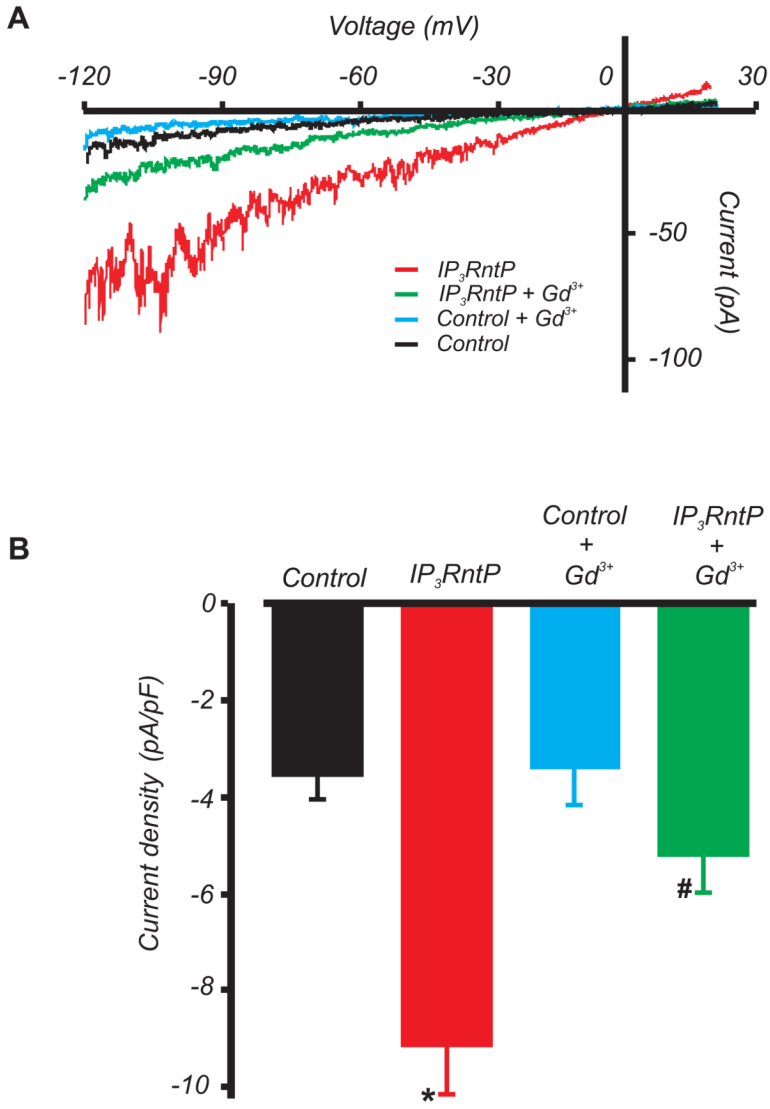

Figure 7. IP_3RntP activates non-selective cation currents in arterial myocytes

A, Exemplary recordings illustrating activation of Gd^{3+}-sensitive I_{Cat} by IP_3RntP (50 μmol/L via pipette). **B,** Mean I_{Cat} density. *P<0.05 vs. control; #P<0.05 vs. IP_3RntP (n=5 and 6 for control and IP_3RntP respectively).

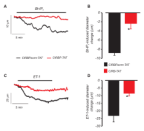

Figure 8. IP_3R to TRPC physical coupling contributes to ET-1 and IP_3-induced vasoconstriction

A, B, CIRBP-TAT (3 μmol/L, 20 min application) attenuates Bt-IP_3 (1 nmol/L, n=4)-induced vasoconstriction in pressurized (60 mmHg) cerebral arteries. **C, D,** CIRBP-TAT (3 μmol/L, 20 min application) attenuates ET-1 (1 nmol/L, n=7)-induced vasoconstriction (*P<0.05 vs. CIRBPscrm-TAT). When applied alone, CIRBP-TAT and CIRBPscrm-TAT similarly reduced mean baseline diameter by ~15 and 13 μm, respectively, consistent with a previous report for the TAT sequence.[25]