IP₃ Constricts Cerebral Arteries via IP₃ Receptor–Mediated TRPC3 Channel Activation and Independently of Sarcoplasmic Reticulum Ca²⁺ Release

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Abstract—Vasoconstrictors that bind to phospholipase C–coupled receptors elevate inositol-1,4,5-trisphosphate (IP₃). IP₃ is generally considered to elevate intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in arterial myocytes and induce vasoconstriction via a single mechanism: by activating sarcoplasmic reticulum (SR)-localized IP₃ receptors, leading to intracellular Ca²⁺ release. We show that IP₃ also stimulates vasoconstriction via a SR Ca²⁺ release–independent mechanism. In isolated cerebral artery myocytes and arteries in which SR Ca²⁺ was depleted to abolish Ca²⁺ release (measured using D1ER, a fluorescence resonance energy transfer–based SR Ca²⁺ indicator), IP₃ activated 15 pS sarcosomal cation channels, generated a whole-cell cation current (I₉₅₁) caused by Na⁺ influx, induced membrane depolarization, elevated [Ca²⁺]ᵢ, and stimulated vasoconstriction. The IP₃-induced I₉₅₁ and [Ca²⁺]ᵢ elevation were attenuated by cation channel (Gd³⁺, 2-APB) and IP₃ receptor (xestospongin C, heparin, 2-APB) blockers. TRPC3 (canonical transient receptor potential 3) channel knockdown with short hairpin RNA and diltiazem and nimodipine, voltage-dependent Ca²⁺ channel blockers, reduced the SR Ca²⁺ release–independent, IP₃-induced [Ca²⁺]ᵢ elevation and vasoconstriction. In pressurized arteries, SR Ca²⁺ depletion did not alter IP₃-induced constriction at 20 mm Hg but reduced IP₃-induced constriction by ∼39% at 60 mm Hg. [Ca²⁺]ᵢ elevations and constrictions induced by endothelin-1, a phospholipase C–coupled receptor agonist, were both attenuated by TRPC3 knockdown and xestospongin C in SR Ca²⁺-depleted arteries. In summary, we describe a novel mechanism of IP₃-induced vasoconstriction that does not occur as a result of SR Ca²⁺ release but because of IP₃ receptor–dependent I₉₅₁ activation that requires TRPC3 channels. The resulting membrane depolarization activates voltage-dependent Ca²⁺ channels, leading to a myocyte [Ca²⁺]ᵢ elevation, and vasoconstriction. (Circ Res. 2008;102:1118-1126.)

Key Words: vascular smooth muscle ■ voltage-dependent calcium channels ■ TRPC channels ■ endothelin-1

Many vasoconstrictors bind to phospholipase (PL)C–coupled receptors, leading to an elevation in intracellular diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). DAG directly modulates ion channels, including transient receptor potential (TRP) channels, and activates protein kinase (PKC), which can phosphorylate a wide variety of proteins that regulate arterial myocyte contractility, including ion channels.¹ ² In contrast, IP₃ binds to IP₃ receptors (IP₃Rs) located on the sarcoplasmic reticulum (SR), leading to SR Ca²⁺ release and an increase in myocyte [Ca²⁺]ᵢ.¹ The resulting activation of Ca²⁺/calmodulin-dependent myosin light chain kinase stimulates vasoconstriction.

The mechanism by which IP₃ regulates arterial contractility is generally well accepted.¹ Indeed, IP₃-induced SR Ca²⁺ release is considered to be the only mechanism by which this second messenger regulates arterial diameter. However, the physiological mechanisms by which IP₃ regulates intracellular Ca²⁺ signaling and arterial diameter are poorly understood, and few studies have directly tested the accepted view. Arterial contractility regulation by IP₃ has primarily been studied by using vasoconstrictors that activate PLC. Because PLC activation elevates both DAG and IP₃ and reduces PIP₂, mechanisms by which IP₃ specifically modulates arterial [Ca²⁺]ᵢ, signaling and diameter require additional study.

Here, we investigated IP₃ regulation of ion channel activity, intracellular Ca²⁺ signaling, and contractility in cerebral artery myocytes and pressurized arteries. We show that IP₃ activates a nonselective cation current (I₉₅₁) in myocytes and induces vasoconstriction via a mechanism that does not require the release of SR Ca²⁺ but involves IP₃R and TRPC3 (canonical transient receptor potential 3) channel activation. IP₃-induced Na⁺ influx produces membrane depolarization, voltage-dependent Ca²⁺ channel activation, an [Ca²⁺]ᵢ elevation, and vasoconstriction. We also show that TRPC3 channel...
activation is required for the \([\text{Ca}^{2+}]\), elevation and vasoconstriction induced by endothelin (ET)-1, a PLC-coupled receptor agonist, and that IP,\(_R\) activation contributes to these responses in SR \(\text{Ca}^{2+}\)-depleted arteries. These data indicate that IP,\(_R\) activation can stimulate vascular contraction through a mechanism that is independent of SR \(\text{Ca}^{2+}\) release.

### Materials and Methods

#### Tissue Preparation
Animal procedures used were approved by the Animal Care and Use Committee at the University of Tennessee. Sprague–Dawley rats (200 to 250g) of either sex were euthanized, and the brains were removed. Posterior cerebral, cerebellar, and middle cerebral arteries were harvested and used for all measurements. Myocytes were isolated as previously described,\(^{6,7}\) maintained at 4°C, and used for experimentation within 8 hours.

#### Adenovirus Construction

cDNA encoding D1ER, a fluorescence resonance energy transfer–based \(\text{Ca}^{2+}\) indicator protein that locates to the endoplasmic reticulum lumen, was kindly provided by Dr R.Y. Tsien (University of California, San Diego).\(^{4}\) A recombinant adenovirus expressing D1ER (adenD1ER) was constructed, as previously described.\(^{5}\) DMEM containing adenD1ER was inserted into the lumen of isolated endothelium-denuded cerebral artery segments that were placed in serum-free DMEM supplemented with 1% penicillin/streptomycin and incubated at 37°C (95% O\(_2\), 5% CO\(_2\)) for 4 days before experimentation.

#### Cytosolic and SR \(\text{Ca}^{2+}\) Imaging

cytosolic \([\text{Ca}^{2+}]\) was measured in isolated myocytes and endothelium-denuded arterial segments using fura-2. Myocytes were imaged using a charge-coupled device camera (Dage-MTI), and arteries were measured using a photomultiplier tube and Ionwizard software using a charge-coupled device camera (CoolSNAPfx charge-coupled device camera (Cairn Research)). For inside-out cation channel experiments, the pipette solution contained (in mmol/L): 140 CsCl, 3 MgCl\(_2\), 0.1 Pipes, 10 glucose (pH 7.4). For conventional whole-cell, pipette solution was used as the pipette solution and the whole-cell pipette solution was used as the bath solution (compositions above). For inside-out \(K_\text{cN}\) channel recordings, bath solution contained (in mmol/L): 140 KCl, 1.6 hydroxyethyltrismethylammonium acetate, 1 EGTA, 2 MgCl\(_2\), 10 Heps, and free \([\text{Ca}^{2+}]\) adjusted to 3 mmol/L as previously described (pH 7.2).\(^{8}\) For cell-attached experiments, the pipette solution was the whole-cell bath solution described above and the bath solution contained (in mmol/L): 140 KCl, 1.8 CaCl\(_2\), 1.2 MgCl\(_2\), 10 Heps, and 10 glucose (pH 7.4). Where appropriate, NaCl was substituted with equimolar \(N\)-methyl-D-glucamine-Cl. Whole-cell currents were measured by applying 940-ms voltage ramps between −120 and +20 mV. Single channel currents were measured at a steady voltage of −60 mV. Single channel activity (NPs) for each condition in each experiment was calculated by analyzing 5 seconds of continuous data by using pClamp 9 (Axon Instruments).

#### Pressurized Artery Membrane Potential Measurements

The membrane potential of pressurized artery segments was measured as previously described.\(^{7}\)

#### Pressurized Artery Diameter Measurements

Pressurized artery diameter was measured using an arteriograph, as previously described.

#### 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). shRNA expressed by each vector is processed by Dicer to generate siRNA that specifically targets exon 6 in TRPC3 (TRPC3shV) or a scrambled sequence (TRPC3scrm).\(^{9}\) Expressed DNA sequences were as follows: for TRPC3shV, GTTCATACTTTACTCCTACTA; and for TRPC3scrm, TGAACATCAGTGCTAGGTTAC.

#### Reverse Permeabilization

Silencing vectors were inserted intracelularly into cerebral artery segments using a reverse permeabilization procedure previously described.\(^{10,11}\) Arteries were placed into serum-free DMEM supplemented with 1% penicillin/streptomycin and incubated at 37°C (95% O\(_2\), 5% CO\(_2\)) for 4 days.

#### Western Immunoblot Analysis

Experiments were performed and data quantified as previously described.\(^{7,8}\) TRPC3 and TRPC6 channel protein bands were first normalized to actin and relative change in expression calculated as: (TRPC3shV/actin)/(TRPC3scrm/actin).

#### Statistical Analysis

Data are presented as means±SE. 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 comparisons. \(P<0.05\) was considered significant.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

### Results

**IP\(_3\) Elevates \([\text{Ca}^{2+}]\) in Myocytes With Intact and Depleted SR \(\text{Ca}^{2+}\)**

In isolated myocytes, from a resting \([\text{Ca}^{2+}]\) of 114±7 mmol/L, Bt-IP\(_3\) (10 μmol/L), a membrane-permeant IP, analog, caused a sustained, mean \([\text{Ca}^{2+}]\) elevation of \(=111\) mmol/L (Figure 1A and 1B). Bt-IP,–induced \([\text{Ca}^{2+}]\) elevations were attenuated by Gd\((\text{III})\) (30 μmol/L), a nonselective cation channel blocker; 2-APB (100 μmol/L), a nonselective cation channel and IP, R inhibitor; and diltiazem (50 μmol/L), a voltage-dependent \(\text{Ca}^{2+}\) channel blocker (Figure 1B). A combination of 2-APB (100 μmol/L) and diltiazem (50 μmol/L) blocked Bt-IP,–induced \([\text{Ca}^{2+}]\) elevations (Figure 1B). These data suggest that both SR \(\text{Ca}^{2+}\) release and sarcolemmal \(\text{Ca}^{2+}\) influx contribute to IP,–induced \([\text{Ca}^{2+}]\) elevations in arterial myocytes.

Because IP, is generally considered to elevate myocyte \([\text{Ca}^{2+}]\), by mobilizing SR \(\text{Ca}^{2+}\), we studied the contribution of SR \(\text{Ca}^{2+}\) release to IP,–induced \([\text{Ca}^{2+}]\) elevations. Thapsigargin (100 nmol/L), a SR \(\text{Ca}^{2+}\)-ATPase blocker, or a combina-
Figure 1. Bt-IP₃ elevates [Ca²⁺]ᵢ in arterial myocytes with intact and depleted SR Ca²⁺. A, Original traces illustrating [Ca²⁺]ᵢ regulation by Bt-IP₃ (10 µmol/L) in myocytes with intact (top) and depleted (bottom) SR Ca²⁺. B, Mean data. Control [Ca²⁺]ᵢ was 114±7 nmol/L (n=53). *P<0.05 compared with control in the same cell, †P<0.05 compared with Bt-IP₃, ‡P<0.05 compared with Bt-IP₃+thapsigargin (Tg) (100 nmol/L). The experimental numbers for bars from left to right were 6, 5, 4, 5, 7, 9, 5, and 4, respectively.

Figure 2. Thapsigargin (100 nmol/L to 5 µmol/L) depletes myocyte SR Ca²⁺. Images illustrate D1ER fluorescence in myocytes of a cerebral artery segment. Original trace is showing the time course of normalized D1ER yellow fluorescent protein (YFP)/cyan fluorescent protein (CFP) ratio change in the same artery in response to thapsigargin and Bt-IP₃ (10 µmol/L). Data are representative of 6 separate experiments.

To determine whether IP₃ elevated [Ca²⁺]ᵢ, by releasing a Ca²⁺ store that was not depleted by SR Ca²⁺-ATPase inhibition or ryanodine receptor channel activation, we measured [Ca²⁺]ᵢ within the entire SR using D1ER, a fluorescence resonance energy transfer–based Ca²⁺ indicator that targets to the endoplasmic reticulum lumen. This methodology also provides a time course of SR Ca²⁺ depletion. Thapsigargin (100 nmol/L) reduced D1ER mean fluorescence ratio to a plateau of 0.87±0.04 within 15 minutes, consistent with a reduction in SR Ca²⁺ concentration ([Ca²⁺]₀, n=6, P<0.05, Figure 2). Coapplication of Bt-IP₃ (10 µmol/L) with thapsigargin (100 nmol/L), or an elevation in thapsigargin concentration to 5 µmol/L, did not further change D1ER ratio (to 0.88±0.04 and 0.85±0.04, respectively, P>0.05 for each, n=6, Figure 2). Collectively, these data indicate that, in cerebral artery myocytes, a 15-minute application of 100 nmol/L thapsigargin depletes Ca²⁺ in the entire SR; that IP₃ does not further reduce [Ca²⁺]₀ when applied in the presence of 100 nmol/L thapsigargin; and that IP₃ elevates [Ca²⁺]ᵢ in arterial myocytes via a SR Ca²⁺ release-independent mechanism.

IP₃ Activates a Iᵣ in Myocytes With Intact and Depleted SR Ca²⁺

To investigate mechanisms by which IP₃ elevates [Ca²⁺]ᵢ in myocytes, we measured plasma membrane Iᵣ. When using the perforated patch-clamp configuration, Bt-IP₃ (10 µmol/L) increased mean whole-cell Iᵣ amplitude from 28 to 120 pA, or ~4.3-fold at ~120 mV (Figure 3B). Bt-IP₃ similarly activated Iᵣ when applied to myocytes pretreated with thapsigargin (Figure 3A and 3B). Iᵣ regulation by IP₃ or
photolytic release of intracellular caged IP$_3$ (both introduced via the pipette solution) was also studied in thapsigargin-treated myocytes by using the conventional whole-cell configuration, with [Ca$^{2+}$], strongly buffered using EGTA. IP$_3$ and release of caged IP$_3$ increased $I_{Ca}$ by 2.6- to 3.4-fold (Figure 3B). Gd$^{3+}$ reversed $I_{Ca}$ activation by Bt-IP$_3$ and caged IP$_3$ (Figure 3B). In contrast, thapsigargin (100 nmol/L to 5 μmol/L) did not alter $I_{Ca}$ (Figure 3B). These data indicate that IP$_3$ activates a $I_{Ca}$ in myocytes with either intact or depleted SR Ca$^{2+}$.

The cell-attached and excised inside-out patch-clamp configurations were used to study IP$_3$-activated single channel currents in myocytes with depleted SR Ca$^{2+}$ (100 nmol/L thapsigargin; >15 minutes). In 8 of 9 cell-attached patches at −60 mV, Bt-IP$_3$ increased the mean activity (NPO) of 0.91±0.13 pA single channels from 0.10±0.02 to 0.75±0.22 (Figure 3C). In inside-out patch-clamp experiments, we ensured that the internal membrane surface was accessible to the bath solution by confirming that KCa channels observed in a K$^+$-containing bath solution were inhibited on switching to the CsCl-containing bath solution used to measure cation channels. In inside-out membrane patches at −60 mV, IP$_3$ (20 μmol/L, n=27), oleoyl acetyl glycerol (OAG) (10 μmol/L, n=8), or IP$_3$ (20 μmol/L, n=8) did not activate single channels. Therefore, these data suggest that an intact myocyte is required for IP$_3$-induced cation channel activation.

We next investigated mechanisms mediating IP$_3$-induced $I_{Ca}$ activation. Xestospongin C (XeC), a membrane permeant IP$_3$R blocker, and heparin, an impermeant IP$_3$R inhibitor (1 mg/mL), reduced mean IP$_3$-induced $I_{Ca}$ in SR Ca$^{2+}$-depleted myocytes from ~95 to ~32 and 18 pA, respectively (Figure 3B). A reduction in extracellular Na$^+$ from 140 to 20 mmol/L also reduced mean IP$_3$-induced $I_{Ca}$ to ~33 pA (Figure 3B). These data indicate that IP$_3$R activation is required for IP$_3$-induced $I_{Ca}$ activation and that Na$^+$ is the principal cation generating $I_{Ca}$.

IP$_3$ Depolarizes and Constricts Pressurized Arteries With Intact and Depleted SR Ca$^{2+}$

Physiological functions of IP$_3$-induced $I_{Ca}$ activation were studied by measuring membrane potential and diameter regulation of pressurized endothelium-denuded arteries. Arteries were studied primarily at low intravascular pressure (20 mm Hg) to: (1) improve success of sustained microelectrode impalement by reducing vasomotion; (2) maintain a relatively negative membrane potential, which creates a larger driving force for cation influx; and (3) reduce negative-feedback regulation of membrane potential and diameter by Ca$^{2+}$ sparks and KCa channels.$^{3,13,14}$ At 20 mm Hg, Bt-IP$_3$ depolarized SR Ca$^{2+}$-depleted arteries by ~16 mV (Figure 4A and 4B). At 20 mm Hg, Bt-IP$_3$ also reduced the diameter of pressurized arteries with intact SR Ca$^{2+}$ by ~12 μm, from a mean diameter of 142±9 μm (Figure 5C). At 20 mm Hg, thapsigargin (100 nmol/L to 3 μmol/L) did not change diameter and did not reduce Bt-IP$_3$-induced vasoconstriction (Figure 5A and 5C). Nimodipine, a voltage-dependent Ca$^{2+}$ channel blocker, reduced Bt-IP$_3$-induced constriction by ~83% (Figure 5B and 5C). At 60 mm Hg, Bt-IP$_3$ reduced the diameter of arteries with intact SR Ca$^{2+}$ by ~11 μm (Figure 5D). At 60 mm Hg, thapsigargin (100 nmol/L) reduced diameter by ~12 μm and attenuated the Bt-IP$_3$-induced constriction by ~39% (Figure 5D). These data indicate that SR Ca$^{2+}$ release is not required for IP$_3$-induced vasoconstriction, IP$_3$, constricts via voltage-dependent Ca$^{2+}$ channel acti-
TRPC3 Channels Are Required for IP3-Induced [Ca2+]i Elevation and Vasoconstriction

Silencing vectors were constructed that express either shRNA targeting TRPC3 exon 6 (TRPC3shV) or scrambled shRNA (TRPC3scrm). Western blot analysis indicated that TRPC3shV reduced TRPC3 channel protein to \( \approx 34\% \) of TRPC3scrm (Figure 6A and 6B). In contrast, TRPC3shV did not alter TRPC6 channel expression in the same arteries (Figure 6A and 6B).

[Ca2+]i was measured in SR Ca2+-depleted myocytes that were isolated from arteries treated with TRPC3shV or TRPC3scrm vectors. TRPC3 knockdown did not alter resting [Ca2+]i (TRPC3scrm, 113 \( \pm \) 4 nmol/L, \( n = 14 \); TRPC3shV, 116 \( \pm \) 3 nmol/L, \( n = 19 \), \( P > 0.05 \)). However, mean Bt-IP3–induced [Ca2+]i elevations in TRPC3shV-treated myocytes were \( \approx 18\% \) of those in TRPC3scrm-treated myocytes (Figure 6C and 6D). In addition, Bt-IP3–induced constrictions in SR Ca2+-depleted, pressurized (20 mm Hg), endothelium-denuded, TRPC3shV-treated arteries were \( \approx 29\% \) of those in TRPC3scrm-treated arteries (Figure 7A through 7C). Collectively, these data indicate that TRPC3 channel activation contributes to the SR Ca2+ release–independent [Ca2+]i elevation and vasoconstriction induced by IP3.

ET-1 Elevates [Ca2+]i and Induces Vasoconstriction Independently of SR Ca2+ Release and via IP3R and TRPC3 Channel Activation

To investigate functional modulation of cerebral artery Ca2+ signaling and diameter by IP3Rs, we studied responses to ET-1, a vasoconstrictor that binds to myocyte PLC-coupled receptors, in endothelium-denuded arteries.15 ET-1 similarly elevated [Ca2+]i when applied to nonpressurized control arteries or arteries with depleted SR Ca2+ (Figure 8B). In SR Ca2+-depleted arteries, XeC did not alter [Ca2+]i when applied alone (3 \( \pm \) 6 nmol/L change, \( n = 5 \), \( P < 0.05 \)) but reduced mean ET-1–induced [Ca2+]i elevation by \( \approx 73\% \) (Figure 8A and 8B). TRPC3 knockdown also reduced mean ET-1–induced [Ca2+]i elevation to \( \approx 32\% \) of that in TRPC3scrm-treated arteries (Figure 8B). In agreement with the [Ca2+]i responses, ET-1 similarly constricted pressurized (20 mm Hg) arteries...
with intact or depleted SR Ca\(^{2+}\) (Figure 8C and 8D). XeC also reduced ET-1–induced vasoconstriction in arteries with depleted SR Ca\(^{2+}\) by \(-53\%\) (Figure 8C and 8D). In addition, TRPC3 knockdown reduced mean ET-1–induced constriction in pressurized (20 mm Hg) arteries to \(-55\%\) of that in TRPC3scrm-treated arteries (Figure 8D). These data indicate that TRPC3 channel activation is necessary for ET-1 to elevate \([\text{Ca}^{2+}]_i\), and induce vasoconstriction in cerebral arteries and that IP3R activation contributes to the SR Ca\(^{2+}\) release–independent responses.

**Discussion**

It is generally well accepted that IP3 contracts arterial myocytes by stimulating SR Ca\(^{2+}\) release. Here, we provide an additional mechanism by which IP3 regulates arterial contractility. In arterial myocytes, a transient or sustained IP3 channel, and voltage-dependent Ca\(^{2+}\) contractility through SR Ca\(^{2+}\), which TRPC3 channels contribute, leading to membrane depolarization, voltage-dependent Ca\(^{2+}\) channel activation, an \([\text{Ca}^{2+}]_i\), and vasoconstriction. These findings reveal a novel physiological signaling pathway for IP3 in the vasculature.

To study IP3 regulation of myocyte \(I_{\text{Ca}}\), \([\text{Ca}^{2+}]_i\), and contractility through SR Ca\(^{2+}\) release–independent mechanisms, SR Ca\(^{2+}\) was depleted using thapsigargin. A concentration of 100 nmol/L thapsigargin abolished caffeine-induced \([\text{Ca}^{2+}]_i\) transients, indicating that SR containing ryanodine receptor channels was Ca\(^{2+}\)-depleted. We also used D1ER to measure \([\text{Ca}^{2+}]_i\) in the entire SR and to obtain a time course of the thapsigargin-induced reduction in \([\text{Ca}^{2+}]_i\). Data indicated that a 15-minute application of 100 nmol/L thapsigargin depleted SR Ca\(^{2+}\). D1ER measurements also indicated that when IP3 was applied with thapsigargin, the \(I_{\text{Ca}}\) activation, \([\text{Ca}^{2+}]_i\), elevation, and vasoconstriction did not occur as a result of a further change in \([\text{Ca}^{2+}]_i\). In addition, in conventional whole-cell experiments, IP3 activated a \(I_{\text{Ca}}\) in myocytes that were exposed to thapsigargin and internally perfused with a pipette solution containing 5 mmol/L EGTA, which would deplete Ca\(^{2+}\) in all SR compartments. Collectively, these data indicate that IP3 activates a \(I_{\text{Ca}}\) via a SR Ca\(^{2+}\) release–independent mechanism.

Store-operated cation currents have been reported in myocytes from several vessels, including mesenteric artery, portal vein, and aorta. The regulation, ion selectivity, and conductance of myocyte store-operated cation currents vary depending on the anatomic origin of the vasculature. In the present study, thapsigargin (100 nmol/L to 5 μmol/L) did not activate a myocyte \(I_{\text{Ca}}\) and did not alter the diameter of arteries pressurized to 20 mm Hg but constricted arteries pressurized to 60 mm Hg. These data indicate that cerebral artery diameter regulation by SR Ca\(^{2+}\) depletion is pressure-dependent. These data are also consistent with previous evidence that SR Ca\(^{2+}\) depletion constricts cerebral arteries by inhibiting Ca\(^{2+}\) sparks and \(K_{\text{Ca}}\) channels, which are activated by an elevation in intravascular pressure. In nonpressurized rabbit and rat cerebral arteries, store depletion also did not induce depolarization and caused an \([\text{Ca}^{2+}]_i\), elevation that did not stimulate contraction. Here, thapsigargin caused a myocyte \([\text{Ca}^{2+}]_i\), transient that declined to a steady-state \([\text{Ca}^{2+}]_i\), elevation of \(-6\) nmol/L. Consistent with these findings, SR Ca\(^{2+}\)-ATPase inhibition also likely elevates cytosolic \([\text{Ca}^{2+}]_i\), by removing a SR Ca\(^{2+}\) sink. In contrast, in myocytes of...
rabbit pial arteriole fragments and murine cerebral artery myocytes, SR Ca\(^{2+}\) depletion activated an inward current.\(^ {22,23} \) Different findings of these studies may be attributable to experimental approach, species, vessel size, and anatomic origin. However, it is unclear why in the present study the thapsigargin-induced [Ca\(^{2+}\)] transient did not cause an associated transient vasoconstriction in pressurized arteries. Conceivably, this may occur because of Ca\(^{2+}\) compartmentalization in a noncontractile location that can activate CREB, as previously proposed.\(^ {20,24} \)

At 20 mm Hg, SR Ca\(^{2+}\) depletion did not alter IP\(_3\) or ET-1–induced vasoconstriction. In contrast, at 60 mm Hg, SR Ca\(^{2+}\) depletion reduced IP\(_3\)-induced vasoconstriction by \(\approx 39\%\). These data indicate that vasoregulation by IP\(_3\) is pressure-dependent. In addition, findings indicate that over this pressure range, IP\(_3\)R activation promotes vasoconstriction primarily by activating a \(I_{\text{Cat}}\), which is contrary to the conventional view of IP\(_3\)-induced vasoconstriction. The increasing contribution of SR Ca\(^{2+}\) release to the IP\(_3\)-induced vasoconstriction likely occurs because pressure-induced arterial depolarization activates voltage-dependent Ca\(^{2+}\) channels, which elevates cytosolic [Ca\(^{2+}\)]\(_{\text{SR}}\) and [Ca\(^{2+}\)]\(_{\text{SR}}\). Thus, data suggest that arterial depolarization increases the contribution of SR Ca\(^{2+}\) release to the IP\(_3\)-induced [Ca\(^{2+}\)] elevation and vasoconstriction. In isolated myocytes, SR Ca\(^{2+}\) depletion reduced the IP\(_3\)-induced [Ca\(^{2+}\)] elevation by \(\approx 25\%\). An explanation for this result is that isolated myocytes are more depolarized than myocytes in arteries at low pressure. Thus, isolated myocytes have a higher [Ca\(^{2+}\)]\(_{\text{SR}}\) and IP\(_3\)-induced SR Ca\(^{2+}\) release contributes to the [Ca\(^{2+}\)] elevation.

IP\(_3\) photoreleased IP\(_3\) and membrane permeant IP\(_3\) all activated a \(I_{\text{Cat}}\) in arterial myocytes via a mechanism that did not require the stimulation of SR Ca\(^{2+}\) release. \(I_{\text{Cat}}\) activation by IP\(_3\) was blocked by IP\(_3\)R inhibitors, cation channel blockers, and a reduction in extracellular Na\(^{+}\). In intact myocytes, IP\(_3\) activated 15 pS (0.91 pA at \(-60\) mV) single-channel currents. Our data suggest that, in cerebral artery myocytes, IP\(_3\) does not directly activate cation channels, and that DAG does not act as a cofactor for activation. In intact cerebral artery myocytes, DAG stimulates cation channels via PKC activation.\(^ {25} \) The lack of effect of OAG in inside-out patches suggests that activated PKC translocates from the cytosol to the membrane to stimulate cation channels. These data indicate that IP\(_3\)R activation mediates IP\(_3\)-induced \(I_{\text{Cat}}\) activation. In contrast, in rabbit portal vein myocytes excised patches, IP\(_3\) activated 2 pS Ca\(^{2+}\)-selective channels.\(^ {26} \) In rabbit coronary artery myocytes, IP\(_3\) only activated Ca\(^{2+}\)-selective channels if patches were also exposed to OAG.\(^ {27} \) Variability in \(I_{\text{Cat}}\) regulation by PLC products or SR Ca\(^{2+}\) depletion in myocytes of anatomically diverse arteries may occur because of cell type–specific TRP channel expression.\(^ {29-32} \) Rat cerebral artery myocytes express several TRP channels, including TRPC1, -3, -4, and -6 and TRPM4 and TRPV4.\(^ {10,21,29,33,34} \) Although TRPC3 channels are Na\(^{+}\) - and Ca\(^{2+}\)-permeant (\(P_{\text{Na}}/P_{\text{Ca}}\approx 1:1.5\)), Na\(^{+}\) influx would be predominant with physiological extracellular cation gradients, consistent with observations made here.\(^ {2,3} \) The IP\(_3\)-activated channels described here are also similar in conductance to TRPC3 channels.\(^ {28} \) We show that TRPC3 channel knockdown attenuates both the ET-1– and IP\(_3\)-induced [Ca\(^{2+}\)] elevation in myocytes and vasoconstriction in pressurized arteries. Whether the IP\(_3\)-activated channels in myocytes are formed from homomultimers or heteromultimers containing TRPC3 remains to be resolved, particularly

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**Figure 7.** TRPC3 knockdown reduces vasoconstriction induced by Bt-IP\(_3\). Original traces illustrating Bt-IP\(_3\)-induced (10 nmol/L) diameter responses in pressurized (20 mm Hg) arteries treated with TRPC3scrm (A) or TRPC3shV (B). C, Mean Bt-IP\(_3\)-induced diameter responses in pressurized (20 mm Hg) arteries treated with TRPC3scrm (n = 6) or TRPC3shV (n = 6). *P < 0.05 compared with TRPC3scrm.
because vascular myocyte heteromultimeric TRPC channels have been described. In cerebral artery myocytes, UTP, another PLC-coupled receptor agonist, activates TRPC3 channels but the mechanisms mediating this effect are unclear.34 Here, XeC reduced both the ET-1–induced \([\text{Ca}^{2+}]_i\) elevation and the constriction in SR \([\text{Ca}^{2+}]_i\)-depleted arteries. Voltage-dependent \([\text{Ca}^{2+}]_i\) channel blockers also robustly reduced the IP3-induced \([\text{Ca}^{2+}]_i\) elevation and vasoconstriction. Thus, data suggest that IP3 is one second messenger mediating vasoconstrictor-induced \(I_{\text{Ca}}\) activation, IP3R activation mediates this response, and IP3-induced \([\text{Ca}^{2+}]_i\) influx occurs primarily through depolarization-induced, voltage-dependent \([\text{Ca}^{2+}]_i\) channel activation.

A major finding of the present study is that IP3 constricted arteries not by stimulation of SR \([\text{Ca}^{2+}]_i\) release but by a mechanism which required IP3, R activation but not the stimulation of SR \([\text{Ca}^{2+}]_i\) release. We show that IP3 activation by IP3 stimulates an \(I_{\text{Ca}}\). The resulting \(Na^+\) influx induces membrane depolarization, leading to voltage-dependent \([\text{Ca}^{2+}]_i\) channel activation, an \([\text{Ca}^{2+}]_i\), elevation, and vasoconstriction. We also show that TRPC3 channel activation contributes to the IP3-induced \([\text{Ca}^{2+}]_i\) elevation and vasoconstriction. These findings supplement the conventional view that IP3 regulates arterial diameter only by stimulating SR \([\text{Ca}^{2+}]_i\) release.

**Sources of Funding**

This study was supported by NIH grants HL077678 and HL67061 (to J.H.J.); HL064981 (to C.M.W.); and HL063886 and HL072902 (to A.H.). Q.X. and A.A. are recipients of American Heart Association Postdoctoral Fellowships (Southeast Affiliate).

**Disclosures**

None.

**References**


IP₃ Constricts Cerebral Arteries via IP₃ Receptor–Mediated TRPC3 Channel Activation and Independently of Sarcoplasmic Reticulum Ca²⁺ Release
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Circ Res. 2008;102:1118-1126; originally published online April 3, 2008;
doi: 10.1161/CIRCRESAHA.108.173948

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/102/9/1118

An erratum has been published regarding this article. Please see the attached page for:
http://circres.ahajournals.org/content/suppl/2008/04/03/CIRCRESAHA.108.173948.DC1

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/04/03/CIRCRESAHA.108.173948.DC1

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IP$_3$ Constricts Cerebral Arteries via IP$_3$ Receptor-Mediated TRPC3 Channel Activation and Independently of Sarcoplasmic Reticulum Ca$^{2+}$ Release: Correction

In the article that appeared on page 1118 of the May 9, 2008, issue, the composition of the pipette solution used to record cation currents in the whole-cell configuration was incomplete. In addition to containing EGTA (5 mmol/L), the pipette solution also contained CaCl$_2$ to produce a free Ca$^{2+}$ concentration of 100 nmol/L. The inclusion of CaCl$_2$ in the pipette solution was omitted from the Materials and Methods section of the article. This methodological amendment does not alter any of the conclusions made in the article. The authors regret the error.

This error has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/reprint/102/9/1118

Reference


DOI: 10.1161/RES.0b013e3181b12da8
Supplemental Documentation

Materials and Methods

Smooth Muscle Cell Isolation

Individual smooth muscle cells were dissociated from arteries using a HEPES-buffered isolation solution containing: (in mM) 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl₂, 10 HEPES, and 10 glucose (pH 7.3 with NaOH), as previously described ¹. 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 18 minutes at 37 °C. Arteries were then immediately transferred to isolation solution containing 1 mg/ml collagenase, 100 μM CaCl₂ and 1 mg/ml BSA for 9 minutes at 37 °C. Arteries were subsequently washed in ice-cold isolation solution for 10 minutes and triturated using a polished glass pasteur pipette to yield single smooth muscle cells. Cells were allowed to adhere to a glass coverslip in the bottom of a chamber for 10 minutes prior to experimentation.

Adenovirus Construction

Linearized recombinant pShuttle and adenoviral backbone DNA (pAd59.2-100, Gene Transfer Vector Core, University of Iowa) were co-transfected in HEK 293 cells ². Recombinant adenoviral vectors were purified using Virakit Adeno4 from Virapur and titrated via tissue culture infection dose assay in HEK293 cells. AdenD1ER was amplified by the University of Iowa Gene Transfer Vector Core (Iowa City, IA) to high titer (1×10¹⁰ PFU/ml).
**Pressurized artery membrane potential measurement**

Prior to obtaining membrane potential recordings, arteries were maintained at either 10 or 50 mmHg for 2 hours to ensure steady-state myogenic tone had occurred. Membrane potential was measured by inserting glass microelectrodes filled with 3 M KCl (50-90 mΩ) into the adventitial side of pressurized arteries. Membrane potential was recorded using a WPI FD223 amplifier and digitized using pClamp 9.2 software (Axon Instruments) and a personal computer. Criteria for successful intracellular impalements were: (1) a sharp negative change in potential upon insertion; (2) stable voltage for at least 1 min after entry; (3) a sharp positive voltage deflection upon exit from the recorded cell, and (4), a <10 % change in tip resistance after the impalement.

**Cytosolic Ca\(^{2+}\) imaging**

Arterial wall \([\text{Ca}^{2+}]_i\), and isolated myocyte \([\text{Ca}^{2+}]_i\), were calculated using the following equation \(^3\):

\[
[\text{Ca}^{2+}]_i = K_d ((R - R_{min}) / (R_{max} - R)) \beta
\]

where \(R\) is the 340/380 nm ratio, \(R_{min}\) and \(R_{max}\) are the minimum and maximum ratios determined in Ca\(^{2+}\)-free and saturating Ca\(^{2+}\) solutions, respectively, \(\beta\) is the ratio of Ca\(^{2+}\) free to Ca\(^{2+}\) replete emissions at 380 nm excitation, and \(K_d\) is the dissociation constant for fura-2 (224 nM, \(^3\)). \(R_{min}\), \(R_{max}\), and \(\beta\) were determined by increasing Ca\(^{2+}\) permeability with ionomycin (10 µM) and perfusing arteries with 10 mM Ca\(^{2+}\) or Ca\(^{2+}\)-free PSS (no added Ca\(^{2+}\) with 10 mM EGTA), as described previously \(^4-6\).
**Pressurized artery diameter measurements**

An arterial segment 1–2 mm in length was cannulated at each end in a temperature-controlled 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. Arteries were observed with a charge-coupled device (CCD) camera attached to an inverted microscope (Nikon TE 200). Arterial diameter was measured by using the automatic edge-detection function of IonWizard software (Ionoptix; Milton, MA) and digitized at 1 Hz using a personal computer. Steady-state changes in intravascular pressure were achieved by elevating and lowering an attached reservoir and monitored using a pressure transducer. No intraluminal flow was present during the experiments. Tested compounds were applied via chamber perfusion.

**Western Immunoblotting**

Cerebral arteries were homogenized with a glass homogenizer in Laemmli sample buffer (2.5% SDS, 10% glycerol, 0.01% bromphenol blue and 5% β-mercaptoethanol in 100mM Tris·HCl, pH 6.8) and then centrifuged at 4000 x g for 10 min. Protein concentration in the supernatant was estimated by dotting 5 µl lysate aliquots on a nitrocellulose membrane, which were then stained with amido black solution and quantified spectrophotometrically at 630 nm. Proteins (30µg/lane) were separated by 4-15% gradient SDS-polyacrylamide gels, and transferred onto PVDF membranes using a Mini Trans Blot Cell (Bio-Rad, Hercules, CA). Membranes were first washed in Tris-buffered solution (TBS) with 0.1% Tween 20 (TBS-T) and then blocked in the same solution supplemented with 5% nonfat dry milk (blocking solution) for 1 hour. Membranes were initially probed with polyclonal anti-TRPC3 (1:500) overnight. After three washes with
TBS-T, membranes were incubated for 1 h with goat anti-rabbit secondary horseradish peroxidase (HRP)-conjugated antibodies (1:20,000, Pierce Bio.) and then developed for 1 min using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Membranes were incubated in stripping buffer (in mM: Tris base 62.5, SDS 70, β-mercaptoethanol 100, pH 6.7 adjusted with HCl) for 1 h at 37 ºC and re-probed with polyclonal anti-TRPC6 antibodies (1:500, Alomone). To normalize TRP channel expression, membranes were finally re-probed with monoclonal anti-actin (Chemicon) followed by HRP-conjugated anti-mouse IgG (Pierce Bio.). Negative controls were obtained by pre-incubating primary antibodies with antigenic peptide. Bands intensities were quantified by digital densitometry using Quantity One version 4.4.1 software.

**Chemicals**

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co (St Louis, MO). 2,3,6-Tri-O-Butyryl-myo-Inositol 1,4,5-Trisphosphate-Hexakis(propionoxymethyl) Ester (Bt-IP$_3$) and 1,2,4-Tri-O-Butyryl-myo-Inositol 3,5,6-Trisphosphate-Hexakis(propionoxymethyl) Ester (L-Bt-IP$_3$) were purchased from SiChem (Bremen, Germany). 2-nitrophenyl ethyl ester D-myo-inositol 1,4,5-trisphosphate (NPE-caged IP$_3$) was purchased from Calbiochem. XeC and 2-APB were purchased from Cayman Chemical (Ann Arbor, MI).
References


Supplemental Figure 1. Thapsigargin (Tg., 100 nM) abolishes caffeine-induced [Ca\textsuperscript{2+}]	extsubscript{i} transients in a cerebral artery, but does not block responses to 60 mM K\textsuperscript{+} (60 K), which induces voltage-dependent Ca\textsuperscript{2+} influx. Caffeine (Caf., 10 mM). Data is representative of 4 separate experiments.
Supplemental Figure 1