Inositol 1,4,5-Trisphosphate (IP₃) Receptor Up-regulation in Hypertension Is Associated with Sensitization of Ca²⁺ Release and Vascular Smooth Muscle Contractility

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Background: The role of the vascular IP₃ receptor (IP₃R) in hypertension is unknown.

Results: IP₃R are up-regulated in vascular smooth muscle (VSM) in hypertension through the calcineurin-NFAT pathway.

Conclusion: Up-regulated IP₃R in VSM sensitize Ca²⁺ release and enhance contraction.

Significance: Up-regulated vascular IP₃R may contribute to vascular resistance in hypertension.

Hypertension is a complex disease that is the clinical manifestation of multiple genetic, environmental, nutritional, and hormonal pathologies that predispose individuals to cardiovascular disease, including heart failure and stroke (1, 2). In recent years, it has become clear that structural alterations in arteries, referred to as remodeling, increase their stiffness and contribute to the development of hypertension (3, 4). Furthermore, arterial wall injury leads to a phenotypic switch of VSM from a contractile to a proliferative phenotype, which is associated with alterations to the VSM Ca²⁺ signaling machinery (5).

The major drop in hydrostatic pressure in the vasculature occurs at the level of resistance arteries. Because blood flow is proportional to the vessel radius to the 4th power, as described by Poiseuille’s law, small changes in the luminal diameter of resistance arteries will dramatically alter resistance to blood flow with a pronounced impact on blood pressure. Peripheral resistance depends on the diameter of resistance arteries, which hinges in part on the contractile state of the VSM cells (6, 7). The resting myogenic tone is set by the summed responses of the Ca²⁺ handling and contractile machineries to various inputs, including sympathetic neurotransmitters (norepinephrine) (8), endothelium-derived agents (NO, endothelin) (6), endocrine factors in the blood stream (angiotensin II) (2), and intraluminal pressure (9).

Several channels and transporters contribute to Ca²⁺ signaling and contraction in VSM, with two primary pathways being the agonist-activated PLC-IP₃R pathway and the voltage-gated L-type Ca²⁺ channel (LTCC) (7). The pore-forming subunit of the LTCC channel in VSM is α₉Ca (10). The fundamental role of these two Ca²⁺ signaling pathways in fueling vasoconstriction during hypertension is attested to clinically by the effective lowering of blood pressure following pharmacological block of the LTCC and α-adrenergic receptors (2). Consistently, norepinephrine produces greater vasoconstriction in hypertensive compared with normotensive individuals (11). However, the molecular mechanisms of this enhanced vasomotor response are unclear.

The abbreviations used are: VSM, vascular smooth muscle; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; NFAT, nuclear factor of activated T cells; Ca²⁺, calcium ion; LTCC, L-type Ca²⁺ channel; PLC, phospholipase C; PMCA, plasma membrane Ca²⁺ pump; AHT, angiotensin II-induced hypertension; SAL, saline; ciIP₃, caged IP₃; O₂⁻, O₂⁻-isosopropylidene-6-O-(2-nitro-4,5-dimethoxy) benzyl-myoinositol 1,4,5-trisphosphate-hexakis(propionoxylmethyl) ester; eNOS, endothelial nitric oxide synthase; NOS, nitric oxide synthase; Ang II, angiotensin II; SBP, systolic blood pressure; MA, mesenteric arteries; PSS, physiological salt solution; PE, phenylephrine; HBS, HEPES-buffered saline; 2-APB, 2-aminoethoxydiphenyl borate; W/V, von Willebrand factor; SOCE, store-operated Ca²⁺ entry; PMCA, plasma membrane Ca²⁺ pump; Nif, nifedipine; Thaps, thapsigargin; CHX, cycloheximide; ACD, actinomycin D; CsA, cyclosporine A.
Vascular IP3 Receptor in Hypertension

The role of the LTCC in modulating the myogenic response of VSM is well established through clinical, genetic, and physiological studies (7, 10, 12–15). In contrast, the contribution of the IP3-R remains poorly characterized. However, several lines of evidence support a role for the PLC-IP3 axis in regulating the myogenic tone. The IP3-R has been implicated in Ca2+-dependent contraction in response to vasoconstrictors central to blood pressure regulation, including norepinephrine and angiotensin II (16–18). In addition, G-protein-coupled receptor-dependent Ca2+ release has been implicated in vasoconstriction (19), and activation of α1-adrenergic receptors increases the percentage of VSM exhibiting Ca2+ oscillations (20). Furthermore, PLC/IP3-dependent Ca2+ release plays an important role in regulating myogenic tone caused by increased intraluminal pressure in isolated arteries (21, 22), and stretch of isolated VSM cells results in enhanced IP3 levels, leading to Ca2+ release (23, 24). Finally, PLC inhibition or Ca2+-store depletion results in vasodilation or a decreased myogenic response (25–27). Collectively, these findings suggest that the activity of in situ IP3-R is influenced by blood pressure and vasoactive agonists impinging on the VSM cells of resistance arteries.

Here, we report up-regulation of the IP3-R in resistance-size mesenteric arteries in hypertensive mouse and rat models. IP3-R up-regulation could be recapitulated in cultured VSM cells by depression-mediated activation of the LTCC, leading to induction of the Ca2+-dependent calcineurin-NFAT signaling pathway. Importantly, IP3-R up-regulation results in sensitization of both IP3-dependent Ca2+ release and VSM contraction. Hence, up-regulation of vascular IP3-R is poised to contribute to enhanced Ca2+ signaling and vasoactivity during hypertension.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. C57BL/6 mice were obtained at 10 weeks of age from Harlan Laboratories (Indianapolis, IN) and maintained in a temperature-controlled room in a 12-h/12-h light/dark cycle with free access to food and water. Wistar Kyoto rats and spontaneously hypertensive rats (SHR) were obtained at 12–14 weeks of age from Taconic Farms (Germany). Sprague-Dawley rats were obtained from Harlan Laboratories (Madison, WI) at the same age.

Minipump Implantation and Blood Pressure Measurements

Mice were anesthetized by 2.5% isoflurane inhalation. Osmotic minipumps (Alzet 1002, Durect Corp.) loaded with angiotensin II (Ang II; Bachem) to accomplish an infusion dose of 2 ng/min/g for 2 weeks or an equal volume of vehicle (0.9% saline) were implanted subcutaneously. After recovery from anesthesia, mice were housed in individual cages and allowed free access to food and water (28). The systolic blood pressure (SBP) was recorded by tail cuff plethysmography (Kent Scientific) before (day 0) and on days 7 and 14 after osmotic minipump implantation. As described earlier (12, 13), blood pressure was recorded by intra-arterial catheter in anesthetized normotensive and hypertensive rats directly prior to studies.

Vascular Reactivity Assays

Second order mesenteric arteries (MA) were isolated from saline (SAL) and Ang II-infused hypertensive (AHT) mice. Arteries were cleaned of adhered fat and connective tissue in ice-cold physiological salt solution (PSS) of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO4, 24 mM NaHCO3, 0.026 mM EDTA, 1.17 mM NaH2PO4, 5.5 mM glucose, and 1.6 mM CaCl2. Vessels were cannulated on both ends with tapered glass micropipettes in a microvessel perfusion system (Living Systems) containing PSS bubbled with a 95% O2, 5% CO2 gas mixture and maintained at 37 °C. Arteries were perfused with PSS at an intraluminal pressure of 60 mm Hg with no outflow. The PSS in the chamber was exchanged every 15 min during 1 h of equilibration. After equilibration, the viability of MA was verified by observing a contractile response to 60 mM KCl (28). Phenylephrine (PE) concentration-response curves (10−8 to 10−4 M; half-log increments) were obtained in the absence or presence of the IP3-R blocker, 2-aminooethoxycyclohexyl borate (2-APB) (50 μM). Changes in outer vessel diameter were recorded using an upright microscope/Spot RT camera and analyzed with automated edge detection and data acquisition software, DMTvas (Danish Myo Technology). The contractile response to PE was calculated as percentage change in diameter from the initial resting diameter.

Cell Culture and Transfection

Embryonic rat aortic smooth muscle-derived A7r5 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were seeded onto a 100-mm dish (BD Biosciences) and grown in Dulbecco’s modified Eagle’s medium-high glucose supplemented with 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 10% fetal bovine serum (Sigma). Cells were kept at 37 °C in a humidified atmosphere with 5% CO2, and fresh medium was added every 48 h until the start of studies.

The plasmid pEGFPC1-NFATc1 was a generous gift from G. Crabtree (Stanford University). A7r5 cells were transiently transfected with pEGFP-NFATc1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 3 days post-transfection, cells were washed with prewarmed Ca2+/Mg2+-free PBS and maintained for 30 min at 37 °C and 5% CO2 in HEPES-buffered saline (HBS): 145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 10 mM Hepes, 2 mM CaCl2, 10 mM glucose, pH 7.4. Fluorescent confocal imaging was performed on a Zeiss LSM 710 confocal microscope (Carl Zeiss) using an EC Plan Neofluar ×40/1.3 numerical aperture oil differential interference contrast objective. GFP signal was excited with a 488-nm laser, and emissions were collected through a bandwidth of 492–558 nm. Images were analyzed using ZEN 2008 software (Carl Zeiss).

Western Blotting

Mesenteric Arteries—MA from SAL and AHT mice were carefully isolated and cleaned of adherent fat and connective tissue. Arteries from each mouse were homogenized on ice in radioimmune precipitation assay buffer (Thermo Scientific) containing protease inhibitor mixture (Roche Applied Science). Large tissue debris and cell fragments were removed by centrifug-
against IP3R1 (1:1000; NeuroMab), STIM1 (1:1000; Abcam), (12, 13). In Western blots, monoclonal antibodies were directed against IP3R1 (1:10,000; EMD Millipore). Polyclonal antibodies were directed against IP3R1 (1:2000; NeuroMab), or Orai1 (1:1000; ProSci Inc.). After exposure to primary antibody, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled sheep anti-mouse IgG or donkey anti-rabbit IgG (GE Healthcare) in TBS-T containing 5% nonfat dried milk. The bands were identified as chemiluminescence and exposed to x-ray films. The density of immunoreactive bands was determined using ImageJ software (National Institutes of Health) and normalized to the GAPDH density for each lane (28). Similar procedures were used to detect rat arterial proteins on Western blot (12, 13).

**A7r5 Cells**—Total proteins were extracted from A7r5 cells (90–100% confluent) and subjected to Western blot analysis as described above. Forty μg of protein was loaded per well, and expression of Ca²⁺-handling proteins was detected using specific antibodies against IP3R1 (1:1000; Millipore), αC (1:1000; Millipore), TRPC1 (1:200; Santa Cruz Biotechnology), TRPC4 (1:200; Novus Biologicals), STIM1 (1:1000; Cell Signaling Technology), Orai1 (1:1000; Sigma), and PMCA1 (1:500; Santa Cruz Biotechnology). The density of bands was determined using ImageJ and normalized to the α-smooth muscle actin density for each lane.

**RT-PCR**

**Mesenteric Arteries**—Total RNA was isolated from MA of SAL and AHT mice using the RNeasy minikit (Qiagen). First strand cDNA synthesis was performed with the iScript™ cDNA synthesis kit (Bio-Rad). After reverse transcription, real-time PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad) containing a hot start iTaq™ DNA polymerase and a 0.4 mM concentration of each primer, according to the manufacturer’s instructions. PCRs contained 1 μg of total RNA, 0.2 mM dNTP, Phusion GC Buffer (5×), and Phusion DNA Polymerase (New England Biolabs), and a 0.4 μM concentration of each primer in a total volume of 20 μl. Reactions were run at 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s and a 10-min final extension using the following primers: IP3R1, 5'-CCCATCCTAACGGAAAGCAGC-3' (forward) and 5'-GGGTTCAGCTTTCCAGAAC-3' (reverse); αC, 5'-TTTACACCCGCTCTACGGC-3' (forward) and 5'-GGGGGAAAGTTACGAAGGT-3' (reverse); TRPC4, 5'-ACTGGCAATCTCAGCTGG-3' (forward) and 5'-GGCGGAACCATTGCTTAGG-3' (reverse); α-actin, 5'-TCTGCCCTTACACACACTG-3' (forward) and 5'-CTAGCTGTTGAAGTCTGTG-3' (reverse); vWF, 5'-CTAGCTGTTGAAGTCTGTG-3' (forward) and 5'-CTAGCTGTTGAAGTCTGTG-3' (reverse); GAPDH, 5'-CCAGAAGTGCGA-3' (forward) and 5'-GAGATGAGCCTG-3' (reverse).

**Vascular IP₃ Receptor in Hypertension**

For multicol PCR using populations of enzymatically isolated mouse mesenteric VSM cells, MA were enzymatically digested, and 60–80 VSM cells were aspirated into borosilicate glass micropipette filled with 1× phosphate-buffered saline under the microscope. Total RNA preparation and cDNA synthesis was performed using the Single Cell real-time RT-PCR assay kit (Signosis) per commercial instructions. cDNA products were amplified by nested PCR using gene-specific primer pairs. The following primer pairs were used for first roundPCR amplification: IP3R1, 5'-CCCATCCTAACGGAAAGCAGC-3' (forward) and 5'-GGGTTCAGCTTTCCAGAAC-3' (reverse); αC, 5'-TTTACACCCGCTCTACGGC-3' (forward) and 5'-GGGGGAAAGTTACGAAGGT-3' (reverse); TRPC4, 5'-ACTGGCAATCTCAGCTGG-3' (forward) and 5'-GGCGGAACCATTGCTTAGG-3' (reverse); α-actin, 5'-TCTGCCCTTACACACACTG-3' (forward) and 5'-CTAGCTGTTGAAGTCTGTG-3' (reverse); vWF, 5'-CTAGCTGTTGAAGTCTGTG-3' (forward) and 5'-CTAGCTGTTGAAGTCTGTG-3' (reverse); GAPDH, 5'-CCAGAAGTGCGA-3' (forward) and 5'-GAGATGAGCCTG-3' (reverse).
**Vascular IP₃ Receptor in Hypertension**

(reverse); β-actin, 5’-ATCTGTGGCATTCCATGAAACTC-3’ (forward) and 5’-AGGAGCCAGGGCAGTAATCTCCT-3’ (reverse).

The amplified products were separated by electrophoresis on 1.2% agarose gels and analyzed as described above. For real-time PCR, each PCR (25 μl) contained 12.5 μl of IQ SYBR Green Supermix (Bio-Rad), 400 nm primers, and an equal volume of cDNA template. Reactions were performed in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems) at 95 °C for 10 min and 40 cycles of 20 s at 95 °C, 20 s at 65 °C, and 45 s at 72 °C.

**Ca²⁺ Imaging**

Cells were seeded onto 35-mm poly-D-lysine-coated glass coverslips (MatTek Corp.) and incubated at 37 °C and 5% CO₂ in complete culture media for 4 days. Cells were incubated with the ratiometric Ca²⁺ dye Fura-2-AM (2 μM Invitrogen) and placed in the incubator for 45 min in the dark. After incubation, cells were washed with prewarmed Ca²⁺/Mg²⁺-free PBS and allowed to rest in HBS for 10 min. The coverslips were then transferred to an inverted epifluorescence microscope (Olympus) equipped with a UPlanSapo ×40/0.95 numerical aperture oil objective and connected to a CCD camera (CoolSNAP HQ²). The fluorescence emission of individual cells was measured at 510 nm following excitation at 340 and 380 nm using an EasyRatioPro calcium imaging system (PTI). The fluorescence ratio (F₃₄₀/F₃₈₀) was recorded in real time at 0.5-s intervals as a relative indicator of intracellular Ca²⁺ concentration. The ratios were calibrated by determining Fₘₐₓ and Fₘᵢₙ values in the presence of 0.1% saponin (Fₘₐₓ) and 10 mM EGTA (Fₘᵢₙ).

**Caged IP₃**

Ca²⁺ transients were analyzed in A7r5 cells loaded with the membrane-permeant caged IP₃ analog ciIP3, purchased from SiChem. Briefly, cells were cultured on glass coverslips and kept in culture as described under “Ca²⁺ Imaging.” Prior to Ca²⁺ imaging, cells were washed with prewarmed Ca²⁺/Mg²⁺-free PBS and incubated in HBS containing 2 μM ciIP3 for 45 min in the dark, followed by incubation with a 2 μM concentration of the cell-permeant dye Ca-Green-1, AM (Invitrogen), which was added to the loading solution for a further 45 min before washing and allowing at least 30 min for de-esterification. Imaging of changes in [Ca²⁺]ᵢ was accomplished using an inverted epifluorescence microscope (Olympus) equipped with a UPlanSapo ×40/0.95 numerical aperture oil objective and connected to a CCD camera (CoolSNAP HQ²). IP₃ was released by a photothermal destruction of the cage with brief exposures (100 ms every 1.5 s) of the cells to UV light (340 nm). The fluorescence of individual cells was collected simultaneously at 510 nm by excitation of Ca-Green-1 at 488 nm using an EasyRatioPro calcium imaging system (PTI). The fluorescence signals are expressed as ratios (Fₘₐₓ/Fₘᵢₙ) of the mean fluorescence F of each cell relative to the mean resting fluorescence F₀.

**Monitoring of A7r5 Contraction**

Assessment of cell contraction was performed on A7r5 cells cultured on glass coverslips as described above. Attached cells were washed with prewarmed Ca²⁺/Mg²⁺-free PBS and allowed to settle in HBS for 10 min prior to live cell imaging. Cell contraction was visualized using an inverted microscope (Olympus) equipped with a LucPlan FLN ×40/0.60 numerical aperture objective. Images were acquired with a CCD camera (Olympus DP72) and processed using DP2-BSW software (Olympus Soft Imaging Solutions). Cell contraction was quantified by morphometric analysis using ImageJ. Briefly, the addition of PE induces the formation of contractile fibers, which appear as protruded edges on the surface of contracted cells. To quantify contractile responses to PE, contractile fibers were quantified using the edge detection function in ImageJ on thresholded time lapse images.

**Statistics**

Data represent means ± S.E. of the mean for the number (n) of animals or in vitro preparations indicated in parentheses. Student’s t test was used to compare two data sets, and one-way/two-way analysis of variance and Bonferroni’s post hoc test were used for multiple group comparisons. p ≤ 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Hypertension Is Coupled to Increased Expression of Vascular IP₃ R**—To determine the expression level of vascular IP₃ R during hypertension, male C57BL/6 mice were infused subcutaneously with Ang II (2 ng/g/min, AHT) for 2 weeks, resulting in average SBP values of 109 ± 2, 136 ± 3, and 152 ± 2 mm Hg at 0, 7, and 14 days, respectively (n = 12 each; Fig. 1A). In contrast, SBP values were unchanged from the preload value of 105 ± 3 mm Hg in SAL-infused mice (n = 12 each; Fig. 1A). At 14 days, hypertension correlates with increased protein expression (2.39 ± 0.19-fold) of the type 1 IP₃ R (IP₃ R₁) in MA (Fig. 1, B and C). Localization and functional studies in renal and mesenteric VSM and in A7r5 cells argue that IP₃ R₁ is the primary isoform (29–31). Consistently, quantitative RT-PCR reveals a 1.83 ± 0.11-fold increase of IP₃ R₁ transcript in arteries of AHT compared with SAL mice (Fig. 1D).

**PE-induced Contractions Are Enhanced in MA of AHT Mice**—To assess the functional significance of IP₃ R up-regulation in arteries exposed to hypertension, we sought to determine the contribution of IP₃-dependent Ca²⁺ release to vascular contractile responses to the vasocostrictor agonist, PE. Isolated, pressurized (60 mm Hg) second order MA branches of AHT mice overexpressing IP₃ R₁ showed accentuated contractions to PE, an α₁-adrenoreceptor agonist that relies on IP₃-dependent Ca²⁺ release for contraction (Fig. 1E) (32). The PE-induced contractions in MA from AHT mice averaged 38% higher at agonist concentrations of ≥10⁻⁶ M (Fig. 1F). The concentration of PE required for half-maximal contraction (ED₅₀) did not differ significantly between MA of SAL (6.9 × 10⁻⁷ M) and AHT mice (6.7 × 10⁻⁷ M). Subsequently, we observed that the nonselective IP₃ R antagonist, 2-APB (33), suppressed contractions to PE in MA of SAL and AHT mice (Fig. 1E). Comparison of the amplitude of 2-APB-sensitive contraction between MA of SAL and AHT mice revealed a higher component in arteries from hypertensive animals (Fig. 1, F and G). These results argue that IP₃-dependent Ca²⁺ release contributes...
more significantly to PE-induced vascular contractions in MA of hypertensive animals, supporting a functional role for the observed IP$_3$R up-regulation in increasing vascular reactivity.

Specific Up-regulation of IP$_3$R and $\alpha_{1C}$ in Arteries of AHT Mice—We explored the possibility that Ang II-induced hypertension causes a nonspecific remodeling of Ca$^{2+}$-handling proteins in VSM by comparing the expression of a panel of proteins implicated in VSM contraction between MA of SAL and AHT mice. It is well known that the $\alpha_{1C}$ pore-forming subunit of the vascular LTCC up-regulates during hypertension (10, 12, 13). This event may partly account for the increased sensitivity of hypertensive subjects to the antihypertensive effect of clinical calcium channel antagonists; these drugs only mildly lower blood pressure in normotensive subjects (34–37). Indeed, Western blots confirmed that $\alpha_{1C}$ up-regulation is coupled to increased IP$_3$R1 expression in MA of AHT mice (Fig. 2A). We also evaluated the expression of two agonist-activated TRPC channels implicated in vascular reactivity, TRPC1 and TRPC4 (34–36, 38). TRPC1 expression was unchanged after Ang II infusion, but a pronounced up-regulation of TRPC4 was detected in MA of AHT mice (Fig. 2A). However, we failed to detect TRPC4 transcript in multicell PCRs limited to mouse mesenteric VSM cells. In this experiment, PCRs using cDNA derived from whole MA revealed transcripts coding for IP$_3$R1, $\alpha_{1C}$, TRPC4, $\alpha$-actin, and vWF; the latter two proteins are VSM and endothelium-specific markers, respectively (Fig. 2B, top). However, only IP$_3$R1, $\alpha_{1C}$, and $\alpha$-actin transcripts were detected in amplified products from isolated VSM cells (Fig. 2B, bottom). Thus, we ruled out TRPC4 as a direct contributor to VSM contraction, and our data suggest that this channel may only be expressed in the endothelium of small arteries, as suggested by others (34, 35).

Finally, we observed no difference in expression levels of STIM1 and Orai1 between MA of SAL and AHT mice. This finding is significant, because we presumed that the enhanced block of PE-induced contractions by 2-APB in arteries of AH mice reflected an increased contribution of IP$_3$-dependent Ca$^{2+}$ release to VSM reactivity in hypertension (1, 2). However, 2-APB also can inhibit store-operated Ca$^{2+}$ entry (SOCE) (33, 39, 40), which relies on the endoplasmic reticulum Ca$^{2+}$ sensor STIM1 and the plasma membrane channel Orai1 (41). The comparable expression of STIM1 and Orai1 between MA of SAL and AHT mice is consistent with a relatively minor role of SOCE in VSM contraction and argues that the observed inhibition with 2-APB primarily relates to block of IP$_3$Rs.
Vascular IP₃ Receptor in Hypertension

We also tested if IP₃R up-regulation is specific to the AHT mouse model by evaluating IP₃R1 expression in two rat models of hypertension, the SHR and aortic-banded rat (12, 13). The MA of the genetically hypertensive SHR were compared with the normotensive Wistar Kyoto rats (WKY) and SHR exposed to hypertension, we used the embryonic rat aortic smooth muscle-derived A7r5 cell line. Notably, pressure-induced depolarization of small arteries and arterioles is recognized as a common abnormality of hypertension, which can induce the up-regulation of LTCCs and trigger enhanced vascular reactivity and vessel remodeling (12, 13). This loss of resting membrane potential (Eₘ) extends to VSM cells of the renal, mesenteric, and skeletal muscle beds in experimental models of hypertension in which vessels are exposed to high intraluminal pressures (10, 12, 42–45). To mimic a key stimulus of vascular remodeling in hypertension, we exposed aortic A7r5 cells to incrementally higher concentrations of 20 (K20), 40 (K40), and 60 (K60) mM KCl for 24 h, to progressively depolarize and activate the SMCs as an in vitro surrogate for the hypertensive environment. These K⁺ concentrations would be predicted to shift Eₘ by +43 mM (K20), +61.5 mM (K40), and +72.4 mM (K60) based on the Nernst potential. All three concentrations of KCl up-regulated the IP₃R protein in A7r5 by 24 h, whereas expression levels of PMCA and smooth muscle α-actin were stable (Fig. 3A). Further studies used the lowest depolarizing stimulus of K20 that caused a 1.7-fold increase in IP₃R expression (Fig. 3E). Importantly, up-regulation of IP₃R was induced by depolarization rather than the osmotic challenge of K20, because enhanced IP₃R was not observed in A7r5 aortic cells exposed to equiosmolar NaCl or sucrose (Sucrose) (Fig. 3, A, B, and E). However, up-regulation of IP₃R by 20K was prevented in A7r5 cells incubated in 10 μM nifedipine (Ni), a specific blocker of the LTCC (Fig. 3, A, B, and E). This finding argues that voltage-dependent Ca²⁺ influx through LTCC mediates IP₃R up-regulation.

Similar to arteries exposed to hypertension, depolarization-induced IP₃R up-regulation in A7r5 cells was coupled to an increased expression of LTCC α₁C subunit (1.6 ± 0.07-fold; Fig. 3B), arguing that depolarization of A7r5 cells faithfully replicates the remodeling of Ca²⁺ signaling pathways observed in arteries from hypertensive animals.

Because SOCE has been previously implicated in VSM proliferation and migration (46), we tested the expression levels of STIM1 and Orai1 following depolarization in A7r5. Consistent with our findings in MA of AHT mice, STIM1 expression levels were stable (Fig. 3C), and we were unable to detect Orai1 expression on Western blots from A7r5 cells. Furthermore, no change in the expression levels of TRPC1 was detected in A7r5 cells exposed to KCl (Fig. 3C), whereas TRPC4 expression was not detected.

We then tested whether Ca²⁺ influx through LTCC is specifically required for IP₃R up-regulation or whether a nonspecific cytoplasmic Ca²⁺ rise is sufficient to trigger and sustain IP₃R1 enrichment in VSM cells. We raised intracellular Ca²⁺ levels by inhibiting the endoplasmic reticulum Ca²⁺-ATPase (SERCA) using the specific antagonist thapsigargin (Thaps) (47). Due to a continuous ill defined Ca²⁺ leak pathway at the endoplasmic reticulum membrane, Thaps results in a slow rise in cytoplasmic Ca²⁺ levels. This passive store depletion activates SOCE, thus accentuating the Ca²⁺ rise (43, 48). Indeed, Thaps treatment results in high basal Ca²⁺ levels (Fig. 4C) and was sufficient to induce significant IP₃R up-regulation (1.5 ± 0.14-fold; Fig. 3, D and E). Although depolarization-dependent Ca²⁺ influx through LTCC was more efficient at inducing IP₃R up-regulation, the results from Thaps argue that a general cyto-
Calcium Influx Triggers IP₃R Expression through Calcineurin-NFAT Signaling—The establishment of the A7r5 cell model allowed us to identify the signal transduction cascade that mediates depolarization-induced IP₃R up-regulation. Studies using quantitative RT-PCR revealed a 2.2 ± 0.4-, 2.1 ± 0.3-, and 1.5 ± 0.3-fold increase of α₁C, IP₃R₁, and the type 3 IP₃R (IP₃R₃) transcripts, respectively, in A7r5 cells exposed to K20 for 24 h (Fig. 3F). Similar results were observed in semiqualitative PCR experiments (supplemental Fig. 1), whereas the type 2 IP₃R (IP₃R₂) transcript was not detected.
Block of protein translation with 10 μg/liter cycloheximide (CHX) prevented K20-induced up-regulation of IP₃R at 24 h (Fig. 3, D and E). Subsequently, nonspecific block of translation with 10 μM actinomycin D (ACD) also prevented K20-induced IP₃R enrichment (Fig. 3, C and D). Given that the Ca²⁺-calmodulin dependent phosphatase, calcineurin, promotes IP₃R abundance in neurons (49), we treated A7r5 cells next with 10 μM cyclosporine A (CsA), a specific calcineurin blocker. CsA also abrogated the increased expression of IP₃R caused by K20 (Fig. 3E).

Calcineurin regulates gene expression by dephosphorylating the transcription factor NFAT, which translocates to the nucleus to promote transcription (50). To directly support a role for NFAT in IP₃R up-regulation, we imaged NFATc1 localization in control or K20-depolarized A7r5 cells. Control A7r5 cells transfected with GFP-NFATc1 exhibit a cytosolic distri-
bution (Fig. 3G). The addition of K20 induced NFATc1 translocation to the nucleus within 15–20 min. Depolarization-induced NFATc1 translocation was prevented by block of LTCC by Nif or by inhibition of calcineurin activity with CsA (Fig. 3G). However, NFATc1 translocation to the nucleus persisted in K20-exposed A7r5 cells pretreated with either CHX or ACD, suggesting that these agents prevent depolarization-induced up-regulation of IP₃R by acting at a site downstream of NFATc1 activation. Notably, we verified that CHX, ACD, and CsA per se do not blunt IP₃R up-regulation by directly inhibiting 20K-induced Ca²⁺ influx through LTCCs, whereas Nif blocked the response as expected (Fig. 4, A and B). Consistent with our earlier finding that exposure of A7r5 cells to Thaps induces IP₃R expression, Thaps treatment also resulted in NFATc1 translocation to the nucleus (Fig. 3G). These data support the concept that Ca²⁺ influx through LTCCs activates calcineurin, resulting in translocation of NFATc1 to the nucleus to trigger IP₃R R₁ transcription.

Given the important role of cytoplasmic Ca²⁺ in mediating IP₃R up-regulation, we were interested in determining the effect of the different treatments on basal Ca²⁺ levels in A7r5 cells. Compared with untreated cells, exposure of A7r5 cells for 24 h to the osmolarity controls (NaCl and sucrose) did not alter basal Ca²⁺ levels (Fig. 4C). In contrast, 20K and Thaps significantly increased basal Ca²⁺ levels at 24 h (Fig. 4C), findings that correlate well with IP₃R up-regulation under these conditions (Fig. 3E). Interestingly, the observed increase in basal Ca²⁺ following 20K treatment was inhibited when cells were pretreated with Nif, CHX, ACD, or CsA (Fig. 4C). These results argue that the increased basal Ca²⁺ levels observed after prolonged KCl treatment are not due to Ca²⁺ influx through LTCCs but rather depend critically on IP₃R up-regulation.

**IP₃R Up-regulation Sensitizes IP₃-dependent Ca²⁺ Release** —To test the consequence of IP₃R up-regulation on IP₃-dependent Ca²⁺ release in response to agonist stimulation, we performed a PE concentration-response curve, which revealed significantly larger Ca²⁺ transients at PE concentration of ≥5 μM in K20-treated cells compared with control (Con) (Fig. 5A). This enhanced Ca²⁺ transient would be predicted to promote VSM contraction. However, the enhanced Ca²⁺ release in response to PE could be due to the remodeling of other components of the signal transduction cascade leading to Ca²⁺ release during the 24-h incubation period and as such may not directly reflect IP₃R function.

To directly assess IP₃R function under conditions where the receptor is up-regulated, we followed the kinetics of IP₃-dependent Ca²⁺ release in A7r5 cells loaded with caged IP₃ (ciIP₃) and exposed to UV illumination to rapidly release IP₃, thus mimicking the rapid responses of the receptor/PLC signaling system. Cells were also loaded with the Ca²⁺-sensitive dye Cal-Green-1 to visualize the ensuing Ca²⁺ dynamics (Fig. 5B). We uncaged ciIP₃ repeatedly for 100 ms every 1.5 s, which resulted in an initial global sustained Ca²⁺ transient in both control and K20-treated cells (Fig. 5B). In a subset of control and K20-treated cells, this was followed by Ca²⁺ oscillations presumably triggered by the oscillatory IP₃ uncaging (Fig. 5B). All aspects of IP₃R function exhibited dramatic sensitization in cells with depolarization-induced enriched IP₃R. As expected, maximal Ca²⁺ release measured in both cells that oscillate and those that respond with a monotonic Ca²⁺ rise was significantly increased (Fig. 5C). In addition, A7r5 cells exposed to K20 for 24 h to up-regulate IP₃Rs were significantly more likely to oscillate compared with control cells; their oscillation frequency was enhanced dramatically as well as the duration of the oscillations (Fig. 5D).

The increased amplitude of Ca²⁺ release in response to IP₃ uncaging is consistent with an increased number of IP₃Rs (Fig. 5C). However, the pronounced oscillatory behavior of the K20-treated cells argues that IP₃R sensitivity is also enhanced in cells with up-regulated IP₃R expression. This predicts that K20-treated cells would release Ca²⁺ at lower IP₃ threshold concentrations. To test whether this is the case, we evaluated the time to threshold, which was defined as the time required to produce a Ca²⁺ release signal significantly above the resting baseline (empirically determined to F/F₀ ≥ 1.04). The time to threshold is significantly shorter in K20-treated versus control cells, consistent with the conclusion that IP₃R sensitivity is enhanced in A7r5 cells showing overabundant IP₃R.

Therefore, up-regulation of the IP₃R is functionally significant and sensitizes Ca²⁺ release. However, the mechanisms regulating this increased sensitivity require additional investigation. For example, IP₃R up-regulation could be sufficient to mediate the increased sensitivity of IP₃-dependent Ca²⁺ release. Alternatively, the increased mass of the IP₃R could be coupled to post-translational modifications or protein-protein interactions that mediate the enhanced sensitivity of the receptor. Nonetheless, because Ca²⁺ release in our experiments was induced using caged IP₃, the observed enhanced sensitivity of

**FIGURE 5. IP₃ R up-regulation sensitizes IP₃-dependent Ca²⁺ release and VSM contraction.** A, PE concentration-response curve for Ca²⁺ release in control (Con) A7r5 cells or similar cells depolarized by K20 for 24 h to up-regulate IP₃R. Cells were loaded with Fura2-AM and bathed in Ca²⁺-containing HBS, and images were acquired with an epifluorescence microscope. At low PE concentrations, the two groups of cells had similar fluorescence amplitudes. At PE concentrations above 5 μM, K20-pretreated cells showed higher fluorescence amplitude than control. Values are the mean of fluorescence intensity of 15–20 cells per group. B–E, kinetics of IP₃-dependent Ca²⁺ release evoked by UV photorelease of ciIP₃ in A7r5 cells bathed in Ca²⁺-containing HBS and loaded with ciIP₃ and Ca-Green-1. CIIP₃ was uncaged by repeated exposure of cells to UV illumination (100 ms every 1.5 s), and the fluorescence of Ca-Green was recorded in real time. B, in a subset of cells, ciIP₃ uncaging caused an initial global sustained Ca²⁺ transient followed by Ca²⁺ oscillations in both control and 20K-pretreated cells. Cells in both groups also responded to ciIP₃ uncaging by a monotonic Ca²⁺ rise without Ca²⁺ oscillations. C, the maximal IP₃-dependent Ca²⁺ release was increased in K20-pretreated cells exhibiting enriched IP₃R (n = 8; *p < 0.05). D, the percentage of oscillating cells (n = 8–10; **p < 0.0001), oscillation frequency/min (n = 5–8; ***p < 0.0001), and duration of oscillations (n = 5–7; **p < 0.005) were enhanced in K20-pretreated cells. E, the time to threshold Ca²⁺ signal was shortened in K20-pretreated cells compared with control (n = 8; **p < 0.0001). F, contractile responses of A7r5 cells to PE (20 μM) in Ca²⁺-containing HBS were evaluated by imaging of contractile fiber formation. PE responses in a transient contractile response in control cells, whereas the contractile response in 20K-pretreated cells is more pronounced and sustained. G, statistical analysis of the maximal contractile response to PE of control and K20-pretreated A7r5 cells (n = 4; **p < 0.05). Error bars, S.E.
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IP$_3$-dependent Ca$^{2+}$ release cannot be due to modulation of the signal transduction cascade upstream of IP$_3$ production.

Consistent with our findings, an increased IP$_3$ binding capacity has been reported in the aorta of SHR (51). In addition, in cerebral arteries, the IP$_3$R was shown to activate Ca$^{2+}$ influx through TRPC3, leading to depolarization and muscle contraction (40). This mechanism, however, is unlikely to contribute to PE-induced Ca$^{2+}$ influx in A7r5 cells that do not express any TRPC3 channels, based on our Western blot analysis (supplemental Fig. 2) and as reported by others (52, 53).

Up-regulation of IP$_3$R Enhances VSM Contractility VSM contraction establishes vascular tone and peripheral resistance. Because IP$_3$R up-regulation is associated with enhanced Ca$^{2+}$ signaling in A7r5 cells, we wondered whether this finding correlates with increased contraction. Thus, we examined the contractile responsiveness of A7r5 cells to PE. A7r5 cells were of the elongated form, and nearly 100% of them contracted upon the addition of PE (20 μM). The extent of contraction was quantified using an imaging approach outlined under “Experimental Procedures,” which exploited the appearance of contractile fibers following PE addition. PE results in a transient contractile response in control cells (Fig. 5F), whereas A7r5 cells with up-regulated IP$_3$R (KCl 20 mM) show a significantly more pronounced contractile response that is sustained for the duration of the recording (Fig. 5F). Consistently, the mean value of maximal contraction was increased 2 ± 0.02-fold in K20-treated cells when compared with control (Fig. 5G).

Collectively, our results show that increased expression of the IP$_3$R in arterial VSM is consistently associated with hypertension. Depolarization-induced IP$_3$R up-regulation in A7r5 VSM cells is associated with a higher resting Ca$^{2+}$ level, enhanced response to agonist stimulation, and more robust and sustained contraction. Hence, IP$_3$R up-regulation observed in VSM cells during hypertension is likely to be an important contributor to enhanced vascular reactivity. As such, the IP$_3$R represents an attractive potential drug target for the treatment of hypertension.

An increased myogenic response and enhanced reactivity to vasoconstrictor agonists are characteristics of hypertension and contribute significantly to increased blood pressure (54). Multiple factors may act in concert to contribute to the enhanced vascular tone, including an overabundance of Ca$^{2+}$-conducting channels, such as α$_{1C}$ and TRPC3, alterations in the Ca$^{2+}$ sensitivity of the contractile apparatus in VSM (55), and structural remodeling of arteries (54). With the recognition that the VSM is the mediator of the myogenic response and that a rise in cytoplasmic Ca$^{2+}$ is essential for smooth muscle contraction, it is significant, as shown in this study, that the IP$_3$R up-regulates in VSM during hypertension, and this event is associated with enhanced IP$_3$R-mediated Ca$^{2+}$ signaling and VSM contraction. Enrichment of IP$_3$R can be recapitulated in cultured VSM cells by depolarization-induced opening of LTCC and Ca$^{2+}$-dependent activation of the calcineurin-NFAT pathway.

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