The nuclear factor of activated T-cells (NFAT), originally identified in T-cells, has since been shown to play a role in mediating Ca\(^{2+}\)-dependent gene transcription in diverse cell types outside of the immune system. We have previously shown that nuclear accumulation of NFATc3 is induced in ileal smooth muscle by platelet-derived growth factor in a manner that depends on Ca\(^{2+}\) influx through L-type voltage-dependent Ca\(^{2+}\) channels. Here we show that NFATc3 is also the predominant NFAT isoform expressed in cerebral artery smooth muscle and is induced to accumulate in the nucleus by UTP and other G\(_{q/11}\)-coupled receptor agonists. This induction is mediated by calcineurin and is dependent on sarcoplasmic reticulum Ca\(^{2+}\) release through inositol 1,4,5-trisphosphate receptors and extracellular Ca\(^{2+}\) influx through L-type, voltage-dependent Ca\(^{2+}\) channels. Consistent with results obtained in ileal smooth muscle, depolarization-induced Ca\(^{2+}\) influx fails to induce NFAT nuclear accumulation in cerebral arteries. We also provide evidence that Ca\(^{2+}\) release by ryanodine receptors in the form of Ca\(^{2+}\) sparks may exert an inhibitory influence on UTP-induced NFATc3 nuclear accumulation and further suggest that UTP may act, in part, by inhibiting Ca\(^{2+}\) sparks. These results are consistent with a multifactorial regulation of NFAT nuclear accumulation in smooth muscle that is likely to involve several intracellular signaling pathways, including local effects of sarcoplasmic reticulum Ca\(^{2+}\) release and effects attributable to global elevations in intracellular Ca\(^{2+}\).

Nuclear factor of activated T-cells (NFAT)\(^{1}\) was originally identified as the transcription factor responsible for mediating the Ca\(^{2+}\)-dependent transcription of genes involved in T-cell activation (1, 2) but has since been shown to play a role in mediating Ca\(^{2+}\)-dependent gene transcription in diverse cell types outside of the immune system, including neurons (3), endothelial cells (4, 5), cardiac muscle (6), skeletal muscle (7, 8), and smooth muscle (9, 10). The potential physiological roles for this transcription factor are also diverse and include the developmental regulation of slow twitch/fast twitch skeletal muscle fiber types (11) and smooth muscle cell precursor migration and vascular development during embryogenesis (12). NFAT has also been implicated in the pathogenesis of cardiac (6) and skeletal (7, 8) muscle hypertrophy and might be predicted to play a similar role in smooth muscle hypertrophy associated with, for example, atherosclerosis and bladder dysfunction.

NFAT represents a family of transcription factors composed of four well characterized members, designated NFATc1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), and NFATc4 (NFAT3). A fifth putative member of the family (NFAT5) is a calcineurin-insensitive, constitutively nuclear phosphoprotein that has limited sequence similarity to other members of the NFAT family (13).

NFAT activation is regulated primarily through control of its subcellular localization (2). Elevation of global Ca\(^{2+}\) produced by a variety of mechanisms activates the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, calcineurin (14). Subsequent calcineurin-mediated dephosphorylation of specific NFAT serine residues induces a conformational change in the NFAT molecule that exposes nuclear localization signals, allowing import of NFAT into the nucleus (15–20). The distribution of NFAT between nuclear and cytoplasmic compartments is dynamically regulated by the activity of nuclear kinases, which oppose the action of calcineurin (3, 28–31). Additional mechanisms, which modulate the ability of calcineurin to associate with and/or dephosphorylate NFAT in the cytosol, provide regulation at the level of nuclear import (17, 32–34). In the nucleus, NFAT associates with a transcriptional co-activator, an interaction that is required for significant NFAT-mediated transcriptional activity. NFAT family members have been shown to cooperatively bind to DNA with variety of cofactors, including AP-1 (24–26), GATA (6, 8, 27), and MEF2 (11), and in this way integrate Ca\(^{2+}\)/calcineurin signaling with other signaling pathways, such as Ras, Rac, and protein kinase C.

A sustained, global increase in intracellular Ca\(^{2+}\) has generally been considered a defining feature of NFAT-activating stimuli (35–38). More recent evidence has shown that temporal modulation of Ca\(^{2+}\) signals in the form of Ca\(^{2+}\) oscillations or waves increases the coupling efficiency of Ca\(^{2+}\) signals to NFAT activation in nonexcitable cells (39, 40). Surprisingly, transient increases in intracellular Ca\(^{2+}\) induced by a depolar-
izing stimulus and mediated by flux through L-type voltage-dependent Ca\textsuperscript{2+} channels (VDCC), have also been shown to effectively stimulate a sustained increase in NFAT activity in hippocampal neurons (3). In contrast, and counter to expectations, sustained increases in intracellular Ca\textsuperscript{2+} induced by depolarization fail to induce nuclear accumulation of the NFATc3 isoform in ideal smooth muscle, although PDGF, which activates multiple intracellular pathways, is a potent stimulus for NFATc3 nuclear translocation in this tissue (10).

Smooth muscle exhibits a diverse array of Ca\textsuperscript{2+} signals, including Ca\textsuperscript{2+} waves that traverse the length of the cell and display distinctive frequency and amplitude properties (41–44), and localized transient releases of Ca\textsuperscript{2+} through sarcoplasmic reticulum (SR) ryanodine receptors (RyRs) in the form of Ca\textsuperscript{2+} sparks (45). The role that various Ca\textsuperscript{2+} signals and Ca\textsuperscript{2+}-dependent transcription factors may play in the physiological or pathological regulation of gene expression in this phenotypically plastic tissue is largely unknown. Here we show that, in native cerebral artery smooth muscle cells, UTP and other G\textsubscript{q11}-coupled receptor agonists induce a calcineurin-mediated nuclear accumulation of NFATc3. This induction is dependent on SR Ca\textsuperscript{2+} release through IP\textsubscript{3} receptors (IP\textsubscript{3}R) and further depends on extracellular Ca\textsuperscript{2+} influx through L-type VDCC. We also show that UTP may act to induce NFATc3 nuclear accumulation, at least in part, by suppressing Ca\textsuperscript{2+} sparks, suggesting a novel inhibitory role for Ca\textsuperscript{2+} sparks in the regulation of Ca\textsuperscript{2+}-sensitive transcription factors.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Epidermal growth factor (EGF) and platelet-derived growth factor BB (PDGF-BB) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); pinacidil was from RBI (Research Biochemicals—Eugene, OR); rat TNF was from Calbiochem, and 2-aminoethoxydiphenyl borate (2-APB) was obtained from Sigma Chemical Co., St. Louis, MO. 4-AM in physiological saline solution and 0.05% pluronic acid for 60 min was applied to induce Ca\textsuperscript{2+}-positive nuclei, multiple fields for each vessel were imaged and counted by two independent observers under double-blind conditions. For quantification, a cell was considered positive if co-localization (yellow) was observed in the nucleus, whereas a cell was considered negative if no co-localization (green only) was visualized.

**Confocal Ca\textsuperscript{2+} Measurements**—All imaging experiments were performed at room temperature. Arteries were loaded with 10 μM fluo-4-AM in physiological saline solution and 0.05% pluronic acid for 60 min and exposed to a high concentration of forskolin solution for 30 min to allow flus- de-esterification. The vessel ends were anchored beneath two stainless steel hooks to maintain the artery at the bottom of the chamber and provide a fixed imaging area. Arteries were illuminated with a krypton/argon laser at 488 nm and imaged using a Noran Oz laser-scanning confocal microscope. NFATc3 was detected by monitoring Cy5 fluorescence using an excitation wavelength of 650 nm and an emission wavelength of 670 nm. Specificity of immune staining was confirmed by the absence of fluorescence in arteries incubated with primary or secondary antibodies and with blocking of NFATc3 nuclear staining. Arteries were imaged and analyzed using custom software written in our laboratory by Dr. Adrian Bonev (using IDL 5.2; Research Systems Inc., Boulder, CO), which allows for off-line quantification of fluorescence changes in selected regions of a sample corresponding to boxes of defined dimensions positioned by eye within the sample. Ca\textsuperscript{2+} spark amplitude (ΔF/ΔF\textsubscript{0}) was obtained by determining the fluorescence intensity within a 2.37-μm\textsuperscript{2} (1.54 μm (7 pixels) × 1.54 μm (7 pixels)) area corresponding to a detected spark event (ΔF), and dividing by a base line (F\textsubscript{0}) that was determined by averaging 50 images without Ca\textsuperscript{2+} spark activity. Ca\textsuperscript{2+} spark frequency under a given condition was calculated by measuring the number of sparks that occurred in a 58.1 × 54.5-μm area (~20 cells) scanned for 10 s. For detection of Cy5\textsuperscript{2+} waves, images of the vessel wall (116.2 × 108.0 μm, or 512 × 480 pixels) were recorded every 531.9 ms (1.88 images/s). Under each condition, at least two different representative areas of the same artery were scanned for 10 s. Ca\textsuperscript{2+} sparks were the mean pixel value of images acquired at 1.88 images/s, before and after application of each drug. The same area was not scanned more than once to avoid introducing Ca\textsuperscript{2+} signaling artifacts due to laser-induced cell damage.

**Statistical Analysis**—Results are expressed as means ± S.E., where applicable. All statistical analysis was performed using GraphPad software (Prism 3.0). Statistical significance was determined using one-way analysis of variance analysis followed by Bonferroni or Tukey-Kramer tests (for comparisons between up to five groups or at least six groups, respectively).
UTP induces nuclear translocation of NFATc3 as evidenced by presence in the cerebral vasculature and has been previously shown (46). The predominant expression of NFATc3 in smooth muscle has led us to focus on this isoform, although it is likely that other NFAT isoforms may play important roles in smooth muscle, as suggested by others (9, 12, 47).

**Induction of NFATc3 Nuclear Accumulation by G_q11-coupled Vasoconstrictor Agonists—UTP is an important vasoactive substance in the cerebral vasculature and has been previously shown to activate the NFATc1 isoform in cultured smooth muscle cells (47). In intact cerebral arteries, treatment with UTP induces nuclear translocation of NFATc3 as evidenced by colocalization of NFATc3 with the fluorescent nucleic acid dye, YOYO-1 (Fig. 2A). These results are summarized in Fig. 2B, which shows that the number of NFATc3-positive nuclei in intact cerebral arteries is increased from 7.9% in untreated vessels to 43.2% in UTP-treated arteries. Similar results for control (5.8%) and UTP-treated conditions (50.7%) were obtained for endothelium-denuded arteries, indicating that this action of UTP on NFATc3 subcellular distribution is a direct effect on smooth muscle.

Other G_q11-coupled vasoconstrictors and peptide ligands for certain tyrosine kinase-linked growth factor receptors are capable of inducing NFATc3 translocation in cerebral artery smooth muscle, although the robustness of the response varies with the agonist used. Endothelin-1 is as effective as UTP in inducing NFATc3 nuclear accumulation (Fig. 2C), whereas angiotensin II and the peptide ligand EGF are much less effective. Prostaglandin F_2α induces a small, but significant, increase in NFAT nuclear accumulation that is comparable in magnitude with that induced by angiotensin II and EGF. PDGF, which is a smooth muscle mitogen and potent activator of NFATc3 nuclear accumulation in ileal smooth muscle (10), is ineffective in cerebral artery smooth muscle.

**Calcineurin Dependence of UTP-induced NFATc3 Nuclear Accumulation—**Calcineurin activity is sensitive to inhibition by the chemically unrelated immunosuppressive agents FK506 and cyclosporin A, which inhibit calcineurin by distinct mechanisms (48). To determine whether UTP-induced NFATc3 nuclear accumulation is calcineurin-dependent, we treated intact cerebral arteries with each of these agents prior to and/or concurrent with UTP treatment. Inhibition of calcineurin activity with either of these compounds completely abrogates UTP-induced NFATc3 nuclear accumulation (Fig. 3), indicating that this process is calcineurin-dependent.

**Role of Intracellular Ca^{2+} Stores in UTP-induced NFATc3 Nuclear Accumulation—**Calcineurin activity is strictly dependent on Ca^{2+}/calmodulin (49). In intact cerebral arteries, UTP induces an increase in global intracellular Ca^{2+} characterized by an initial Ca^{2+} spike followed by a sustained elevated plateau phase (Fig. 4A; see also Ref. 41). Although the magnitude of each phase is somewhat variable between and within vessels, this biphasic response is a consistent property of UTP-induced Ca^{2+} elevation. To determine whether SR-mediated Ca^{2+} release is involved in UTP-induced NFATc3 nuclear accumulation, we pretreated cerebral arteries with the SR Ca^{2+}-ATPase inhibitor, thapsigargin, to deplete SR luminal Ca^{2+}. This treatment prevents the increase in global Ca^{2+} induced by UTP (Fig. 4B), indicating that intracellular calcium stores are required for this effect. To determine whether this calcium release from the SR induced by UTP contributes to UTP-induced NFATc3 nuclear accumulation, NFATc3 subcellular distribution was monitored immunohistochemically in cerebral arteries pretreated with thapsigargin. In these experiments, we employed cerebral arteries that had first been denuded of endothelium, as described under “Experimental Procedures.” In endothelium-denuded cerebral arteries, prior depletion of Ca^{2+} stores completely prevents UTP-induced NFATc3 nuclear accumulation (Fig. 4C). Under these conditions, thapsigargin alone has no effect on NFATc3 subcellular distribution. These data indicate that UTP mediates its effects on NFATc3 subcellular distribution, at least in part, through release of SR Ca^{2+}.

**Role of Extracellular Ca^{2+} Influx through L-type VDCC**—In UTP-induced NFATc3 Nuclear Accumulation—In nonexcitable cells, the sustained increase in intracellular Ca^{2+} required to maintain NFAT in the nucleus is provided by a capacitative mechanism by which depletion of intracellular Ca^{2+} stores is coupled to extracellular Ca^{2+} influx (50, 51). In smooth muscle, where a potential role for capacitative Ca^{2+} entry pathways remains speculative (55), the principle mediator of extracellular Ca^{2+} influx is the L-type VDCC (56).

To determine whether influx of Ca^{2+} through VDCC is required for UTP-induced NFATc3 nuclear accumulation, we employed two complementary approaches. First, we inhibited VDCC directly using the dihydropyridine, nisoldipine, which potently and specifically blocks these channels (57). We also indirectly decreased VDCC activity using pinacidil, an agent that activates ATP-sensitive K^+ channels, resulting in membrane potential hyperpolarization and diminished VDCC activ-
Tissue: 10

The temperature at the following concentrations was applied for 30 min at room temperature following UTP treatment (10 μM, 30 min at room temperature) in cerebral arteries. Cells were co-stained with the DNA-binding dye YOYO-1; yellow indicates nuclear co-localization of NFATc3 (red) and YOYO (green). White bars, 20 μm. B, summary data showing percentage of cells exhibiting NFATc3 nuclear accumulation in intact cerebral vessels and endothelium-denuded vessels, treated with UTP as in A. In cerebral arteries exhibiting NFATc3 nuclear accumulation in response to various vasoconstrictors. Each substance was applied for 30 min at room temperature at the following concentrations: 10 μM UTP, 100 ng/ml angiotensin II (AgII), 5 nM endothelin-1 (Et-1), 290 nM prostaglandin F2α (PP2α), 20 ng/ml EGF, 40 ng/ml PDGF. **, p < 0.001, UTP and Et-1 compared with control; ***, p < 0.0005, PP2α compared with control (m, number of mice; v, number of vessels; i, analyzed images; c, total number of cells counted).

We have previously shown that extracellular Ca2+ influx through VDCC is required for UTP-induced NFATc3 nuclear accumulation in native arterial smooth muscle. We have previously shown that extracellular Ca2+ influx through VDCC is required for UTP-induced NFATc3 nuclear accumulation in native arterial smooth muscle. We have previously shown that extracellular Ca2+ influx through VDCC is required for UTP-induced NFATc3 nuclear accumulation in native arterial smooth muscle.

Ca2+ influx through VDCC is a common mechanism of action of Gq/11-coupled vasoconstrictor agonists. The subsequent release of SR Ca2+ sparks, which create high local concentrations of Ca2+ capable of activating closely juxtaposed, large conductance, Ca2+-activated K+ (BK) channels (45). The resulting increase in K+ conductance promotes membrane hyperpolarization, thus reducing the activity of voltage-gated Ca2+ channels and opposing vasoconstriction. In contrast to the inhibitory effect that blockers of IP3Rs exert on IP3R-mediated release of Ca2+ from smooth muscle SR.

In addition to IP3Rs, the SR membrane also contains a ryanothidine-sensitive class of Ca2+-activated Ca2+-release channels, known as RyRs. These channels play an important role in regulating vascular tone through transient release of Ca2+ in the form of Ca2+ sparks, which create high local concentrations of Ca2+ capable of activating closely juxtaposed, large conductance, Ca2+-activated K+ (BK) channels (45). The resulting increase in K+ conductance promotes membrane hyperpolarization, thus reducing the activity of voltage-gated Ca2+ channels and opposing vasoconstriction. In contrast to the inhibitory effect that blockers of IP3Rs exert on IP3R-mediated release of Ca2+ from smooth muscle SR.

The activity of UTP and other Gq/11-coupled receptor agonists is not simply the result of simultaneous elevation of intracellular Ca2+ and activation of protein kinase C. Differential Effects of IP3R- and RyR-mediated Intracellular Ca2+ Release—Stimulation of phospholipase C-mediated hydrolysis of phosphoinositol bisphosphate to yield IP3 and diacylglycerol constitutes a common mechanism of action of Gq/11-coupled vasoconstrictor agonists. The subsequent release of IP3 through IP3Rs increases global [Ca2+], and contributes to the Ca2+ required for contraction. To confirm a role for IP3-mediated Ca2+ release in UTP-induced NFATc3 nuclear accumulation, we employed the structurally dissimilar, cell-permeable IP3R inhibitors, 2-APB and xestospongin C (59, 60). Treatment with either of these agents completely prevents the nuclear accumulation of NFATc3 induced by treatment with UTP (Fig. 6A). Taken together with the results from SR Ca2+ depletion, these data indicate that induction of NFATc3 nuclear accumulation by UTP is dependent on IP3R-mediated release of Ca2+ from smooth muscle SR.
membrane potential was ionically clamped with 60 mM potassium, ryanodine exerted a similar potentiating effect on UTP-induced NFATc3 nuclear accumulation, strongly suggesting that membrane potential depolarization attributable to RyR inhibition is not the primary mechanism by which ryanodine exerts its enhancing effect on NFAT3 nuclear accumulation. To directly confirm that increased Ca\(^{2+}\) influx associated with membrane depolarization does not augment UTP-induced NFATc3 nuclear accumulation, we treated cerebral arteries with UTP and high K\(^+\). Membrane depolarization with high K\(^+\) had no significant effect on UTP-induced NFATc3 translocation (Fig. 6C). Ryanodine alone and ryanodine plus high K\(^+\) were also without effect (Fig. 6C).

The absence of a membrane potential component to the potentiating action of RyR blockade suggests that Ca\(^{2+}\) flux through RyRs in the form of Ca\(^{2+}\) sparks, acting independently of BK channel activation, may have an inhibitory effect on UTP-induced NFATc3 nuclear accumulation. Accordingly, increasing spark frequency in the presence of UTP would be predicted to exert an inhibitory effect on UTP-induced NFATc3 accumulation. To test this hypothesis, we pretreated cerebral arteries with 300 mM caffeine, a concentration that induces a reproducible increase in Ca\(^{2+}\) spark frequency (Fig. 7B; see also Refs. 61 and 62). This caffeine-induced increase in spark frequency is accompanied by a complete abrogation of UTP-induced NFATc3 nuclear translocation (Fig. 7C). This postulated inhibitory effect of the spark pathway may also help to explain the failure of high K\(^+\) alone to stimulate NFATc3 nuclear accumulation (see Fig. 5C), since, in addition to increasing global intracellular Ca\(^{2+}\), high K\(^+\) increases SR Ca\(^{2+}\) load, resulting in an increase in Ca\(^{2+}\) release through RyRs that is reflected in a substantial increase in spark frequency (Fig. 7B). Finally, treatment with UTP alone induces a profound decrease in spark frequency with a time course that closely parallels the nuclear accumulation of NFATc3 (Fig. 7, A and D). These data suggest that inhibition of Ca\(^{2+}\) sparks may constitute an important component of the mechanism of action of UTP with respect to induction of NFATc3 nuclear accumulation.

Ca\(^{2+}\) waves give rise to oscillatory Ca\(^{2+}\) signals similar to those that have been shown to increase the efficiency of NFAT3 signaling properties associated with treatment of mouse cerebral arteries with UTP. Spatial and temporal changes in intracellular Ca\(^{2+}\) were determined in intact cere-

![Fig. 4. Dependence of UTP-induced NFATc3 nuclear accumulation on SR Ca\(^{2+}\). A, biphasic increase in global F/F\(_0\) induced by UTP (10 \(\mu\)M, reflecting an initial transient increase in \([\text{Ca}^{2+}]\), followed by a sustained elevated plateau phase. Representative trace from six separate arteries represents average fluo-4 fluorescence changes with time calculated from images of the vessel wall (116.2 \(\times\) 108.0 \(\mu\)M or 512 \(\times\) 480 pixels) recorded every 531.9 ms (1.88 images/s). B, elimination of the UTP-induced Ca\(^{2+}\) transient by pretreatment with the SR Ca\(^{2+}\)-ATPase inhibitor, thapsigargin (200 nM, 30 min). C, summary data illustrating abrogation of UTP-induced NFATc3 nuclear accumulation by treatment with thapsigargin. Endothelium-denuded arteries were pretreated with thapsigargin (200 nM) for 30 min, followed by concomitant incubation with UTP (10 \(\mu\)M, 30 min). Thapsigargin (200 nM, 60 min) alone has no effect on NFATc3 nuclear accumulation.

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**Fig. 3.** Calcineurin dependence of UTP-induced NFATc3 nuclear accumulation in intact vascular smooth muscle. A, representative immunofluorescence images of cerebral vascular segments showing inhibition of UTP-induced nuclear accumulation of NFATc3 by the calcineurin inhibitors cyclosporin A (1 \(\mu\)M, CsA) or FK506 (1 \(\mu\)M). Arteries were treated with UTP (10 \(\mu\)M) for 30 min at room temperature in the continued presence (or absence) of inhibitors. Cells were co-stained with the DNA-binding dye YOYO-1; yellow indicates nuclear co-localization of NFATc3 (red) and YOYO (green). White bars, 20 \(\mu\)M. B, summary data, quantified as described under “Experimental Procedures,” from multiple repeats of experiments described for A. ***p < 0.001, UTP compared with control, UTP + CsA and UTP + FK506 (m, number of mice; v, number of vessels; i, analyzed images; c, total number of cells counted).
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Fig. 5. Dependence of UTP-induced NFATc3 nuclear accumulation on Ca2+ influx through VDCC. A, representative images of cerebral arteries stained with anti-NFATc3 antibody (red) and with the DNA-binding dye YOYO-1 (green) as described in the legend to Fig. 1, showing inhibition of UTP-induced (10 μM, 30 min) nuclear accumulation of NFATc3 by treatment with the VDCC inhibitor nisoldipine (100 nm) or the ATP-dependent potassium channel opener, pinacidil (1 μM). B, summary data, quantified as described under “Experimental Procedures,” from multiple repeats of experiments described for A. C, summary data from multiple repeats of experiments employing depolarizing stimuli (60 mM K+ and the Ca2+ ionophore, ionomycin (1 μM) for 30 min (room temperature) in the presence or absence of the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) (100 nm), depicting the failure of these treatments to induce NFATc3 nuclear accumulation in cerebral arteries (n, number of mice; i, analyzed images; c, total number of cells counted).

We have shown that UTP and a number of other Gq/11-coupled vasoconstrictor agonists effectively induce the nuclear accumulation of NFATc3 in cerebral artery smooth muscle. UTP-induced NFATc3 nuclear accumulation is blocked by the chemically unrelated compounds cyclosporin A and FK506, which inhibit calcineurin activity by distinct mechanisms, indicating that this action of UTP is dependent on calcineurin. We have also found that release of Ca2+ from intracellular stores and influx of extracellular Ca2+ are both required for the induction of NFATc3 nuclear accumulation by UTP, since inhibition of either pathway completely abrogates UTP-induced NFATc3 nuclear accumulation. A specific role for Ca2+ release through IP3Rs is strongly suggested, based on the inhibition of UTP action by the cell-permeable IP3R inhibitors, xestospongin C and 2-APB.

Surprisingly, it appears that release of SR Ca2+ by RyR exerts an inhibitory effect on NFATc3 nuclear accumulation, since blocking these receptors with ryanodine potentiates UTP-induced NFATc3 nuclear translocation. The potentiating effect of RyR inhibition, which does not appear to reflect ryanodine-induced membrane depolarization, is consistent with experi-
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FIG. 6. Differential effects of IP₃Rs and RyRs on UTP-induced NFATc3 nuclear accumulation. A, inhibition of UTP-induced NFATc3 nuclear accumulation by blockers of IP₃ receptors. Cerebral arteries were treated with UTP (10 μM) with or without xestospongin C (10 μM) or 2-APB (100 μM). All treatments were for 30 min except for xestospongin C, which was applied 30 min prior to the addition of UTP. *** p < 0.001, UTP compared with all groups. B, potentiation of UTP-induced NFATc3 nuclear accumulation by the selective RyR blocker, ryanodine (Ry; 10 μM) in the presence or absence (+ HK) of ryanodine-induced changes in membrane potential. All treatments were for 30 min except for ryanodine (10 μM), which was applied 20 min prior to the addition of UTP (10 μM). HK (high K⁺), 60 mM potassium (n, number of mice; v, number of vessels; i, analyzed images; c, total number of cells counted).

ments showing that increasing Ca²⁺ spark frequency with micromolar caffeine inhibits UTP-induced NFATc3 nuclear accumulation, as well as data indicating that UTP profoundly decreases spark frequency. Collectively, these data provide strong support for the idea that Ca²⁺ sparks may exert a novel inhibitory effect on NFATc3 nuclear accumulation in addition to their well characterized role in providing negative feedback regulation of vascular tone through activation of BK channels (45).

The role of Ca²⁺ waves in the action of UTP on NFATc3 subcellular localization is less clear. UTP induces a global elevation in [Ca²⁺], as well as a striking increase in the frequency of smooth muscle cells exhibiting Ca²⁺ waves or wave-like features. Ca²⁺ waves are thought to be due to IP₃R-mediated Ca²⁺ release (42, 63, 64) and may further depend on Ca²⁺-dependent Ca²⁺ release through ryanodine receptors (41). Therefore, it was not unexpected that inhibitors of IP₃R or RyR prevented Ca²⁺ waves. However, treatments that inhibit RyR-potentiated UTP-induced NFATc3 nuclear accumulation, unlike inhibitors of IP₃R, which had a profound inhibitory effect on NFATc3 nuclear accumulation. Since inhibitors of IP₃R and RyR block waves but have opposite effects on NFATc3 nuclear accumulation, it is unlikely that Ca²⁺ waves are required, although it is conceivable that they may contribute to the efficacy of UTP. In contrast, the UTP-induced IP₃R-mediated global Ca²⁺ transient, which was not inhibited by ryanodine (Fig. 5D), is probably necessary and sufficient for NFATc3 nuclear accumulation in cerebral artery smooth muscle cells.

The differential effects of SR Ca²⁺ release through IP₃R and RyR suggests that local Ca²⁺ action at release sites may be an important component of the observed effects. In this view, IP₃R-mediated Ca²⁺ release is coupled to cellular elements that promote calcineurin activation and NFAT dephosphorylation, whereas RyR-mediated Ca²⁺ release may act through associated or closely apposed molecular components to negatively regulate NFAT import or promote its nuclear export.

FIG. 7. Ca²⁺ spark frequency and NFATc3 nuclear accumulation in cerebral artery smooth muscle. A, intact cerebral arteries loaded with fluo-4 depicting Ca²⁺ sparks under control conditions (left) and in the presence of 10 μM UTP (right). Average fluorescence of 30 images in 58.1 × 54.5 μm (256 × 240 pixels) areas for each condition. Labeled boxes (1.54 × 1.54 μm or 7 × 7 pixels) indicate the locations of detected events. Note the greater frequency of spark events under control conditions (left) than in the presence of UTP (right). Three representative events are depicted below each respective image, plotted as F/F₀, as a function of time. Spark events are qualitatively similar in the presence and absence of UTP, and spark amplitudes are not affected by UTP (675 and 168 events analyzed for control and UTP, respectively). White bars represent 20 μm. B and C, summary data showing average spark frequency (B) and NFATc3 nuclear accumulation (C) under control conditions and upon treatment with 10 μM UTP alone, UTP plus caffeine (300 μM), and 60 mM potassium (HK) alone (only in B). D, UTP induces NFATc3 nuclear accumulation and decreased Ca²⁺ spark frequency with a similar time dependence in smooth muscle cells of intact cerebral arteries. For spark frequency data, eight vessels from six different animals were studied, and for each time point between 8 and 21 different representative areas were each scanned for 10 s. For immunofluorescence data, a minimum of 600 cells, 10 vessels, and 3 animals were analyzed for each time point.
Alternatively, it is possible that inhibition of RyR-mediated Ca\textsuperscript{2+} efflux may simply serve to increase Ca\textsuperscript{2+} efflux through IP\textsubscript{3}R. Under identical experimental conditions, no Ca\textsuperscript{2+} waves are observed in individual myocytes (not shown).

In addition to highlighting potentially important tissue-specific differences in NFAT regulation, our results provide the first evidence that two distinct intracellular Ca\textsuperscript{2+} release mechanisms, represented by IP\textsubscript{3}R and ryanodine receptors, can have opposing regulatory effects on a Ca\textsuperscript{2+}-dependent transcription factor.

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