Rho-kinase-mediated suppression of K_{DR} current in cerebral arteries requires an intact actin cytoskeleton

Kevin D. Luykenaar, Rasha Abd El-Rahman, Michael P. Walsh, and Donald G. Welsh

Departments of 1Physiology and Biophysics and 2Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada

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Luykenaar KD, El-Rahman RA, Walsh MP, Welsh DG. Rho-kinase-mediated suppression of K_{DR} current in cerebral arteries requires an intact actin cytoskeleton. Am J Physiol Heart Circ Physiol 296: H917–H926, 2009. First published February 13, 2009; doi:10.1152/ajpheart.01206.2008.—This study examined the role of the actin cytoskeleton in Rho-kinase-mediated suppression of the delayed-rectifier K\(^+\) (K_{DR}) current in cerebral arteries. Myocytes from rat cerebral arteries were enzymatically isolated, and whole cell K_{DR} currents were monitored using conventional patch-clamp electrophysiology. At +40 mV, the K_{DR} current averaged 19.8 ± 1.6 pA/pF (mean ± SE) and was potently inhibited by UTP (3 × 10^{-5} M). This suppression was observed to depend on Rho signaling and was abolished by the Rho-kinase inhibitors H-1152 (3 × 10^{-7} M) and Y-27632 (3 × 10^{-5} M). Rho-kinase was also found to concomitantly facilitate actin polymerization in response to UTP. We therefore examined whether actin dynamics played a role in the ability of Rho-kinase to suppress K_{DR} current and found that actin disruption using either cytochalasin D (1 × 10^{-5} M) or latrunculin A (1 × 10^{-8} M) prevented current modulation. Consistent with our electrophysiological observations, both Rho-kinase inhibition and actin disruption significantly attenuated UTP-induced depolarization and constriction of cerebral arteries. We propose that UTP initiates Rho-kinase-mediated remodeling of the actin cytoskeleton and consequently suppresses the K_{DR} current, thereby facilitating the depolarization and constriction of cerebral arteries.

pyrimidine nucleotides; Rho signaling; potassium channels; vascular smooth muscle; delayed-rectifier potassium current

A network of resistance arteries controls the distribution of blood flow through the cerebral circulation. Arterial tone is determined by smooth muscle contractility and is regulated by a number of physiological factors, including metabolic state (9), humoral and neural stimuli (27), intraluminal pressure (10, 17), as well as endothelial factors (16). Many of these stimuli initiate changes in vascular tone via G protein-coupled receptors and the activation of downstream signaling pathways. Effector proteins within such transduction pathways can influence vascular smooth muscle contractility by altering the Ca\(^{2+}\) sensitivity of the myofilaments (29) and/or the activity of ion channels that control membrane potential (E\(_m\)) and voltage-gated Ca\(^{2+}\) entry (22).

The Rho pathway is a primary signaling pathway regulating vascular smooth muscle contraction. Rho signaling is initiated via receptors coupled to G\(_{12/13}\), resulting in the activation of the small GTPase RhoA and its principal downstream effector Rho-kinase (3, 28). Rho-kinase directly phosphorylates and inactivates myosin light chain phosphatase, ultimately increasing the phosphorylation state of myosin and facilitating contraction (30). Recent studies suggest that Rho-kinase is also capable of modulating ion channels and E\(_m\) (8, 19). In particular, we found that Rho-kinase was essential to the depolarization and constriction of cerebral arteries in response to agonists such as UTP. Electrophysiological measurements revealed that depolarization involved the Rho-mediated inhibition of a delayed-rectifying K\(^+\) (K_{DR}) current (19).

The mechanism by which Rho-kinase suppresses the K_{DR} current remains unclear. One possibility is that Rho-kinase may directly phosphorylate voltage-gated K\(^{+}\) (K\(_V\)) channels underlying the K_{DR} current to reduce open probability. Currently, however, there is no experimental evidence in support of such a mechanism. Alternatively, Rho-kinase may indirectly mediate K_{DR} channel suppression by targeting the actin cytoskeleton. A number of vascular studies have implicated Rho-kinase in remodeling of the actin cytoskeleton, where it facilitates the polymerization of actin structures (1, 5, 33). Examination of the signaling events underlying this process has revealed that Rho-kinase likely phosphorylates and activates LIM-kinase, an endogenous inhibitor of coflin, a protein that catalyzes the disassembly of filamentous actin (F-actin; see Ref. 12). Intriguingly, past studies have demonstrated that both Kv1.2 and Kv1.5, key pore-forming subunits of the cerebral arterial K_{DR} current, can couple to actin via the cytoskeletal-binding proteins cortactin and α-actinin2, respectively (11, 21). In consideration of these observations, it is conceivable that Rho-kinase may regulate K_{DR} current by modifying cytoskeletal elements to influence channel activity, thereby producing the electrical and vasomotor responses associated with the constriction of cerebral arteries.

In this study, we examined the roles of Rho signaling and the actin cytoskeleton in enabling vasoconstrictors to suppress the K_{DR} current and elicit arterial depolarization. Electrophysiological measurements confirmed that the K_{DR} current was potently inhibited by UTP through a mechanism dependent on Rho-kinase. We found that Rho-kinase facilitated actin polymerization in response to UTP and that a functional actin cytoskeleton was necessary for K_{DR} current suppression. Furthermore, disrupting the actin cytoskeleton limited the ability of UTP to depolarize and constrict cerebral arteries, similar to the effects of Rho-kinase inhibition. Our findings suggest that Rho-kinase likely modifies actin cytoskeletal structure to reduce K_{DR} channel activity, thereby facilitating the depolarization and constriction of cerebral arteries.

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MATERIALS AND METHODS

Animal procedures and tissue preparation. Animal procedures were approved by the University of Calgary Animal Care and Use Committee. Female Sprague-Dawley rats (10–12 wk of age; ~150 grams) were killed via carbon dioxide asphyxiation. Following death, the brain was isolated and stored in ice-cold PBS (pH 7.4) solution containing (in mM): 138 NaCl, 3 KCl, 10 Na2HPO4, 2 NaH2PO4, 5 glucose, 0.1 CaCl2, and 0.1 MgSO4. Middle cerebral arteries were enzymatically digested to isolate smooth muscle cells. In brief, arterial segments were equilibrated for 10 min at 37°C in an isolation medium containing (in mM): 60 NaCl, 80 sodium glutamate, 5 KCl, 2 MgCl2, 10 glucose, and 10 HEPES with 1 mg/ml albumin (pH 7.4). Tissue samples were subsequently incubated for 15 min in the same medium supplemented with 0.5 mg/ml papain and 1.5 mg/ml dithioerythritol, followed by a 10-min incubation in medium containing 100 μM Ca2+, 0.7 mg/ml type F collagenase, and 0.4 mg/ml type H collagenase. The tissue was then washed repeatedly in ice-cold isolation medium and triturated with a fire-polished pipette to disperse smooth muscle cells. Cell samples were stored in cold isolation medium for electrophysiological study the same day.

Electrophysiology. Conventional patch-clamp electrophysiology was used to measure KDR currents as previously described (20). Patch pipettes were pulled from borosilicate glass and fire-polished to resistances of 4–7 MΩ. Pipettes were then coated with wax to minimize capacitance and backfilled with pipette solution containing (in mM): 110 potassium gluconate, 30 KCl, 0.5 MgCl2, 5 HEPES, 10 EGTA, 5 Na2-ATP, and 1 GTP (pH 7.2). Cells were voltage clamped in a bath solution containing (in mM): 120 NaCl, 3 NaHCO3, 4.2 KCl, 1.2 KH2PO4, 2 MgCl2, 0.1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4). A 1 M NaCl-agar salt-bridge around the reference electrode was used to minimize offset potentials. Whole cell currents were recorded on an Axopatch 200B amplifier (Molecular Devices, MDS Analytical Technologies, Mississauga, ON), filtered at 1 kHz, digitized at 5 kHz, and analyzed with Clampfit 8.2 software. Cell capacitance was measured with the cancellation circuitry in the voltage-clamp amplifier and averaged 16.8 ± 0.7 pF. All experiments were performed at room temperature (20–22°C). Cells were voltage-clamped at −60 mV and equilibrated for 15 min before experimentation. Whole cell KDR currents were monitored under control conditions and following the addition of UTP (3 μM). To examine Rho-kinase signaling and the function of the actin cytoskeleton, myocytes were preincubated in relaxation medium and triturated with a fire-polished pipette to disperse smooth muscle cells. Cell samples were stored in cold isolation medium for electrophysiological study the same day.

Arterial diameter and Ems. Segments of unbranched middle cerebral arteries (~2 mm in length) were cannulated and mounted in a customized arteriograph chamber (J.B. Pierce Laboratory, New Haven, CT) and superfused with warm (37°C) physiological salt solution (pH 7.4) containing (in mM): 119 NaCl, 4.7 KCl, 20 NaHCO3, 1.7 KH2PO4, 1.2 MgSO4, 1.6 CaCl2, and 10 glucose. Arteries were maintained under no-flow conditions and at low intraluminal pressure (15 mmHg) to minimize myogenic mechanisms during the examination of agonist responses. Endothelial-dependent mechanisms were eliminated by passing air bubbles through the arterial lumen. Arterial diameter was monitored using an automated edge detection system (IonOptix, Milton, MA). Smooth muscle Ems was recorded by inserting a glass microelectrode (120–150 MΩ) filled with 1 M KCl in the vessel wall and assessing the voltage difference across the membrane using an intracellular electrometer (Warner Instruments, Hamden, CT). Successful cell impalements consisted of: 1) a sharp negative Ems deflection upon entry, 2) a stable recording following entry, and 3) a sharp return to baseline upon electrode removal. Cerebral arteries were equilibrated for 30 min at 37°C before experimentation. Before experimentation, the contractile ability of each vessel was determined by a brief exposure to KCl (6 × 10−2 M). To ascertain whether Rho-kinase and the cytoskeleton were involved in UTP-induced constriction, changes in arterial diameter and smooth muscle Ems were measured under control conditions, in response to UTP, and following the addition of H-1152, cytochalasin D, or latrunculin A.

Chemicals, drugs, and enzymes. H-1152, Y-27632, cytochalasin D, and latrunculin A were purchased from Calbiochem (La Jolla, CA). Buffer reagents, collagenases (type F and H), UTP, and 4-AP were obtained from Sigma (St. Louis, MO). Papain was acquired from Worthington (Lakewood, NJ).

Statistical analyses. Data are expressed as means ± SE, and n indicates the number of vessels or cells. Paired t-tests were performed to statistically compare the effects of a given condition/treatment on arterial diameter, Ems, or whole cell current. If more than two conditions or treatments were being compared, a repeated-measures ANOVA was used. When appropriate, a Tukey-Kramer pairwise comparison was used for post hoc analysis. P values ≤0.05 were considered statistically significant.

RESULTS

KDR current and Rho-kinase regulation. To better define the mechanisms enabling pyrimidine nucleotides to inhibit the KDR current, we began our investigation by isolating the current and again demonstrating its susceptibility to UTP inhibition. With the use of whole cell patch-clamp electrophysiology, the KDR current was readily identified in smooth muscle cells isolated from rat cerebral arteries. As shown in Fig. 1A, KDR currents were measured by stepping to a series of increasingly positive potentials from a holding potential of −60 mV. The current typically activated at potentials positive to −40 mV and averaged 19.8 ± 1.6 pA/pF at +40 mV. To assess voltage dependence, tail currents were measured at −40 mV to obtain an accurate indication of the proportion of channels open following a given voltage step. Plotting normalized tail currents against voltage shows activation was detectable at voltages positive to −40 mV and was near maximal at...
+30 mV (Fig. 1B). Applying a Boltzmann function to the data establishes a voltage for half-maximal activation of 3.7 ± 1.0 mV, consistent with previously reported values for the K<sub>DR</sub> current in cerebral arterial smooth muscle (19, 20).

K<sub>DR</sub> current amplitude was reduced significantly following the application of UTP (Fig. 1, C and D). As evident in the I-V relationships, 3 × 10<sup>-5</sup> M UTP inhibited the K<sub>DR</sub> current by 37.0% as measured at +40 mV. This suppression was not associated with changes in whole cell capacitance nor was it attributable to current rundown over time (19). To emphasize that modulation occurs through a Rho-kinase pathway, we measured the effect of UTP following Rho-kinase inhibition (Fig. 2). Representative recordings in Fig. 2A show that K<sub>DR</sub> suppression did not occur in the presence of 3 × 10<sup>-7</sup> M H-1152, resulting in the absence of any significant net change in the I-V relationship of K<sub>DR</sub> (Fig. 2B). The subsequent addition of the K<sub>V</sub> channel blocker 4-AP (5 × 10<sup>-3</sup> M) significantly reduced the current (Fig. 2A and B), verifying the presence of channels known to be regulated by UTP (19).

Similar to the effect of H-1152, Rho-kinase inhibition using Y-27632 (3 × 10<sup>-5</sup> M) prevented K<sub>DR</sub> current suppression (n = 3; data not shown).

**Rho-kinase modulation of the actin cytoskeleton and K<sub>DR</sub>**

To test whether the regulation of K<sub>DR</sub> current may sequentially involve activation of Rho-kinase and changes in actin structure, we first assayed the state of actin in cerebral arteries. As shown in Fig. 3, latency revealed that a significant amount of F-actin was present, but not regulated, by capping, as well as severing, filamentous actin. Figure 4, A and C, shows that the addition of cytochalasin D (1 × 10<sup>-5</sup> M), despite not having a significant impact on baseline current, completely prevented current suppression by UTP. Similar results were obtained when the experiment was repeated with latrunculin A, which binds globular actin and prevents its incorporation into filaments (Fig. 4B). The mean data shows that latrunculin A (1 × 10<sup>-8</sup> M) did not affect baseline current amplitude but prevented inhibition by UTP (Fig. 4D). The substantial block subsequently achieved by 4-AP demonstrates that 4-AP-sensitive channels are present, but not regulated, following actin disruption.

**Rho-kinase-mediated depolarization and constriction of cerebral arteries**

Given the electrophysiological observations, it would be expected that interfering with either Rho signaling or actin dynamics would limit the ability of UTP to depolarize and constrict cerebral arteries. As shown in Fig. 5, A and B,
arteries preconstricted with UTP dilated to the Rho-kinase inhibitor H-1152 in a concentration-dependent manner. The effect of H-1152 was noticeable at $1 \times 10^{-7}$ M and induced nearly 50% dilation at $3 \times 10^{-4}$ M. We subsequently measured the effect of Rho-kinase inhibition on the ability of UTP to depolarize cerebral smooth muscle. As shown in Fig. 5, C and D, UTP typically depolarized cerebral arteries from a resting $E_m$ of $-46.5 \pm 1.2$ to $-32.9 \pm 1.9$ mV. H-1152 ($1 \times 10^{-5}$ M) significantly attenuated the depolarization by 12 mV, since $E_m$ was measured at $-44.8 \pm 3.0$ mV. Because arteries were stripped of endothelium and maintained at low pressure (15 mmHg), the effects of H-1152 on diameter and $E_m$ were not the result of effects on myogenic tone or endothelial-dependent mechanisms.

Role of the actin cytoskeleton in depolarization and constriction. Actin disruption dilated cerebral arteries preconstricted by UTP. As shown in Fig. 6, A and B, 50% dilation was achieved with $3 \times 10^{-6}$ M cytochalasin D and $1 \times 10^{-5}$ M induced a near complete return to baseline diameter. $E_m$ recordings revealed that cytochalasin D also reversed the depolarization induced by UTP, shifting smooth muscle $E_m$ from $-33.2 \pm 1.2$ to $-44.6 \pm 3.2$ mV (Fig. 6, C and D). In sharp contrast, when we repeated the previous experiment using latrunculin A, we found it affected neither the constriction nor the depolarization associated with UTP (Fig. 7). Because latrunculin A disrupts actin by binding G-actin and preventing its incorporation into growing filaments, effects on diameter and electrical responses may not have been detected because agonist-induced alterations in cytoskeletal structure were complete. To address this possibility, we tested whether pretreatment with latrunculin A may have an effect on agonist responses. The experiment shown in Fig. 8A shows that a 30-min preincubation with latrunculin A ($1 \times 10^{-5}$ M) indeed attenuated the concentration-dependent constriction to UTP. The mean data indicate a significant rightward shift in the sensitivity to UTP (Fig. 8B). When vessels were pretreated with latrunculin A, depolarization to UTP was also significantly attenuated ($-37.6 \pm 0.9$ to $-45.5 \pm 0.9$ mV; Fig. 8, C and D). The above findings are consistent with the idea that disruption of the actin cytoskeleton with cytochalasin D or latrunculin A may limit the extent to which KDR current inhibition contributes to depolarization and constriction.

DISCUSSION

In this study, we further define the mechanisms by which pyrimidine nucleotides elicit constriction of cerebral arteries to effectively reduce blood flow. Electrophysiological examination of smooth muscle cells isolated from cerebral arteries revealed a $K_{DR}$ current that was potently inhibited by UTP through a signaling mechanism involving Rho-kinase. On the basis of previous reports, we questioned whether suppression of the $K_{DR}$ current was dependent on the actin cytoskeleton. We found that Rho-kinase activity was requisite for UTP-induced actin polymerization in cerebral arteries and that interfering with actin dynamics prevented Rho-kinase from regulating the $K_{DR}$ current. The initiation of Rho-kinase-mediated alterations in the cytoskeleton and subsequent inhibition of the $K_{DR}$ current appears to be central in enabling agonists such as UTP to depolarize and constrict cerebral arteries.

$K_{DR}$ current and rho-kinase regulation. Pyrimidine nucleotides, such as UTP, are endogenous signaling compounds secreted by a number of cell types found in the blood and within the arterial wall (18). When released in close proximity to vascular smooth muscle, pyrimidine nucleotides bind to the
P2Y class of receptors to initiate a sustained constriction (7, 15). Cerebral arteries express P2Y2, P2Y4, and P2Y6 receptor subtypes, and we have previously investigated the mechanisms enabling UTP to constrict these vessels (19). Detailed examination revealed that smooth muscle depolarization and subsequent voltage-gated Ca\(^{2+}\) entry contributed substantially to constriction. We further determined that depolarization was facilitated by the inhibition of an outward K\(^{+}\) conductance, the KDR current. Consistent with the view that P2Y receptors can couple to G12/13 trimeric G proteins to initiate Rho signaling (26), Rho-kinase activity was found to be essential to the suppression of KDR current and to the associated depolarization and constriction.

In the present study, we have verified that UTP elicits KDR current inhibition via Rho-kinase. Using conventional whole cell patch-clamp electrophysiology, we readily identified a current with features characteristic of a KDR current: 1) slow time-dependent activation in response to depolarizing potentials, 2) slow deactivation kinetics, and 3) a voltage for half-maximal activation of 3.7 ± 1.0 mV. Additionally, both 4-AP-sensitive and -insensitive components could be distinguished. The 4-AP-sensitive conductance likely includes a heteromultimeric channel formed by Kv1.2, Kv1.5, and Kv1.6 subunits (24) and is of particular relevance, since it is the conductance that is regulated by UTP (19). The 4-AP-insensitive current appears to be less susceptible to agonist regulation in the cerebral circulation and may consist of members of Kv2 and Kv7 subfamilies (2, 23).

The stimulation of voltage-clamped cerebral myocytes with UTP elicited KDR current suppression with a magnitude and time course similar to earlier reports. We have previously implicated Rho signaling in this response, since suppression was abolished following the targeted inactivation of RhoA and Rho-kinase by C-3 enoyl-0methylase and Y-27632, respectively (19). Recent studies have demonstrated that Y-27632 can affect protein kinase C-δ activity (6, 31), a secondary effect that would complicate our previous interpretation of the ability of Y-27632 to prevent KDR current suppression and arterial depolarization. To address this concern, we opted to use H-1152, since it has been reported to be a more selective and potent Rho-kinase inhibitor (25). H-1152 was found to be more potent than Y-27632, with a concentration of 3 x 10\(^{-7}\) M consistently preventing KDR current suppression in isolated cells. Although both Y-27632 and H-1152 act by competing with ATP binding to Rho-kinase, the comparable effects of two structurally distinct compounds strengthen the evidence that Rho-kinase is essential in enabling UTP to elicit KDR current suppression. It is apparent that, in addition to the well-described inhibition of myosin phosphatase in Ca\(^{2+}\) sensitization, Rho-kinase can also impact ion channel activity.

The actin cytoskeleton and KDR current suppression. Although a dependence on Rho signaling is clear, we questioned the mechanism by which Rho-kinase may influence KDR channel activity. A number of vascular studies have implicated the Rho pathway in the regulation of the cytoskeletal structure, where it can influence both the assembly and disassembly of actin filaments (1, 5, 12, 33). RhoA has been shown to promote actin polymerization by activating profilin, a protein that mediates the addition of actin monomers onto the growing (+) end of filaments (12). Conversely, actin polymerization can
also be achieved through a reduced rate of disassembly at the pointed (−) end of actin filaments, and this is thought to occur upon activation of the Rho-kinase/LIM-kinase/cofilin pathway (12). We assayed the state of actin and found that Rho-kinase indeed facilitates polymerization in intact cerebral arteries stimulated with UTP. Following stimulation, the percentage of F-actin increased, and separate analyses of the G-actin content revealed a corresponding decrease. These effects were not observed when arteries were preincubated with a Rho-kinase inhibitor before agonist application. It may be predicted that such alterations in actin structure would not only impact smooth muscle cell morphology and force generation, but would likely influence the localization and/or regulation of membrane proteins and signaling complexes, including ion channels. Intriguingly, several KV channel subtypes thought to contribute to the K_{DR} current, namely Kv1.2 and Kv1.5, have been shown to be capable of associating with actin through cytoskeleton-binding proteins (11, 21).

In the present study, pharmacological disruption of actin with either cytochalasin D or latrunculin A effectively abolished the ability of UTP to suppress K_{DR} current. Therefore, an intact actin cytoskeleton appears essential to Rho-kinase inhibition of the current. Because Rho-kinase promotes actin polymerization, it is likely that alterations in actin structure are associated with changes in cytoskeleton-channel interactions that reduce channel activity. The exact nature of these interactions, and the possible involvement of intermediary proteins, has yet to be resolved. Suppression of the K_{DR} current could also involve channel internalization rather than changes in channel gating. Several reports have indicated that vascular K_{DR} channels may undergo translocation from the membrane to the cytosol in response to agonists (4, 13). The translocation...
of KDR channels would reduce current density and facilitate arterial depolarization.

Rho-kinase-mediated depolarization and constriction. The functional impact of the preceding mechanism was evident in arterial constrictions to UTP, which displayed a dependence on both Rho-kinase activity and the actin cytoskeleton. Treatment of intact cerebral arteries with H-1152 diluted arteries in a concentration-dependent manner. However, given that Rho-kinase inhibition limits actin polymerization in smooth muscle, part of this dilation is likely because of the disruption of force-generating structures. We therefore directly measured smooth muscle $E_m$ to assess changes in ion channel activity in the absence of effects linked to the force generation. These measurements indicated that Rho-kinase inhibition indeed attenuated UTP-induced depolarization, consistent with an increase in outward conductance that would accompany the relief of KDR current suppression. It should be noted that a significant constriction ($\approx 50\%$) remained following Rho-kinase inhibition, indicating that at least one additional signaling pathway is initiated during constriction to pyrimidine nucleotides. This is not surprising given that UTP is known to elicit Ca$^{2+}$ waves (14), events that have not shown a dependence on Rho signaling.

Fig. 5. UTP-induced depolarization and constriction is dependent on Rho-kinase activity. A: representative trace showing the effects of increasing concentration of H-1152 on an artery preconstricted with UTP. B: summary data of the concentration-dependent effect of H-1152 on a preconstricted artery ($n = 6$). C: representative recordings of smooth muscle membrane potential ($E_m$) measured under control conditions and in the presence of UTP ($H11011 10^{-5} \text{M}$). D: summary data of $E_m$ measured under control conditions and in the presence of UTP ($H11011 10^{-5} \text{M}$) ($n = 6$). Asterisks indicate statistical differences from control (*) and UTP (**).

Fig. 6. Effect of cytochalasin D on UTP-induced depolarization and constriction. A: representative trace showing the effects of increasing concentration of cytochalasin D on an artery preconstricted with UTP. B: summary data of the concentration-dependent effect of cytochalasin D on a preconstricted artery ($n = 6$). C: representative recordings of smooth muscle $E_m$ measured under control conditions and in the presence of UTP ($H11011 10^{-5} \text{M}$). D: summary data of $E_m$ measured under control conditions and in the presence of UTP ($H11011 10^{-5} \text{M}$) ($n = 6$). Asterisks denote statistical differences from control (*) and UTP (**).
Similar to the effects of Rho-kinase inhibition, actin disruption using cytochalasin D or latrunculin A limited the electrical and vasomotor responses to UTP. These findings are congruent with the electrophysiological measurements indicating outward KDR currents are not suppressed significantly under analogous conditions. Intriguingly, the impact of latrunculin A on cerebral vessels depended on the order of application. To observe an effect, vessels had to be pretreated with latrunculin A before UTP stimulation. The application of latrunculin A following the agonist response elicited neither a change in $E_m$ nor diameter. We believe these observations reflect the distinct mechanisms by which cytochalasin D and latrunculin A interfere with actin. Cytochalasins are known to cap the growing (+) barbed end of actin and cleave F-actin to promote depolymerization, the effects of which become apparent during the sustained response to UTP. In contrast, latrunculin A disrupts actin by binding to globular actin and preventing its incorporation into filaments. Because the recruitment of G-actin into filaments is likely complete when a sustained constriction is attained, the subsequent addition of latrunculin would be expected to target residual G-actin with little effect. Although the interconversion between G- and F-actin is likely a dynamic process, the effects observed suggest a specific role for actin dynamics in vascular tone regulation.

Fig. 7. Effect of latrunculin A on UTP-induced depolarization and constriction. A: representative trace showing the effects of increasing concentration of latrunculin A on an artery preconstricted with UTP. B: summary data of the concentration-dependent effect of latrunculin A on a preconstricted artery ($n = 6$). C: representative recordings of smooth muscle $E_m$ measured under control conditions and in the presence of UTP $\pm$ latrunculin A ($1 \times 10^{-5}$ M). D: summary data of $E_m$ measured under control conditions and in the presence of UTP $\pm$ latrunculin A ($n = 6$). *Statistical difference from control.

Fig. 8. Effect of latrunculin A pretreatment on UTP-induced depolarization and constriction. A: representative trace showing the concentration-dependent constriction to UTP in the presence and absence of latrunculin A. B: summary data of arterial constriction to UTP in the presence or absence of latrunculin A ($n = 6$). C: representative recordings of smooth muscle $E_m$ measured in the presence of UTP $\pm$ pretreatment with latrunculin A ($1 \times 10^{-5}$ M). D: summary data of $E_m$ measured in the presence of UTP $\pm$ pretreatment with latrunculin A ($n = 6$). *Statistical difference.
process that may promote gradual depolymerization, we did not observe any time-dependent effect of latrunculin over the course of 1–2 h.

Physiological implications. Our findings indicate that Rho-kinase likely suppresses the \( K_{\text{DR}} \) current by eliciting reorganization of the cortical actin cytoskeleton. We propose that such a mechanism enables UTP to depolarize smooth muscle, thereby facilitating voltage-gated \( Ca^{2+} \) entry and the constriction of cerebral arteries. We have previously hypothesized that pyrimidine nucleotides activate Rho-kinase through the sequential activation of \( P_2Y \) receptors, \( G_{12/13} \), p115 RhoGEF, and RhoA (19). Therefore, cytoskeletal remodeling and \( K_{\text{DR}} \) current suppression are likely to similarly facilitate depolarization and constriction to UDP (19). Furthermore, this type of regulation may not be specific to \( P_2Y \) receptor agonists, since constriction of cerebral arteries to the thromboxane mimetic U-46619 also involves Rho-kinase-dependent \( K_{\text{DR}} \) current suppression are likely to similarly facilitate depolarization and constriction to UDP (19). Moreover, U-46619 elicits a significant suppression of the \( K_{\text{DR}} \) current (32), conceivably involving a mechanism analogous to UTP inhibition. A role for Rho-kinase in the voltage-dependent constriction of mesenteric arteries has also been reported (8), suggesting the capacity of Rho-kinase to regulate electromechanical coupling extends beyond the cerebral circulation. More detailed examination is required to determine whether the modulation of ion channels and \( E_{\text{in}} \) in these tissues is similarly dependent on cytoskeletal remodeling.

In closing, it is clear that the initiation of Rho signaling can alter cerebrovascular tone through multiple, interconnected pathways. In addition to sensitizing the contractile machinery to available \( Ca^{2+} \), Rho-kinase plays an essential role in remodeling the actin cytoskeleton during agonist responses. Our present findings suggest that this influence on actin dynamics enables Rho-kinase to effectively regulate the \( K_{\text{DR}} \) current and impact electromechanical coupling.

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