

Pyrimidine nucleotides suppress K_{DR} currents and depolarize rat cerebral arteries by activating Rho kinase

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Luykenaar, Kevin D., Suzanne E. Brett, Bin Nan Wu, William B. Wiehler, and Donald G. Welsh. Pyrimidine nucleotides suppress K_{DR} currents and depolarize rat cerebral arteries by activating Rho kinase. *Am J Physiol Heart Circ Physiol* 286: H1088–H1100, 2004. First published October 30, 2003; 10.1152/ajpheart.00903.2003.— This study examined whether, and by what signaling and ionic mechanisms, pyrimidine nucleotides constrict rat cerebral arteries. Cannulated cerebral arteries stripped of endothelium and pressurized to 15 mmHg constricted in a dose-dependent manner to UTP. This constriction was partly dependent on the depolarization of smooth muscle cells and the activation of voltage-operated Ca^{2+} channels. The depolarization and constriction induced by UTP were unaffected by bisindolylmaleimide I, a PKC inhibitor that abolished phorbol ester (PMA)-induced constriction in cerebral arteries. In contrast, the Rho-kinase inhibitor Y-27632 attenuated the ability of UTP to both constrict and depolarize cerebral arteries. With patch-clamp electrophysiology, a voltage-dependent delayed rectifying K^+ (K_{DR}) current was isolated and shown to consist of a slowly inactivating 4-aminopyridine (4-AP)-sensitive and an -insensitive component. The 4-AP-sensitive K_{DR} current was potently suppressed by UTP through a mechanism that was not dependent on PKC. This reflects observations that demonstrated that 1) a PKC activator (PMA) had no effect on K_{DR} and 2) PKC inhibitors (calphostin C or bisindolylmaleimide I) could not prevent the suppression of K_{DR} by UTP. The Rho kinase inhibitor Y-27632 abolished the ability of UTP to inhibit the K_{DR} current, as did inhibition of RhoA with C3 exoenzyme. Cumulatively, these observations indicate that Rho kinase signaling plays an important role in eliciting the cerebral constriction induced by pyrimidine nucleotides. Moreover, they demonstrate for the first time that Rho-kinase partly mediates this constriction by altering ion channels that control membrane potential and Ca^{2+} influx through voltage-operated Ca^{2+} channels.

vasoconstrictors; membrane potential; potassium channels; protein kinase C

AN INTEGRATED NETWORK of resistance arteries controls the magnitude and distribution of blood flow within cerebral tissue. Under dynamic conditions, tone within the cerebral network is regulated by multiple stimuli produced by changes in tissue metabolism (14), neural activity (38), blood flow (11), and intravascular pressure (15, 21). Many vasoactive stimuli initiate changes in arterial tone by activating transduction pathways associated with G protein-coupled receptors. It is key proteins within such pathways that in turn influence the Ca^{2+} sensitivity of the myofilament (39) and/or ion channels that control cytosolic Ca^{2+} through changes in resting membrane potential (28).

Pyrimidine nucleotides such as UTP and UDP are released from cells that are part of, or pass through the lumen of, resistance arteries (23, 31). These sources include activated platelets and endothelial cells (25, 36). In the cerebral circulation, the binding of pyrimidine nucleotides to P2Y receptors can both dilate and constrict resistance arteries, depending on which population of receptors is recruited (17, 27). Endothelial P2Y receptors mediate cerebral artery dilation through the enhanced production of endothelium-derived factors including nitric oxide (26, 27, 31). In contrast, it is P2Y receptors on smooth muscle that are responsible for cerebral artery constriction (17, 27).

The mechanisms that enable smooth muscle P2Y receptors to constrict the cerebral vasculature have been poorly defined. Jaggar and Nelson (18), in one of the few investigations to address the issue, theorized a prominent role for electromechanical coupling in the overall transduction process. In greater detail, these investigators argued that the activation of pyrimidine-sensitive P2Y receptors enhances PKC activity via G_q , phospholipase $C\beta$, and the production of diacylglycerol. By altering the phosphorylation state of key proteins, this serine/threonine kinase was subsequently proposed to inhibit outward K^+ conductance and thereby initiate depolarization (18). In cerebral smooth muscle, K^+ conductance is determined by four families of K^+ channels including those that underlie the inwardly rectifying, the ATP-sensitive, the large-conductance Ca^{2+} -activated (BK), and the voltage-dependent delayed rectifying (K_{DR}) K^+ currents (28). With the exception of the inwardly rectifying K^+ channels, all of these K^+ conductances display sensitivity to PKC and thus, in theory, could be targeted and suppressed by pyrimidine nucleotides (1, 4, 5).

This study examined whether, and by what signaling and ionic mechanisms, pyrimidine nucleotides constrict rat cerebral arteries. We report that UTP-induced constriction partly depends on the depolarization of cerebral smooth muscle cells and the activation of voltage-operated Ca^{2+} channels. Contrary to initial expectations, the depolarization and constriction induced by UTP did not depend on PKC but rather arose from the activation of Rho kinase. Such findings indicate that, in addition to regulating myosin light chain phosphatase, Rho kinase likely targets other downstream effectors of smooth muscle cell contraction including ion channels. Electrophysiological measurements indicate that the slowly inactivating 4-aminopyridine (4-AP)-sensitive K_{DR} current is one of the ionic conductances suppressed by UTP through a signaling pathway that involves RhoA/Rho kinase and not PKC. In summary, our findings are the first to establish RhoA/Rho kinase as a mod-

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ulator of ion channels that control smooth muscle membrane potential (E_m). K_{DR} current suppression by RhoA/Rho kinase likely plays an important role in enabling pyrimidine-sensitive P2Y receptors to depolarize and consequently constrict intact cerebral arteries.

MATERIALS AND METHODS

Animal procedures and tissue preparation. All procedures were approved by the University of Calgary Animal Care and Use Committee. Briefly, female Sprague-Dawley rats (10–12 wk of age) were euthanized by carbon dioxide asphyxiation. The brain was carefully removed and placed in cold phosphate-buffered saline containing (in mM) 138 NaCl, 3 KCl, 10 Na_2HPO_4 , 2 NaH_2PO_4 , 5 glucose, 0.1 CaCl_2 , and 0.1 MgSO_4 (pH 7.4). Cerebellar and cerebral arteries were dissected free of the surrounding tissue and cut into 2-mm segments.

Intact cerebral arteries. Cerebral arteries were mounted in a customized arteriograph chamber (J. B. Pierce Laboratory, New Haven, CT) and superfused with warm (37°C) physiological salt solution containing (in mM) 119 NaCl, 4.7 KCl, 20 NaHCO_3 , 1.7 KH_2PO_4 , 1.2 MgSO_4 , 1.6 CaCl_2 , and 10 glucose (pH 7.4). Endothelial cells were removed from all arteries by passing air bubbles through the vessel lumen for 2 min; successful removal was confirmed by the loss of acetylcholine-induced dilations. In keeping with past studies (43, 44), cannulated arteries were pressurized to 15 mmHg. Low intravascular pressure minimizes myogenic tone and enables investigators to study the effects of pyrimidine nucleotides with and without inhibitors in isolation. Arterial diameter was monitored with an automated edge detection system (IonOptix, Milton, MA). Smooth muscle E_m was assessed by inserting a glass microelectrode filled with 1 M KCl (tip resistance = 120–150 M Ω) carefully into the vessel wall. The criteria for successful cell impalement were 1) a sharp negative E_m deflection on entry, 2) a stable recording for at least 1 min after entry, and 3) a sharp return to baseline on electrode removal. Because E_m recordings were typically limited to 3–10 min, a distinct impalement was required to measure E_m under each experimental condition.

Experimental protocol and intact cerebral arteries. Cerebral arteries were equilibrated for 60 min at 37°C. Before experimentation, the vessel's contractile state was determined by briefly (~10 s) exposing the tissue to a 6×10^{-2} M KCl challenge. Dose responsiveness of cerebral arteries to UTP, UDP, and U-46619 was ascertained by increasing agonist concentration every 10 min and monitoring arterial diameter. To study the effects of PKC, Rho kinase, and voltage-operated Ca^{2+} channel inhibition, cerebral arteries were precontracted with agonist and then exposed to bisindolylmaleimide I (10^{-8} – 3×10^{-6} M), Y-27632 (10^{-7} – 3×10^{-5} M), and diltiazem (3×10^{-5} M), respectively. It should be noted that agonist concentration was varied slightly among experiments (UTP, 10^{-6} – 10^{-5} M; U-46619, 10^{-9} – 10^{-8} M) to set the initial constrictor response at 70–90% of maximum.

Preparation of isolated arterial smooth muscle cells. Smooth muscle cells from rat cerebral arteries were enzymatically isolated as previously described (44). In brief, arterial segments were placed in warm (37°C) cell isolation medium containing (in mM) 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl_2 , 10 glucose, and 10 HEPES with 1 mg/ml albumin (pH 7.2) for 10 min. After this equilibration step, arterial segments were initially incubated (37°C) in 0.6 mg/ml papain and 1.8 mg/ml dithioerythritol for 15 min. This was followed by a second incubation (37°C) in isolation medium containing 100 μM Ca^{2+} , 0.7 mg/ml type F collagenase, and 0.4 mg/ml type H collagenase for 10 min. After enzyme treatment, the tissue was washed three times in ice-cold isolation medium and triturated with a fire-polished pipette to release the myocytes. Cells were stored in ice-cold isolation medium for use the same day.

Patch-clamp electrophysiology and isolated smooth muscle cells. Conventional whole cell patch clamp was used to measure K_{DR} currents in cerebral smooth muscle cells. Recording electrodes (resistance 4–7 M Ω) were pulled from borosilicate glass, covered in sticky wax to reduce capacitance, and backfilled with pipette solution containing (in mM) 110 K-gluconate, 30 KCl, 0.5 MgCl_2 , 5 HEPES, 10 EGTA, 5 Na_2ATP , and 1 GTP. Recording pipettes were gently lowered onto a cerebral smooth muscle cell, and negative pressure was briefly applied to rupture the membrane and attain a gigaohm seal. Cells were voltage clamped (–60 mV) in a bath solution containing (in mM) 120 NaCl, 3 NaHCO_3 , 4.2 KCl, 1.2 KH_2PO_4 , 2 MgCl_2 , 0.1 CaCl_2 , 10 glucose, and 10 HEPES. A 1 M NaCl-agar salt bridge between the bath and the Ag-AgCl reference electrode was used to minimize offset potentials. Liquid junction potentials were measured and were <2 mV. Whole cell currents, recorded on an Axopatch 200B amplifier (Axon Instruments, Union City, CA), were filtered at 1 kHz, digitized at 5 kHz, and stored on a computer for subsequent analysis with Clampfit 8.1. Cell capacitance averaged 16.4 ± 0.4 pF and was measured with the cancellation circuitry in the voltage-clamp amplifier. Cells were excluded from analysis if capacitance shifted >0.3 pF during an experiment. All electrical recordings were performed at room temperature.

Experimental procedures and isolated smooth muscle cells. Voltage-clamped cells were equilibrated for 15 min before experimentation. After equilibration, whole cell K_{DR} currents were monitored in the presence and absence of 4-AP (5 mM), UTP (30 μM), or PMA (50 nM). To ascertain whether PKC or Rho kinase signaling was involved in the UTP-induced suppression of K_{DR} , cerebral smooth muscle cells were preincubated for 15 min with bisindolylmaleimide I (1×10^{-7} M), calphostin C (2×10^{-7} M), Y-27632 (3×10^{-5} M), or C3 exoenzyme (10 $\mu\text{g}/\text{ml} + 5 \times 10^{-5}$ M NAD^+) before the addition of UTP. Bisindolylmaleimide I, calphostin C, and Y-27632 were continuously superfused in the bath, whereas C3 exoenzyme- NAD^+ was added to the pipette solution. In general, the net current-voltage relationship was determined at 5-min intervals by measuring the peak current at the end of a 300-ms pulse to voltages between –70 and +40 mV. After each 300-ms pulse, cells were voltage clamped to –40 mV (500 ms) to facilitate the monitoring of tail currents. Tail current amplitude was used to assess steady-state activation and was calculated as the difference between the peak amplitude of the tail and the sustained level of current at –40 mV. Steady-state inactivation was ascertained by measuring peak outward current at +20 mV (200-ms voltage step) after a series of prolonged test pulses (45 s) ranging from –90 to 0 mV. Data were fitted to a Boltzmann distribution function so that the voltages for half-maximal activation and inactivation could be calculated.

RT-PCR analysis and isolated smooth muscle cells. Approximately 300 smooth muscle cells were enzymatically isolated from cerebral and cerebellar arteries and placed in RNase- and DNase-free collection tubes. After total RNA extraction (RNeasy mini kit with DNase treatment; Qiagen, Valencia, CA), first-strand cDNA was synthesized with the Sensiscript RT kit (Qiagen). Subsequently, 2 μl of each first-strand cDNA reaction were used as the template in a PCR reaction containing 1.5 mM MgCl_2 , 0.25 μM forward and reverse primers (University of Calgary), 0.2 mM deoxynucleotide triphosphates; and 2.5 units of recombinant *Taq* DNA polymerase. PCR reactions were hot started (94°C for 3 min) and underwent 35 cycles of 94°C for 1.0 min, 60°C for 0.5 min, and 72°C for 0.75 min. PCR samples were then exposed to a final extension period at 72°C for 10 min. Forward and reverse primers specific to rat P2Y2, P2Y4, and P2Y6 were as follows: P2Y2 (forward) 5'-TTCCACGTCACCCGCACCCCTCTTACT-3', P2Y2 (reverse) 5'-CGATTCCTCAACTCACACATACAAATGATTG-3'; P2Y4 (forward) 5'-CTTCTCTGCCTGGGTGTTTGGTTGGTAGTA-3', P2Y4 (reverse) 5'-TCCCCGTGAAGAGATAGAGCACTGGA-3'; and P2Y6 (forward) 5'-GCCAGTTATGGAGCGGGACAATGG-3', P2Y6 (reverse) 5'-AGGAACAGGATGCTGCCGTGTAGGTTG-3'.

The expected amplicon sizes for P2Y2, P2Y4, and P2Y6 were 539, 473, and 357 bp, respectively. DNA sequencing was used to identify each reaction product. Control experiments confirmed the absence of genomic DNA contamination by assessing where an Rho kinase-specific amplicon could be generated from samples exposed to RNase.

Chemicals, drugs and enzymes. Buffer reagents, UTP, NAD⁺, collagenase (types F and H), and diltiazem were obtained from Sigma (St. Louis, MO). PMA, bisindolylmaleimide I, Y-27632, C3 exoenzyme, and U-46619 were purchased from Calbiochem (La Jolla, CA). PMA, bisindolylmaleimide I, and C3 exoenzyme were dissolved in DMSO, with the final solvent concentration $\leq 0.05\%$.

Statistical analysis. Data are expressed as means \pm SE, and *n* indicates the number of vessels or cells. The EC₅₀ of a given agonist was ascertained by fitting a four-parameter logistic function to the observed data. Paired *t*-tests were performed to statistically compare the effects of a given condition or treatment on arterial diameter, *E_m*, 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

Vasomotor and *E_m* measurements of intact cerebral arteries.

The addition of pyrimidine nucleotides to the superfusate elicited dose-dependent constrictions in cerebral arteries pressurized to 15 mmHg (to minimize myogenic tone) and stripped of endothelium (UTP, EC₅₀ = 3.1×10^{-6} M; UDP, EC₅₀ = 3.2×10^{-6} M; Fig. 1, A and B). The sustained nature of this constriction is indicative of P2Y receptor activation and contrasts sharply with the transient constrictions elicited by agonists known to modulate P2X receptors (2). RT-PCR analysis performed on ~ 300 cerebral smooth muscle cells revealed product bands with the predicted sizes of 539, 473, and 357 bp for P2Y2, P2Y4, and P2Y6 receptors, respectively (Fig. 1C). DNA sequencing confirmed the identity of all PCR products.

Cerebral smooth muscle cells depolarized from -56.8 ± 0.8 to -37.1 ± 1.6 mV in the presence of UTP (Fig. 2, A and B). Diltiazem (3×10^{-5} M), an inhibitor of voltage-operated Ca²⁺ channels, attenuated UTP-induced constriction by $31.3 \pm 4.8\%$, indicating that tone development partly depended on depolarization (Fig. 2, C and D); nifedipine (1×10^{-6} M; *n* = 2) was also noted to attenuate constriction (data not shown). Given that P2Y receptors can activate PKC (18), subsequent experiments examined whether this kinase enabled UTP to both constrict and depolarize the cerebral vasculature. The PKC activator PMA (3×10^{-8} M) initiated a sustained constriction that was abolished by preincubation with bisindolylmaleimide I (10^{-7} M; Fig. 3A). Despite its effectiveness as a PKC inhibitor, UTP-constricted arteries did not dilate to the application of bisindolylmaleimide I at concentrations ranging from 10^{-8} to 3×10^{-7} M (Fig. 3, B and C). Preincubation of vessels with bisindolylmaleimide I (1×10^{-7} M) before the addition of UTP ($5 \mu\text{M}$) was also ineffective at attenuating constriction [diameter response in μm (*n* = 5): control, 77.1 ± 5.7 ; bisindolylmaleimide I, 74.3 ± 7.1]. Bisindolylmaleimide I did not prevent UTP from depolarizing cerebral smooth muscle cells (Fig. 3, C and D).

Contrary to the preceding observations, the Rho kinase inhibitor Y-27632 dilated cerebral arteries precontracted with UTP (Fig. 4, A and B). This attenuating effect was dose dependent, with $74.0 \pm 5.9\%$ (*n* = 7) of the agonist-induced constriction eliminated at a Y-27632 concentration of $3 \times$

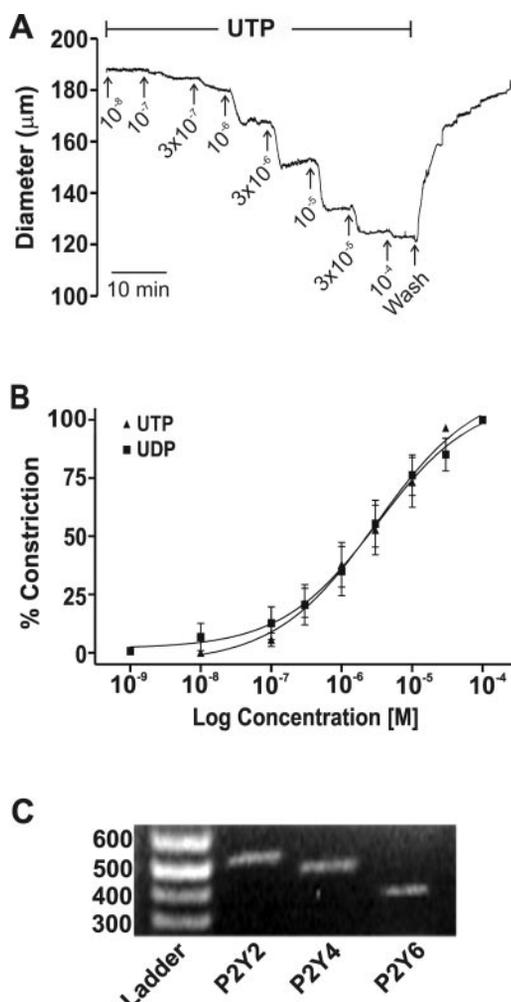


Fig. 1. Pyrimidine nucleotides constrict rat cerebral arteries. A: representative tracing of arterial constriction to an increasing concentration of UTP. B: UTP (*n* = 6) and UDP (*n* = 6) induce a dose-dependent constriction. C: RT-PCR analysis of UTP-sensitive P2Y receptors (P2Y2, P2Y4, and P2Y6) from isolated rat cerebral smooth muscle cells. Ladder markers are measured in base pairs. Data are means \pm SE.

10^{-5} M. In addition to its effects on agonist-induced tone, Y-27632 attenuated the ability of UTP to depolarize cerebral smooth muscle cells by 13.8 ± 2.1 mV (Fig. 4, C and D). The effects of Y-27632 on arterial diameter and *E_m* cannot be ascribed to alterations in myogenic signaling because vessels were maintained at low intravascular pressure (15 mmHg).

Patch-clamp electrophysiology and isolated smooth muscle cells. With conventional whole cell patch-clamp electrophysiology and pipette solutions that minimize BK channel activity, the K_{DR} current was isolated in rat cerebral smooth muscle cells. In general, brief voltage steps positive to -30 mV activated K_{DR} without an induction of inactivation (Fig. 5A). Steady-state activation and inactivation were ascertained by monitoring 1) tail currents (at -40 mV) after a set of 300-ms voltage pulses (range -70 to $+40$ mV) and 2) peak outward current (at $+20$ mV) after a series of prolonged test potentials (45 s, range -90 to -10 mV; Fig. 5B). Data fitted to a Boltzmann function established voltages for half-maximal activation and inactivation of $+1.5$ and -36.2 mV, respectively (Fig. 5C). At $+40$ mV, the K_{DR} current was stable over time:

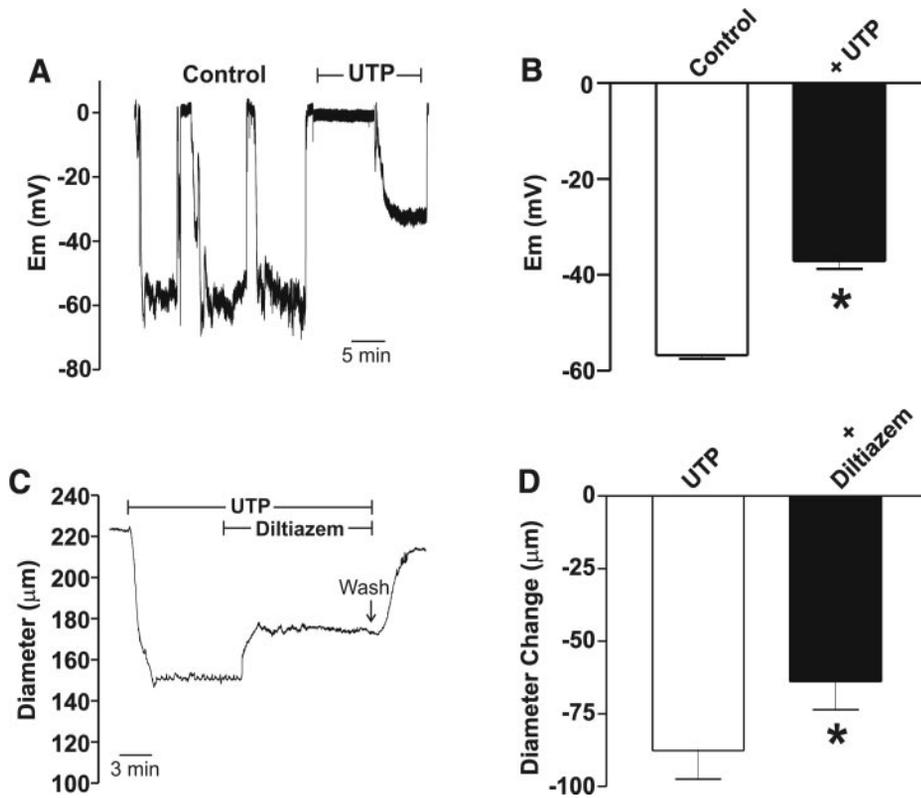


Fig. 2. UTP-induced constriction partly depends on the depolarization of cerebral smooth muscle cells and the activation of voltage-operated Ca^{2+} channels. *A*: representative tracing of membrane potential (E_m) measured from 3 separate smooth muscle cells (at the proximal, middle, and distal end of an isolated vessel) under resting conditions and from 1 smooth muscle cell in the presence of UTP (5×10^{-6} M). *B*: summary data of the effects of UTP on E_m ($n = 8$). *C*: representative tracing of the effects of diltiazem (3×10^{-5} M) on the UTP (2×10^{-6} M)-mediated constriction of rat cerebral arteries. *D*: summary data of the effects of diltiazem ($n = 8$) on the constriction induced by UTP. Data are means \pm SE. *Significant difference from control.

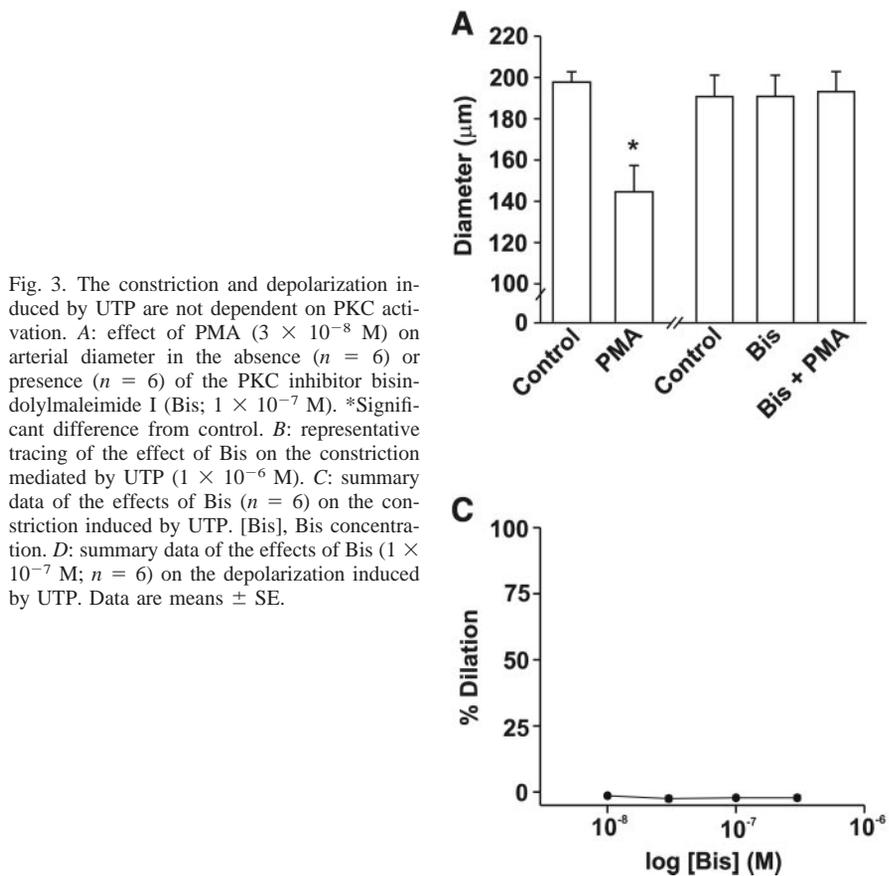
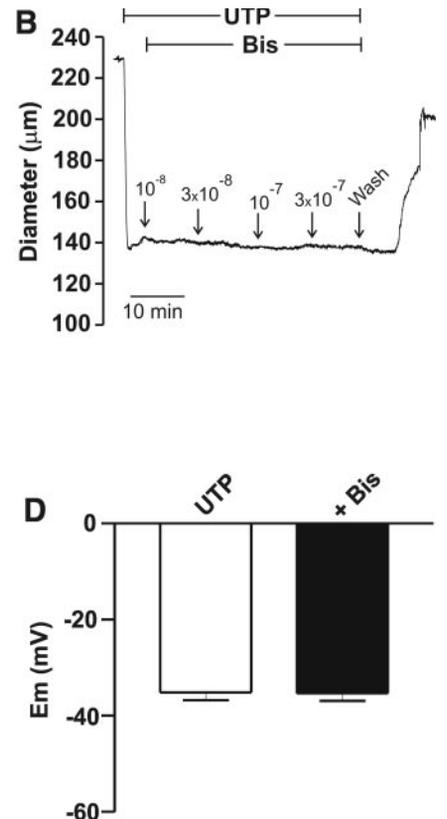


Fig. 3. The constriction and depolarization induced by UTP are not dependent on PKC activation. *A*: effect of PMA (3×10^{-8} M) on arterial diameter in the absence ($n = 6$) or presence ($n = 6$) of the PKC inhibitor bisindolylmaleimide I (Bis; 1×10^{-7} M). *Significant difference from control. *B*: representative tracing of the effect of Bis on the constriction mediated by UTP (1×10^{-6} M). *C*: summary data of the effects of Bis ($n = 6$) on the constriction induced by UTP. [Bis], Bis concentration. *D*: summary data of the effects of Bis (1×10^{-7} M; $n = 6$) on the depolarization induced by UTP. Data are means \pm SE.



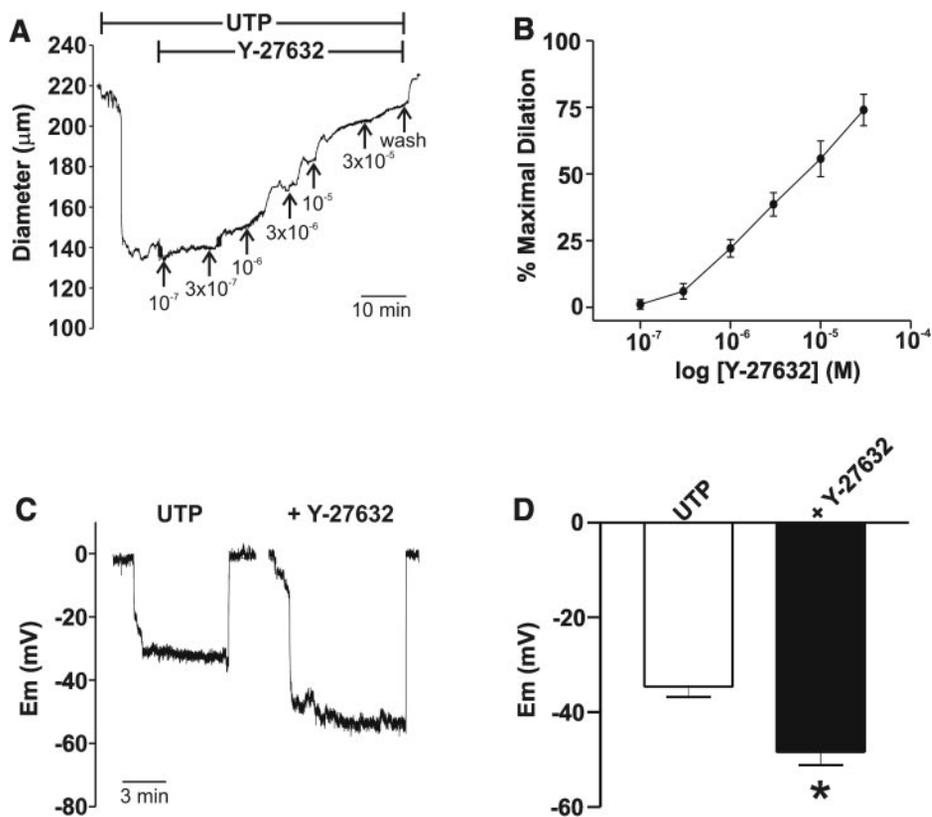


Fig. 4. The depolarization and constriction induced by UTP arise from the activation of Rho kinase. *A*: representative tracing of the effects of Y-27632 (3×10^{-5} M) on the constriction induced by UTP (5×10^{-6} M). *B*: summary data of the effects of Y-27632 ($n = 7$) on the constriction induced by UTP. *C*: representative tracing of the effects of Y-27632 (3×10^{-5} M) on the depolarization induced by UTP (5×10^{-6} M). *D*: summary data of the effects of Y-27632 ($n = 6$) on the depolarization induced by UTP. Data are means \pm SE. *Significant difference from UTP.

a small but significant current run-up was noted at -20 mV ($P \leq 0.05$; Fig. 5D).

K_{DR} currents in cerebral smooth muscle cells are comprised of two slowly inactivating components that vary in their sensitivity to 4-AP (33). In accordance with past studies (1, 33), 4-AP (5×10^{-3} M) partially suppressed the K_{DR} current, thereby revealing both the 4-AP-sensitive and -insensitive components (Fig. 6, *A* and *B*). The slowly inactivating 4-AP-sensitive component of the K_{DR} current represented $37.9 \pm 3.8\%$ of the total whole cell current at $+40$ mV. Superfused ibertoxin had no significant effect on peak outward current, confirming that high pipette concentrations of EGTA minimize the activity of BK channels (Fig. 6C).

UTP modulation of K_{DR} current. As shown in Figs. 1 and 2, pyrimidine nucleotides depolarize and constrict cerebral arteries. Consistent with the view that this depolarization partly depends on the modulation of outward K^+ conductance, UTP (3×10^{-5} M) was observed to inhibit K_{DR} by $35.0 \pm 3.4\%$ at $+40$ mV (Fig. 7, *A* and *B*). Characteristically, K_{DR} current suppression was observable 5–10 min after the addition of UTP, with peak inhibition occurring at 20 min. In the presence of 4-AP (5×10^{-3} M), UTP had no effect on the remaining whole cell K_{DR} current (Fig. 7C). Such observations are consistent with pyrimidine nucleotides modulating the 4-AP-sensitive component of the K_{DR} current.

Subsequent experiments defined whether PKC enables UTP to suppress the 4-AP-sensitive component of the K_{DR} current. PMA initiated a sustained constriction in intact cerebral arteries that was abolished by the PKC inhibitors calphostin C and bisindolylmaleimide I [diameter response in μm : PMA (3×10^{-8} M), 75.7 ± 8.8 ; calphostin C (2×10^{-7} M) + PMA, 1.2 ± 1.5 ; see Fig. 2A for bisindolylmaleimide I]. Despite the

effectiveness of PMA as a PKC activator, this agent (5×10^{-8} M) had no discernible effect on the K_{DR} current (Fig. 8). The ability of UTP to suppress the K_{DR} current was not blocked by preincubating cerebral smooth muscle cells with calphostin C (2×10^{-7} M; Fig. 9, *A* and *C*) or bisindolylmaleimide I (1×10^{-7} M; Fig. 9, *B* and *C*).

In light of recent observations showing that the UTP-sensitive P2Y receptor can activate RhoA and Rho kinase (37), subsequent experiments examined whether these signaling proteins enable UTP to suppress the 4-AP-sensitive component of the K_{DR} current. Under resting conditions, the application of Y-27632 (3×10^{-5} M) elicited a small but statistically insignificant increase in whole cell K_{DR} current (Fig. 10). Although the effects of Y-27632 were small under basal conditions, this Rho kinase inhibitor abolished the ability of UTP to suppress K_{DR} . Inhibiting RhoA activation with C3 exoenzyme ($10 \mu\text{g/ml}$) also eliminated the UTP-induced suppression of K_{DR} (Fig. 11, *A* and *C*). Control experiments confirmed that NAD^+ alone could not prevent UTP from suppressing the K_{DR} current (Fig. 11, *B* and *D*).

U-46619 and intact cerebral arteries. Further experiments tested whether the thromboxane mimetic U-46619 constricts and depolarizes cerebral arteries through a transduction process that also involves Rho kinase rather than PKC. As shown in Fig. 12A, U-46619 elicited a dose-dependent constriction in cerebral arteries stripped of endothelium and pressurized to 15 mmHg to minimize myogenic tone ($\text{EC}_{50} = 1.7 \times 10^{-9}$ M). This constriction was not blocked by bisindolylmaleimide I [diameter in μm ($n = 6$): control, 226.5 ± 8.3 ; U-46619, 137.2 ± 7.4 ; U-46619 + bisindolylmaleimide I, 133.3 ± 8.2] but was attenuated in a dose-dependent manner by Y-27632 (Fig. 12, *B* and *C*). U-46619 initiated a depolarization in

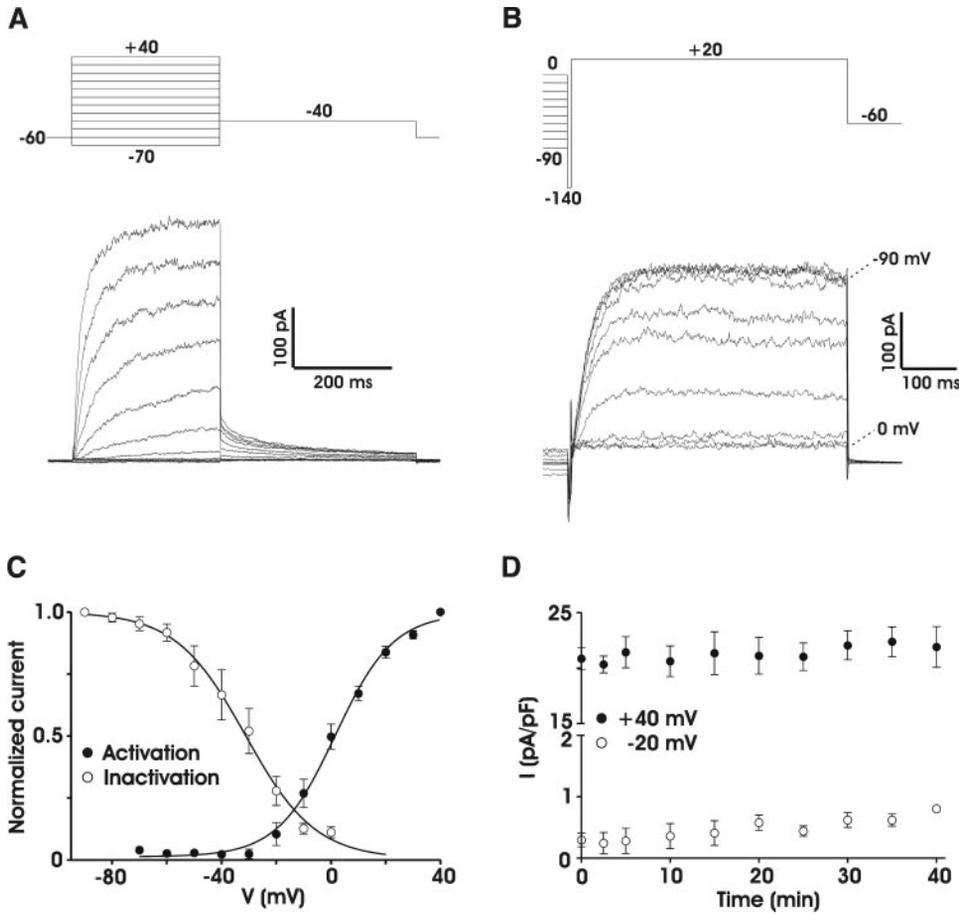


Fig. 5. Delayed rectifier K⁺ (K_{DR}) current in myocytes isolated from rat cerebral arteries. Voltage (V) paradigms (top) were designed to measure steady-state activation (A) and inactivation (B) of the K_{DR} current (I). C: plots of steady-state activation (n = 6) and inactivation (n = 8). Solid lines are Boltzmann distribution functions with half-maximal voltage potentials of +1.5 and -36.1 mV for activation and inactivation, respectively. D: time course of net outward current measured at -20 and +40 mV.

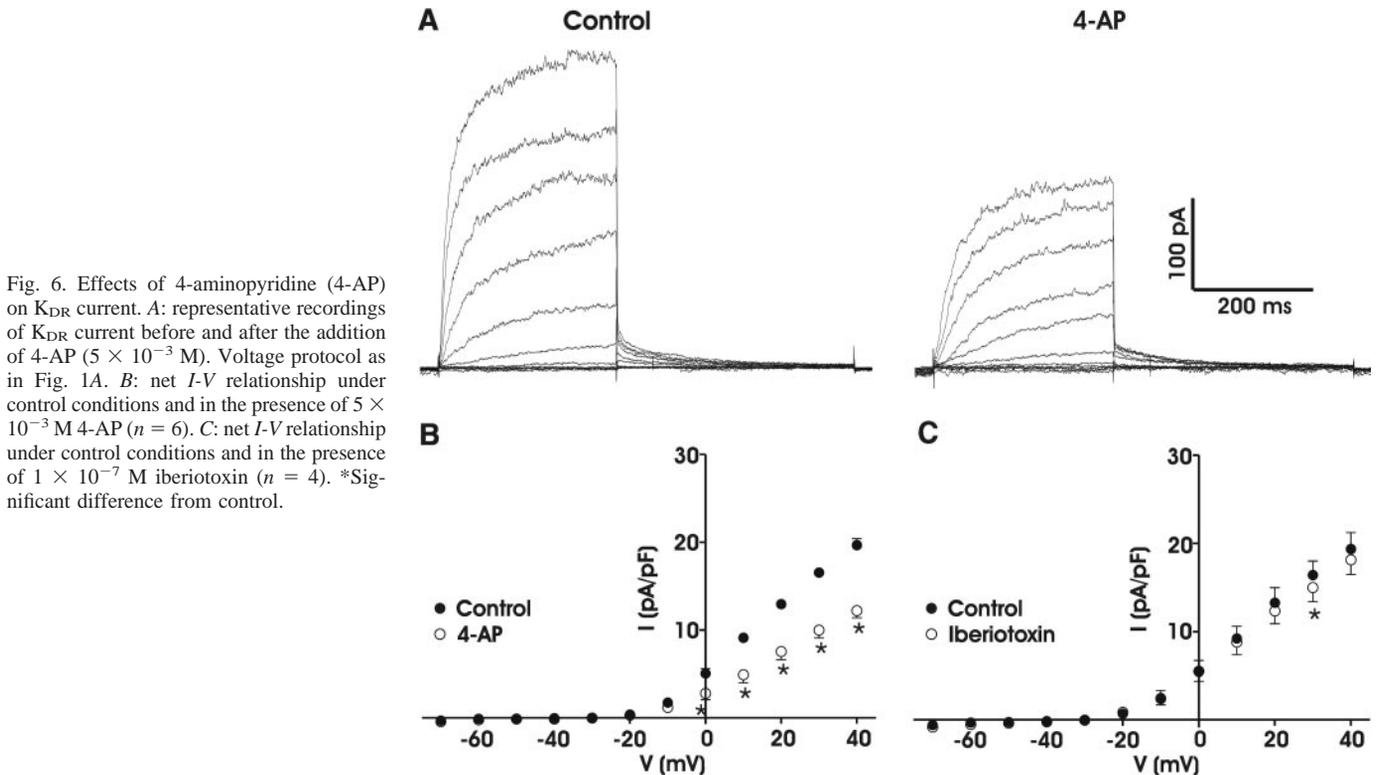
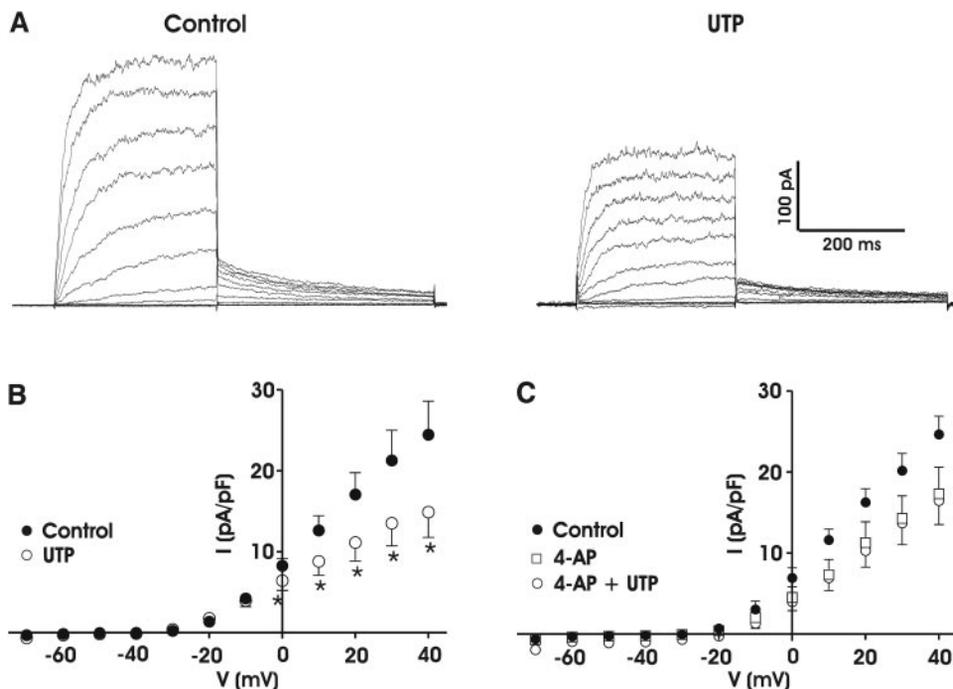


Fig. 6. Effects of 4-aminopyridine (4-AP) on K_{DR} current. A: representative recordings of K_{DR} current before and after the addition of 4-AP (5×10^{-3} M). Voltage protocol as in Fig. 1A. B: net I-V relationship under control conditions and in the presence of 5×10^{-3} M 4-AP (n = 6). C: net I-V relationship under control conditions and in the presence of 1×10^{-7} M iberiotoxin (n = 4). *Significant difference from control.

Fig. 7. UTP inhibits 4-AP-sensitive component of K_{DR} current. *A*: representative recordings of K_{DR} current before and after the addition of UTP (3×10^{-5} M). Voltage protocol as in Fig. 1*A*. *B*: net *I-V* relationship under control conditions and in the presence of UTP ($n = 6$) *C*: net *I-V* relationship under control conditions and in the presence of 4-AP \pm UTP ($n = 6$). *Significant difference from control.



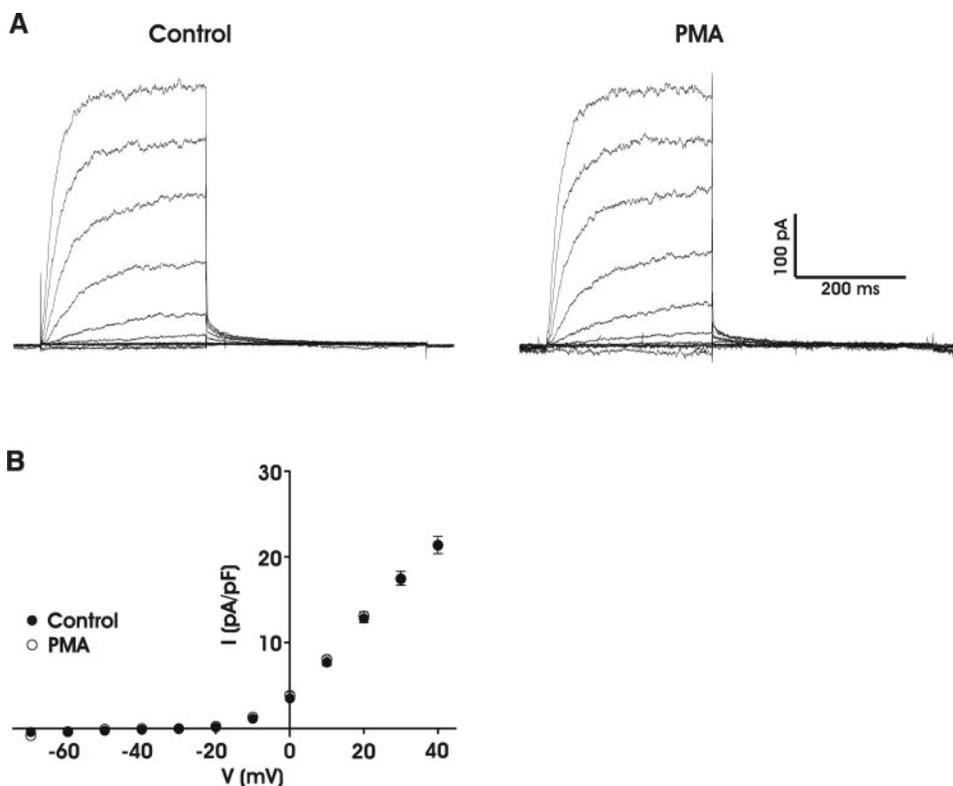
cerebral smooth muscle cells that was unaffected by bisindolylmaleimide I (Fig. 12*D*). In contrast, when bisindolylmaleimide I and Y-27632 were applied together, the depolarization induced by U-46619 was diminished by 9.4 ± 0.6 mV.

DISCUSSION

We present evidence here that UTP constricts rat cerebral arteries through a mechanism that partly depends on smooth

muscle cell depolarization and the activation of voltage-operated Ca^{2+} channels. Surprisingly, the depolarization and constriction induced by UTP was independent of PKC and instead arose from the activation of Rho kinase. Such findings indicate that Rho kinase likely targets ion channels involved in E_m regulation. Electrophysiological measurements demonstrate that one of the ionic conductances regulated by UTP via Rho kinase is the slowly inactivating 4-AP-sensitive K_{DR} current. In summary, it is Rho

Fig. 8. The PKC activator PMA does not inhibit K_{DR} current. *A*: representative recordings of K_{DR} current before and after the addition of PMA (5×10^{-8} M). Voltage protocol as in Fig. 1*A*. *B*: net *I-V* relationship under control conditions and in the presence of PMA ($n = 6$).



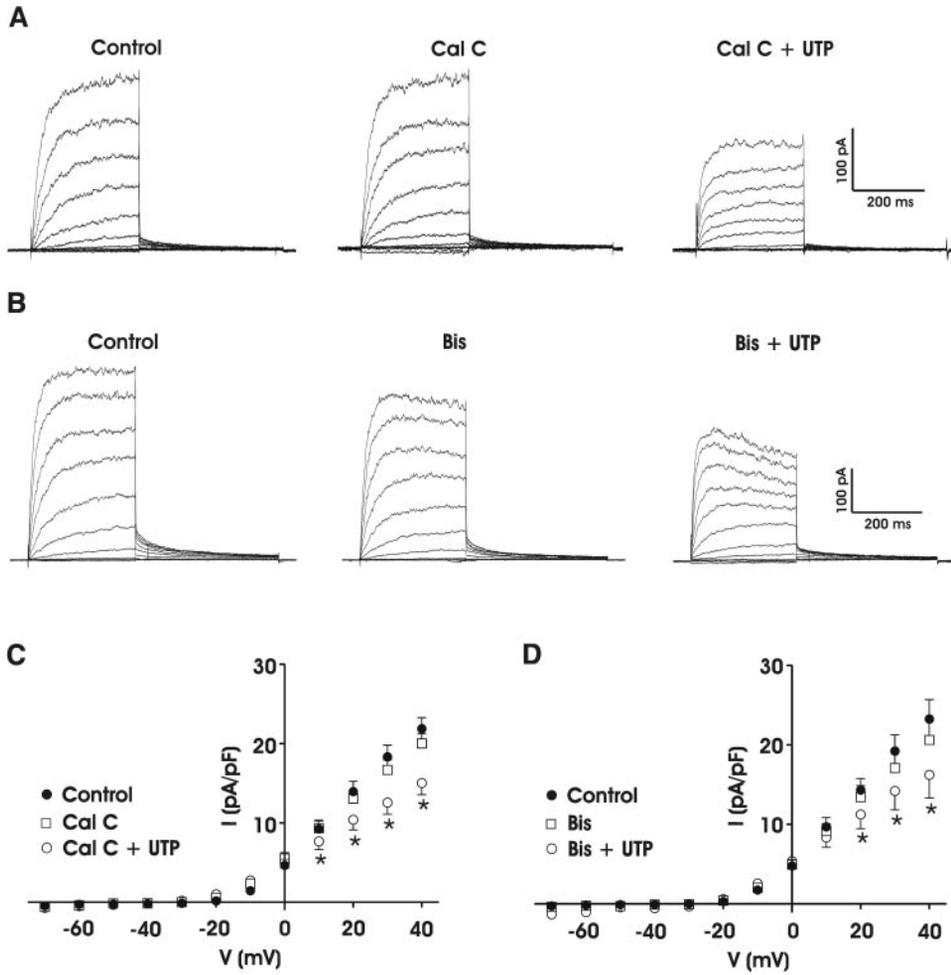


Fig. 9. K_{DR} current suppression by UTP is not dependent on PKC. *A*: representative recordings of K_{DR} current under control conditions and in the presence of calphostin C (Cal C) \pm UTP (3×10^{-5} M). Voltage protocol as in Fig. 1A. *B*: representative recordings of K_{DR} current under control conditions and in the presence of 100 nM Bis \pm UTP (3×10^{-5} M). *C* and *D*: net I - V relationship under control conditions and in the presence of Cal C \pm UTP ($n = 6$; *C*) or Bis \pm UTP ($n = 6$; *D*). *Significant difference from control.

kinase signaling that enables UTP to control both the electrical and the contractile state of cerebral smooth muscle cells.
Pyrimidine nucleotides and cerebral vasculature. Pyrimidine nucleotides such as UTP and UDP are important signaling

compounds released from cells during mechanical stress and cellular injury (23). When secreted in close proximity to cerebral smooth muscle cells, these agents elicit a sustained constrictor response (12, 19, 27). This maintained constriction

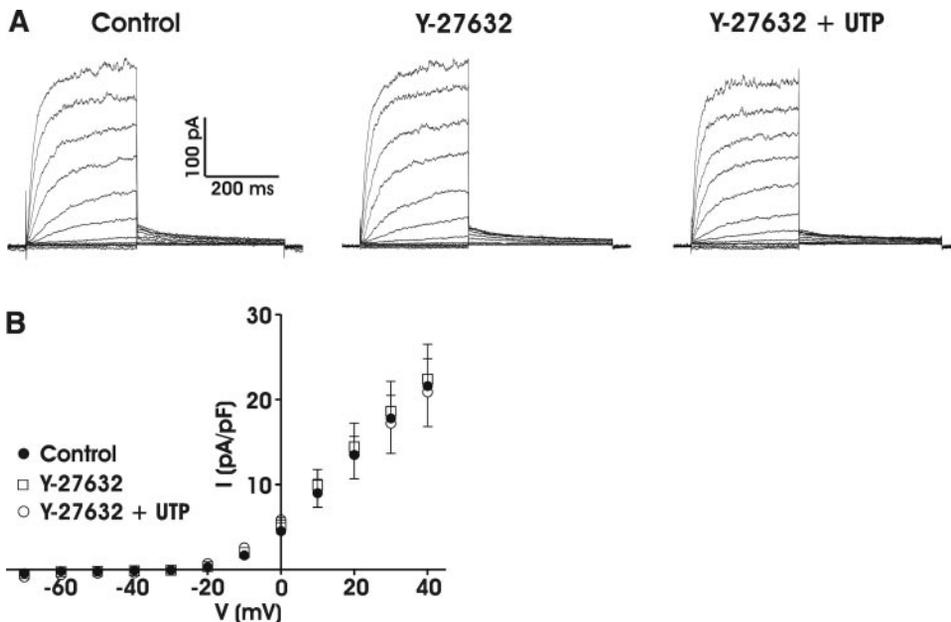


Fig. 10. K_{DR} current suppression by UTP is dependent on Rho kinase activity. *A*: representative recording of K_{DR} current under resting conditions and in the presence of the Rho kinase inhibitor Y-27632 \pm UTP (3×10^{-5} M). Voltage protocol as in Fig. 1A. *B*: net I - V relationship under control conditions and in the presence of Y-27632 \pm UTP ($n = 6$).

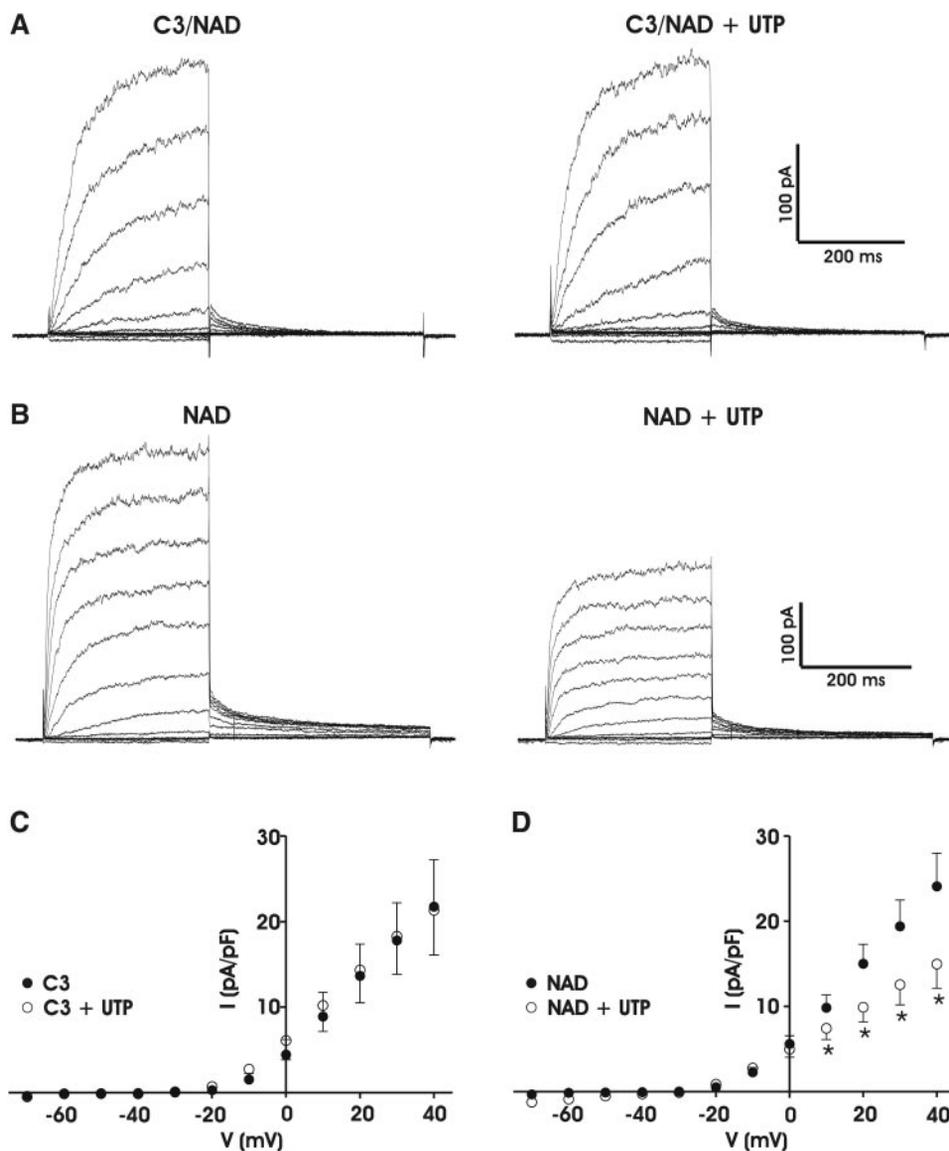


Fig. 11. K_{DR} current suppression by UTP is dependent on RhoA activity. *A*: representative recordings demonstrating the effect of UTP (3×10^{-5} M) on K_{DR} current with C3 exoenzyme (C3; 10 μ g/ml) and NAD^+ (5×10^{-5} M) in the pipette solution. Voltage protocol as in Fig. 1A. *B*: representative recordings of K_{DR} current in the presence of $NAD^+ \pm$ UTP. *C*: net $I-V$ relationship in the presence of C3- $NAD^+ \pm$ UTP ($n = 6$). *D*: net $I-V$ relationship in the presence of $NAD^+ \pm$ UTP ($n = 6$). *Significant difference from control.

was readily observed in the present study and is the result of P2Y receptor activation. Molecular studies have identified at least five subtypes of P2Y receptors that are coupled to trimeric G proteins (3, 31). Pyrimidine nucleotides potently activate three of these subtypes (i.e., P2Y2, P2Y4, and P2Y6), all of which are expressed at the mRNA level in isolated cerebral smooth muscle cells. To elicit constriction, pyrimidine-sensitive P2Y receptors must activate a signaling pathway that enhances the Ca^{2+} sensitivity of the myofilament (39) or increases cytosolic Ca^{2+} through mechanisms that are either dependent or independent of resting membrane potential (28). Two key findings support the view that electromechanical coupling plays an important role in enabling UTP to constrict cerebral arteries. First, the bath application of UTP depolarized cerebral smooth muscle cells by 19.6 ± 1.6 mV. Second, inhibiting depolarization-induced increases in Ca^{2+} channel activity (with diltiazem) attenuated constriction by $>30\%$. At present, there is little consensus as to which signaling mechanisms enable pyrimidine-sensitive P2Y receptors to depolarize and constrict intact cerebral arteries. Of the few studies con-

ducted in this area, Jaggard and Nelson (18) proposed a prominent role for PKC, a serine/threonine kinase coupled to P2Y receptors through G_q , phospholipase C β , and the production of diacylglycerol. This hypothesis was predicated on patch-clamp observations of spontaneous transient outward currents but did not involve a direct examination of intact cerebral arteries (18). Surprisingly, when this study applied a confirmed PKC inhibitor to cerebral arteries precontracted with UTP, there was no measurable effect on either constriction or depolarization.

Although PKC activation is not a key factor in enabling UTP-sensitive P2Y receptors to depolarize or constrict cerebral arteries, these findings should not be interpreted to suggest that arterial constriction is independent of G_q signaling. Indeed, pyrimidine nucleotides have been observed in cerebral arteries to induce Ca^{2+} waves, transitory Ca^{2+} events driven by phospholipase C β activation and the production of inositol (1,4,5)-trisphosphate (18). Although Ca^{2+} waves have been associated with arterial constriction (10, 35), studies have yet to resolve how a periodic event can elicit sustained constriction. We propose that a sustained constrictor response could be achieved

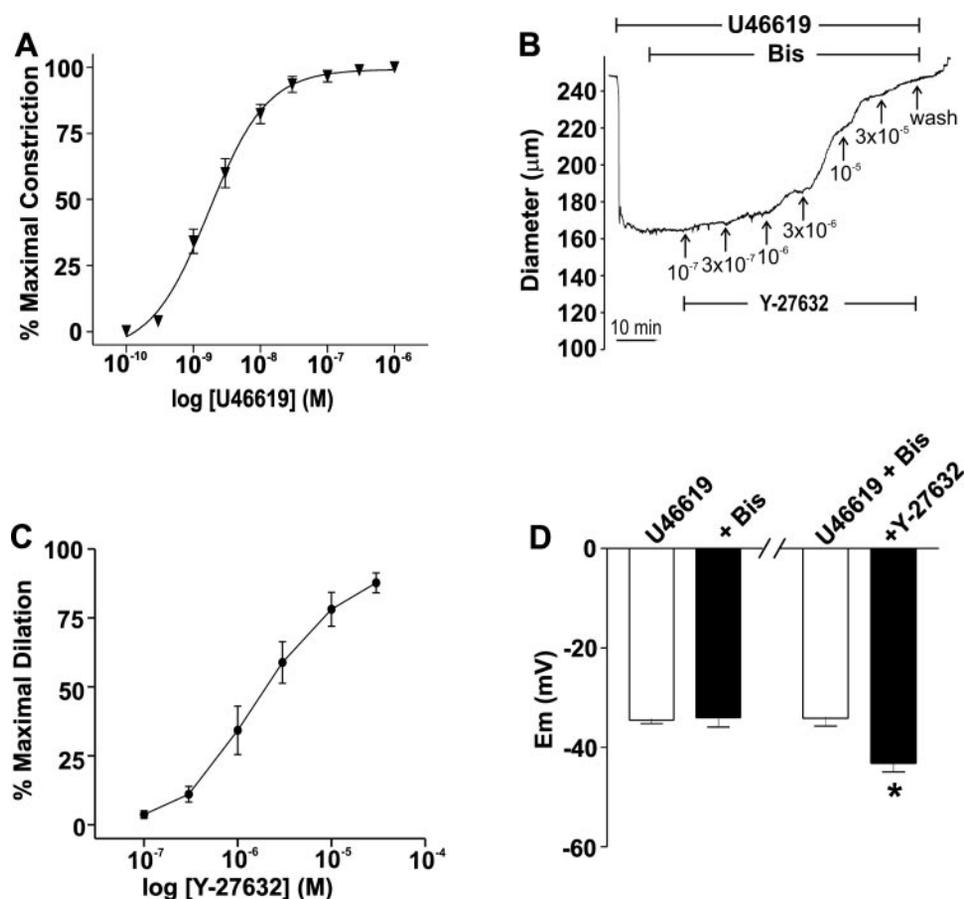


Fig. 12. The constriction and depolarization induced by the thromboxane mimetic U-46619 is dependent on Rho kinase but not PKC. *A*: U-46619 ($n = 6$) induces dose-dependent constriction. *B*: representative tracing of the effects of Bis (1×10^{-7} M) and Y-27632 on the constriction induced by U-46619 (5×10^{-9} M). *C*: summary data of the effects of Y-27632 ($n = 7$) on the constriction induced by UTP. Bis was continuously present in the superfusate throughout these experiments. *D*: summary data of the effects of Y-27632 (3×10^{-5} M; $n = 6$) on the depolarization induced by U-46619. Data are means \pm SE. *Significant difference from U-46619 + Bis.

if Ca^{2+} wave activation coincided with an inhibition of myosin light chain phosphatase. Indeed, it could be the combination of Ca^{2+} waves and phosphatase inhibition (via Rho kinase) that underlies a significant part of the UTP-mediated constriction insensitive to Ca^{2+} channel blockade. The periodic release of Ca^{2+} may also stimulate store-operated channels that in theory could increase cytosolic Ca^{2+} and elicit a constriction independent of membrane potential.

Recent findings in the coronary circulation have shown that pyrimidine-induced contraction can be effectively abolished by the Rho kinase inhibitor Y-27632 (37). From these observations, it was hypothesized that UTP-sensitive P2Y receptors mediate arterial constriction by activating $G_{12/13}$ and its associated signaling pathway including p115 Rho guanine nucleotide exchange factor (GEF), RhoA, and Rho kinase (37). Observations from the current study indicate that Rho kinase signaling is also important in the regulation of cerebral vascular tone. More specifically, we observed that cerebral arteries precontracted with UTP dilated in a dose-dependent manner to Y-27632. The vascular literature characteristically assumes that if Rho kinase inhibition impairs arterial constriction, such an alteration must exclusively reflect a change in the Ca^{2+} sensitivity of the myofilament (37, 40). This assumption arises from the known ability of Rho kinase to target and regulate myosin light chain phosphatase (40, 42). Although an important regulator of pharmacomechanical coupling, Rho kinase could conceivably target other downstream effectors of smooth muscle cell contraction including ion channels involved in membrane potential regulation. Consistent with this view,

Y-27632 was observed to reverse the depolarization initiated by UTP. These physiological findings appear to be the first in the resistance vasculature to support a role for Rho kinase in the regulation of ion channel activity and electromechanical coupling.

To depolarize cerebral smooth muscle cells, P2Y receptors along with Rho kinase must enhance depolarizing inward currents and/or inhibit hyperpolarizing K^+ conductances. Although voltage-operated Ca^{2+} (34), nonselective cation (44), and four distinct K^+ (28) currents have been isolated in cerebral smooth muscle cells, studies have never ascertained whether any of these conductances are sensitive to Rho kinase modulation. Despite this lack of evidence in native tissue, findings in expression systems have shown that voltage-dependent K_{DR} channels may be one of the primary targets (6). This view is based on the work of Cachero et al. (6), who observed the ability of G protein-coupled receptors to suppress $\text{Kv}1.2$ by activating RhoA, the upstream regulator of Rho kinase. Intriguingly, $\text{Kv}1.2$ is one of the key pore-forming subunits of the slowly inactivating 4-AP-sensitive K_{DR} channel in vascular smooth muscle (20, 43). This K_{DR} channel contributes to resting E_m , and its inhibition by vasoconstrictors is known to induce arterial depolarization (7, 16). In light of these observations, a supplemental examination was undertaken to examine the relationship between pyrimidine nucleotides and K_{DR} channel regulation.

K_{DR} current in cerebral smooth muscle cells. Conventional whole cell patch-clamp electrophysiology was used to isolate and examine the K_{DR} current in cerebral smooth muscle cells.

Generally speaking, whole cell K_{DR} current was characterized by 1) measurable activation at voltages positive to -30 mV, 2) slow time-dependent inactivation, and 3) voltages of half-maximal activation and inactivation of $+1.5$ and -36.1 mV, respectively. These documented K_{DR} properties are similar to those in rat mesenteric arteries (16) and indicate that steady-state K_{DR} current will be small at physiological E_m (-30 to -60 mV). Despite their limited magnitude, the high input resistance of vascular smooth muscle cells (5 – 20 G Ω) dictates that such currents will play an important role in controlling membrane potential (28). Like K_{DR} , outward current through inwardly rectifying K^+ channels and inward current through nonselective cation channels are equally small and difficult to monitor with patch-clamp electrophysiology (30, 32, 44). Nevertheless, both currents have been shown to alter the resting E_m of intact cerebral arteries by 10 – 15 mV (22, 44). Quantitative three-dimensional electrical models of the resistance vasculature also indicate that small currents are functionally relevant and important to dynamic vessel function (45).

The K_{DR} current in vascular smooth muscle is divisible into distinct components that include 1) a slowly inactivating 4-AP-sensitive current, 2) a slowly inactivating 4-AP-insensitive current, and 3) a rapidly inactivating 4-AP-sensitive A-type current (K_{TO} ; Refs. 1 and 20). Unlike portal vein, K_{TO} current could not be discerned in cerebral smooth muscle cells. The slowly inactivating currents were, however, readily observable, with the 4-AP-sensitive component representing $37.9 \pm 3.7\%$ of the overall current. Interestingly, this proportion is sizably smaller than that previously reported for portal vein (7) and pulmonary (8) arteries. Although work is limited in the resistance vasculature, observations in portal vein have implicated Kv1.2 and Kv1.5 as the key pore-forming subunits of 4-AP-sensitive channels (20). In contrast, the pore-forming subunits of 4-AP-insensitive channels are thought to include members of the Kv7 family (29).

UTP-induced regulation of K_{DR} current. Intact vessel measurements demonstrated that pyrimidine nucleotides constrict intact cerebral arteries through a mechanism that partly depends on smooth muscle cell depolarization and Ca^{2+} influx through voltage-operated Ca^{2+} channels (18). Consistent with depolarization arising from a reduction in outward K^+ conductance, UTP was observed to potently suppress the K_{DR} current. This suppression of K_{DR} was particularly evident at positive potentials where the current is large and less susceptible to subtle time-dependent changes in current amplitude and seal resistance. The onset of current suppression typically began 5–10 min after agonist application and peaked by 20 min. The

slow nature of agonist-induced current suppression was not entirely unexpected in light of past findings (7) and given that the current experiments were performed on enzymatically isolated cells maintained at room temperature and under conditions that result in significant cellular dialysis. The two slowly inactivating components of K_{DR} current were differentially affected by this pyrimidine nucleotide. More specifically, UTP elicited a near-complete inhibition of the 4-AP-sensitive current whereas the 4-AP-insensitive component remained unaffected. Although this study is the first to demonstrate that pyrimidine-sensitive P2Y receptors target specific K_{DR} channels, this pattern of preferential regulation is not unique. For example, vasoconstrictors such as angiotensin II have been reported to selectively suppress 4-AP-sensitive K_{DR} currents in portal vein (7). Correspondingly, activators of PKA and PKC have been shown to preferentially target the same K_{DR} channels (1, 9). Such observations support the view that 4-AP-sensitive channels are more tightly controlled by phosphorylation events than K_{DR} channels insensitive to this K^+ channel blocker (7).

To inhibit the slowly inactivating 4-AP-sensitive component of the K_{DR} current, UTP-sensitive P2Y receptors must activate a trimeric G protein along with its associated signaling cascade. Traditionally, it has been asserted that this receptor class is coupled to G_q and its downstream effectors including phospholipase $C\beta$ and PKC (24, 41). PKC is a serine/threonine kinase whose activity is known to suppress 4-AP-sensitive K_{DR} channels in portal vein and mesenteric artery (1, 7, 16). Although UTP-sensitive P2Y receptors can in theory stimulate PKC, two key findings indicate that this kinase is not responsible for K_{DR} current suppression in cerebral artery myocytes. First, the PKC activator PMA failed to inhibit K_{DR} when applied at concentrations known to constrict intact cerebral arteries. Second, preincubation of cerebral smooth muscle cells with PKC inhibitors (calphostin C or bisindolylmaleimide I) did not prevent UTP from suppressing the K_{DR} current. These electrophysiological findings nicely parallel functional observations that demonstrated that PKC inhibition did not impair the depolarization of intact cerebral arteries by UTP. Although Rho kinase is conventionally viewed as a regulator of Ca^{2+} sensitivity, it is conceivable for this phosphotransferase protein along with its upstream regulator RhoA to mediate K_{DR} current suppression by UTP (6). Supporting a role for Rho kinase signaling, Y-27632 was observed in this study to prevent UTP from inhibiting K_{DR} in isolated cerebral myocytes. Likewise, the RhoA inhibitor C3 exoenzyme was also effective at preventing pyrimidine nucleotides from regulating K_{DR} .

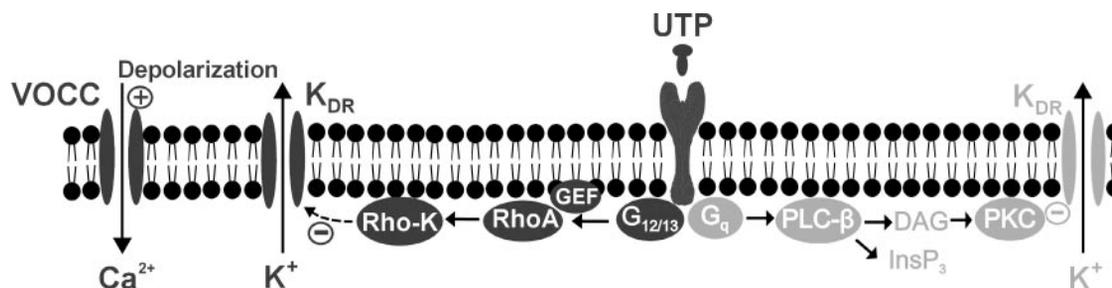


Fig. 13. Schematic representation of the signal transduction pathway proposed to regulate the 4-AP-sensitive K_{DR} current in rat cerebral arteries. G_q and $G_{12/13}$, trimeric G proteins; DAG, diacylglycerol; $InsP_3$, inositol (1,4,5)-trisphosphate; GEF, guanine nucleotide exchange factor; RhoA, monomeric G protein RhoA; Rho-K, Rho kinase; VOCC, voltage-operated Ca^{2+} channel.

Physiological implications. In summary, this study demonstrated that pyrimidine nucleotides constrict intact cerebral arteries through a mechanism that partly involves the depolarization of vascular smooth muscle cells and the activation of voltage-operated Ca^{2+} channels. We report for the first time in cerebral arterial smooth muscle that depolarization is dependent on the activation of Rho kinase and not PKC. One of the ionic conductances inhibited by UTP via Rho kinase is the 4-AP-sensitive K_{DR} current. We hypothesize that pyrimidine-sensitive P2Y receptors activate Rho kinase through a signal transduction cascade that involves $G_{12/13}$, p115 Rho GEF, and RhoA (Fig. 13). It is conceivable that Rho kinase regulation of ion channels might be narrowly limited to the cerebral vasculature or to the preceding receptor class. This view, however, is challenged by recent mesenteric observations showing that Rho kinase inhibition partially attenuates norepinephrine-induced depolarization (13) as well as by the findings shown in Fig. 12, which indicate that the thromboxane mimetic U-46619 also depolarizes and constricts cerebral arteries by activating Rho kinase and not PKC. Further investigation is required to determine whether other ion channels are modulated by Rho kinase and thus contribute to the depolarizations induced by UTP and other constrictor agonists. Indeed, the recent observations by Ghisdal et al. (13) have indirectly implied that this serine/threonine kinase could activate a depolarizing nonselective cation current. In closing, we suggest that in the cerebral circulation Rho kinase regulation may be a common mechanism by which G protein-coupled receptors control both the electrical and the contractile state of vascular smooth muscle.

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GRANTS

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