Conducted dilations initiated by purines in arterioles are endothelium dependent and require endothelial Ca\(^{2+}\)

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Duza, Tasimia, and Ingrid H. Sarelius. Conducted dilations initiated by purines in arterioles are endothelium dependent and require endothelial Ca\(^{2+}\). Am J Physiol Heart Circ Physiol 285: H26–H37, 2003. First published March 13, 2003; 10.1152/ajpheart.00788.2002.—The signaling pathways underlying the regulation of vascular resistance by purines in intact microvessels and particularly in communication of remote vasomotor responses are unclear. One process by which remote regions of arterioles communicate is via transmission of signals axially along the vessel wall. In this study, we identified a pathway for local and conducted dilations initiated by purines. Adenosine (Ado) or ATP (bind P\(_1\) and P\(_2\) purinergic receptors, respectively) was micropipette applied to arterioles (maximum diameter \(\sim 40 \mu m\)) in the cheek pouch of anesthetized hamsters. Observations were made at the site of stimulation (local) or \(\sim 1,200 \mu m\) upstream along the same vessel. P\(_1\) antagonists (pyridoxal phosphate-6-azophenyl-2,4'-disulphonic acid tetrasodium and suramin) inhibited local constriction to ATP, whereas local and upstream dilations were unaffected. In contrast, during inhibition of P\(_1\) receptors (with xanthine amine congener) the local constriction was unchanged, whereas both local and upstream dilations to ATP were inhibited. Hydrolysis of ATP to Ado is implicated in the dilator response as blocking ATP by 10.220.33.3 on July 3, 2017 http://ajpheart.physiology.org/ Downloaded from

Conducted response; endothelium-dependent dilation; microvascular communication

THE TRANSMISSION OF VASOMOTOR SIGNALS axially along the blood vessel wall is one phenomenon by which stimuli sensed by a localized region of the vasculature are communicated to remote regions (typically defined as \(>1,000 \mu m\) upstream). Local and remote changes in resistance acting in concert match the supply of oxygen and other nutrients to localized metabolic needs. The ultimate vasomotor outcomes of axially communicated signals are often referred to as conducted responses and have been observed in response to a wide range of vasoactive molecules (15, 18, 27). ATP is one such metabolically related nucleotide (22) that has also been implicated as an autocrine and paracrine signaling molecule in numerous cell systems (13, 29).

The vascular wall is decorated with purinergic receptors, which are divided into two main families. P\(_1\) purinergic receptors are defined as preferentially binding adenosine (Ado) > AMP > ADP > ATP, whereas the reverse is defined for P\(_2\) purinergic receptors (5). Purines play an essential role in the regulation of vascular resistance. Stimulation of P\(_1\) receptors by Ado is a well-established dilator pathway, although the relative importance of endothelial cells (ECs) versus smooth muscle cells (SMC) is still unclear (31). In contrast, a clear understanding of the function of P\(_2\) receptors and the effects of ATP in the microvasculature remains largely undefined. One reason for this is that ATP can have either vasoconstrictor or vasodilator effects on arterioles (6). The presence of ecto-ATPases, which rapidly degrade ATP, generally maintains ATP between nanomolar and micromolar concentrations in the extracellular space (13, 29). Appreciable levels of ATP can however occur transiently (3, 6, 16) and may underlie one of the mechanisms by which blood flow is regulated.

The goal of this study was to characterize the signaling pathway underlying the local and conducted (upstream) vasomotor response of intact blood-perfused arterioles to ATP. Using the terminal vasculature of hamster cheek pouch as a model, we identified the primary cell type targeted by extracellular purines. We also investigated the importance of P\(_2\) versus P\(_1\) purinergic receptors and the role of EC Ca\(^{2+}\) as a second messenger molecule for both local and conducted dilations.

METHODS

General Methods

All protocols were approved by the Animal Care and Use Committee of the University of Rochester and performed in...
95% N₂ to maintain pH 7.40. MgSO₄, and 30.0 NaHCO₃ and equilibrated with 5% CO₂–
O₂ and dilation to 10⁻⁴ M ACh or 10⁻³ M sodium nitroprusside (SNP) were kept for analysis (5% of all preparations were discarded). Vessel diameter following at least 3 min of superfusion of the entire preparation with 10⁻⁴ M ACh (Ca²⁺
measurement data sets) or 10⁻³ M SNP (all other data sets) was recorded for each observed arteriole and is reported as the maximum.

**Agonist Application**

Pressurized glass micropipettes placed at the vessel wall were used for localized agonist application [ATP, Ado, AMP, ACh, or norepinephrine (NE)] as previously described (28). Flow out of the pipette (~10 μm tip diameter) was achieved by a manometer system (30 cmH₂O ejection pressure). FITC-dextran (100 μM) or 2% Texas red-dextran was added to the contents of the pipette, and brief epi-illumination was used to confirm that flow out of the pipette was over the local site (~100 μm length of vessel on either side of the pipette) only and to verify that the superfusion solution carried the pipette contents away from the upstream site. From the diffusion constants for small molecules (between 10⁻⁶ and 10⁻⁵ cm²/s) such as those applied in this study and the distance between the local application and upstream observation site (>1,000 μm), it is apparent that diffusion is insufficient to account for

![Fig. 1. Schematic (not to scale) of the experimental observation site consisting of a third-order arteriole originating from a feed arteriole. Arrows in the lumen indicate the direction of blood flow. Local and upstream observation sites are shown. Gray arrow at the local site indicates the direction of flow of micropipette contents being washed away from the upstream site by the flowing superfusion solution. A cannulating micropipette is positioned in another third-order arteriole arising from the same parent vessel as the test arteriole. Ocluding rod A is temporarily placed to inhibit blood flow and allow perfusion of the cannulating micropipette contents through the vascular network. During BAPTA loading only, occluding rod B is placed at the position indicated to limit BAPTA loading to the upstream half of the test vessel. Blood remains in the vascular network downstream of this temporary occluding rod.](image)
the conducted response (2, 26). In addition, it has been shown that not all agonists that produce large local dilations (e.g., SNP) initiate upstream responses even when applied for 2 min (26). This provides functional evidence that diffusion or convection of local agonists do not underlie upstream responses. Fluorescent tracers themselves do not affect arteriolar responses (17).

**Endothelium Denudation With Air Embolism**

Cannulating micropipettes were triple beveled to create a sharp tip (diameter 7–10 μm) to facilitate penetration through connective tissue and the arteriolar wall. To selectively remove ECs, an arteriole was cannulated with a pipette containing air. Once the pipette entered the vessel lumen, it was rapidly pressurized to perfuse a local region of the microvascular network with air, which could be verified visually as the vessel was completely cleared of blood by the flow of air. The pipette was depressurized immediately and removed. Within 5–10 min the bubble would disperse into the microvasculature, and blood flow would resume. Air bubbles were rarely seen in venules. Air treatment generally denuded a limited length of vessel (~200 μm). A period of 20–25 min was allowed for vessel tone to reestablish before data collection. Selective disruption of ECs could be verified visually as SMCs were slightly constricted and occasional platelet interactions with the vessel wall could be seen. The following functional response was used as the identifying criterion for an arteriolar segment: selective endothelial disruption: lack of dilator response to ACh despite the preservation of intact smooth muscle functionality defined as its ability to contract (with ATP) and relax (with SNP).

**EC Ca2+ Measurement**

EC Ca2+ measurements were made by using the indicator fura-PE3 as previously described (25). Briefly, after identification of the test arteriole, another third-order arteriole arising from the same parent vessel was cannulated with a triple-beveled micropipette containing 2 μM fura-PE3 (AM) dye solution (Fig. 1). Once the pipette had entered the vessel lumen, it was pressurized to intraluminally perfuse the microvascular network. Areas of perfusion could be verified visually as blood was completely cleared from the vessel by the flow of the dye. Up to two blunted, curved glass-occluding rods were gently placed in upstream or downstream regions to temporarily inhibit blood flow and direct flow of fura-PE3 (AM) down the test arteriole. After 30 min of dye perfusion (total volume ~20 μl), the pipette and occluding rods were removed, and blood flow was allowed to resume. Thirty minutes were allowed for intracellular deesterification of the molecule. The following functional response was used as the criterion for selective chelation of EC Ca2+: inhibition of dilator response to ACh despite preservation of intact smooth muscle functionality defined as it’s ability to contract (with NE) and relax (with SNP).

**ATP and Ado dose response.** Concentrations of 10⁻⁷ M–10⁻³ M ATP or 10⁻⁷ M–10⁻⁴ M Ado were micropipette applied as described above to determine the dose response of arterioles to these purines. Observations were first made at the local site. After a brief recovery period (~10 min), observations were made upstream. In preliminary experiments it was verified that multiple applications and the order of observation (local followed by upstream or vice versa) did not affect the arteriole’s response. Paired local and remote observations were made on the same vessel in all experiments unless specified otherwise. Only one concentration of each agonist was usually tested on each arteriole.

**Role for P₂X, P₂Y, and P₁ receptors in ATP-initiated response.** The P₂ receptor family is divided into two subtypes, P₂X and P₂Y. P₂X receptors are ATP-gated cation channels and allow direct entry of Na⁺ and Ca²⁺. P₂Y receptors are coupled to G proteins and initiate phospholipase-based signal transduction via mobilization of inositol trisphosphate-sensitive Ca²⁺ stores (1, 13). To identify the purinergic receptor being activated by extracellular ATP, the local and upstream vasoconstrictor response to 10⁻⁴ M ATP was first recorded as described above (control data). The entire cheek pouch preparation was then exposed (in separate experiments) to either 1) 10⁻⁵ M pyridoxal-phosphate-6-azophenyl-2',4'-disulfonyl acid tetrasodium (PPADS, P₂X antagonist); 2) 10⁻⁵ M suramin (P₂X and P₂Y antagonist); or 3) 10⁻⁶ M xanthine amine congener (XAC, P₁ antagonist) by adding the agent to the superfusion solution. After 30 min of exposure to the antagonist, the same vessel’s response to ATP was recorded again in the continued presence of the blocker.

**Role for hydrolysis of ATP to Ado during the response to ATP application.** To determine whether hydrolysis of ATP to Ado plays a role in the dilator response to ATP, observations were first made at the local and upstream sites during 10⁻⁴ M ATP application and at the local site only during 10⁻⁴ M AMP and 10⁻⁴ M Ado (in separate arterioles) exposure in the same preparation (control data). The entire tissue preparation was then exposed to 10⁻⁴ M α,β-methylene adenosine 5’-diphosphate (AOPCP, 5’-ectonucleotidase inhibitor) by adding it to the superfusion solution. After 30 min of expo-

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sure to the antagonist, the responses to ATP, AMP, and Ado were recorded again in the continued presence of the blocker.

Identification of the vascular cell type initiating the response to ATP and Ado. To identify the primary cell type (endothelial vs. smooth muscle) responsible for initiation of the vasomotor responses to ATP and Ado, observations were made before and after selective removal of ECs via air embolism in the same preparation. Observations were first made in intact arterioles with local application of 10⁻⁴ M ACh, 10⁻⁴ M ATP, or 10⁻⁴ M Ado (control data), following which ECs were removed as described above. The arteriolar responses of the endothelium-denuded region to local application of ACh, ATP, and Ado were then recorded. Subsequent to collection of all local agonist application data in each animal, the entire preparation was exposed to 10⁻³ M SNP by adding it to the superfusion solution, and the vasomotor response of the endothelium-removed region was recorded.

EC Ca²⁺ response to ATP and Ado. To test whether the arteriolar dilation associated with ATP or Ado stimulation involves EC Ca²⁺ as a signaling intermediate, we measured local and upstream changes in EC Ca²⁺ and vessel diameter initiated by 10⁻⁴ M ATP or 10⁻⁴ M Ado. Paired local and remote observations were not always made on the same vessel in these experiments, because we were limited to collecting data in arteriolar regions that were fara-Pe3 loaded (for Ca²⁺ measurement) and in focus (for vessel diameter measurement). Subsequent to collection of all micropipette agonist application data in each animal, the entire cheek pouch preparation was exposed to 10⁻⁴ M ACh (an agonist known to maximally increase intracellular Ca²⁺ in the endothelium) and EC Ca²⁺ was measured.

Role of change in EC Ca²⁺ in the response to P₂ receptor stimulation. To determine whether the change in EC Ca²⁺ associated with arteriolar dilation to purines is a required signaling intermediate, observations were made in the same arterioles before and after chelation of EC Ca²⁺. Local and upstream responses to 10⁻⁴ M Ado and the local response to 10⁻⁴ M ACh were first recorded under control conditions. After BAPTA was loaded, local responses to Ado, ACh, 10⁻⁴ M NE, and 10⁻³ M SNP applied to the BAPTA-perfused site (+BAPTA) were observed. Ado was also applied to the downstream site that remained blood filled and observations were made at the local site (−BAPTA) and at a BAPTA-loaded upstream site. Regions of the arteriolar architecture that were BAPTA perfused did not completely regain spontaneous tone; hence, 5 min before the beginning of data collection, the superfusion solution was changed to one containing 10⁻⁷ M NE to augment tone. This was done for both control and BAPTA-perfused conditions to ensure that NE itself does not affect local and upstream responses.

Materials

A 20-μl aliquot of 10⁻³ M fura-Pe3 (AM) (TEF Labs, Austin, TX; dissolved in 100% DMSO) was micropipetted into the vessel. A 4-μl aliquot of 12.5 mg/ml Pluronic-127 (TEF Labs, Austin, TX; made in 100% DMSO) stock solutions were diluted and added in 10 ml 0.9% NaCl (dye solution). This resulted in a final concentration of 2 μM fura-Pe3 (AM), 5 μg/ml Pluronic-127, and 2.4 μl/ml DMSO. A 50-μl aliquot of 10⁻³ M BAPTA (AM) (Molecular Probes, Eugene, OR; dissolved in 100% DMSO) and 20 μl of 12.5 mg/ml Pluronic-127 (TEF Labs; made in 100% DMSO) stock solutions were mixed and diluted in 10 ml 0.9% NaCl (Ca²⁺ buffer solution). This resulted in a final concentration of 5 μM BAPTA (AM), 25 μg/ml Pluronic-127, and 7.0 μl/ml DMSO.

All other reagents were obtained from Sigma (St. Louis, MO). Solutions were prepared fresh daily in superfusion solution.

Data Analysis and Statistics

Typically, only one arteriole was studied in each animal. In some experiments (e.g., dose response, AOPCP), up to three arterioles were observed, but a different agonist and/or concentration was tested in each case to avoid introduction of bias for a particular animal in the averaged data set for any given condition. The number of observations (n) refers to the number of arterioles studied. All data are reported as means ± SE. Data are expressed normalized to baseline (340-to-380-nm ratio and percent diameter) or as an absolute diameter change (in μm) over 10-s intervals relative to baseline (averaged over 1 min). Responses from multiple experiments were analyzed by repeated-measures ANOVA with Dunnett’s multiple-comparison post-test or paired Student’s t-test as appropriate to determine statistical differences compared with baseline. Changes were considered significant if P < 0.05.

RESULTS

The number of arterioles studied, resting and maximum vessel diameters, and local to upstream site distance for all experiment sets are summarized in Table 1. The time at which the peak response occurred varied between vessels (by up to ~30 s). In the figures, the averaged time course refers to the mean observation at each time point for multiple arterioles, whereas peak response refers to the mean of the peak response from multiple arterioles. For the sake of clarity, data in the text refer to the mean peak response.

Dose Response to ATP and Ado

Application of 10⁻⁷ M to 10⁻³ M ATP caused a dose-dependent constriction (Fig. 2A) followed by dilation locally (Fig. 2B) and dilation (but no constriction) upstream (Fig. 2C). The peaks occurred 40 ± 10 s, 130 ± 10, and 110 ± 10 s after the onset of ATP application for the local constriction, local dilation, and upstream dilation, respectively. Peak constriction was observed at 10⁻⁴ M ATP. The magnitude of the local and upstream dilator responses to ATP were identical (Fig. 2, B vs. C, filled squares, P > 0.05). Application of 10⁻⁷ M to 10⁻⁴ M Ado caused a dose-dependent dilation, but not constriction, both locally (Fig. 2B) and upstream (Fig. 2C). The times at which the peak dilations occurred were 70 ± 10 s and 110 ± 10 s after the onset of Ado application for the local and upstream response, respectively. On average, the local dilator responses to Ado were slightly larger than the upstream responses (Fig. 2, B vs. C, open squares, P < 0.05). However, the local and upstream dilator responses between ATP and Ado were indistinguishable (P > 0.05). All subsequent experiments were conducted using 10⁻⁴ M ATP or Ado, because this concentration of ATP clearly stimulated P₂ receptors (indicated by constriction) without saturating the vessel’s dilator capacity.
ATP-Induced Constrictions are Via P$_{2X}$ Receptors and Dilations are Via P$_{1}$ Receptors

To identify the role of P$_{2X}$ receptors in the response to ATP, the local (Fig. 3A) and upstream (Fig. 3B) vasomotor responses were observed before (control) and during treatment with PPADS, a selective P$_{2X}$ antagonist. In the presence of PPADS, the local constriction to ATP was abolished (12.4 ± 3.4 vs. 0.2 ± 0.2 μm, P < 0.05; control vs. treatment), whereas the local (8.4 ± 1.3 vs. 8.4 ± 1.0 μm, P > 0.05) and upstream dilations (7.2 ± 1.6 vs. 7.0 ± 1.4 μm, P > 0.05) were unaffected (Fig. 3C).

To determine the role of P$_{2}$ receptors in the local (Fig. 4A) and upstream (Fig. 4B) responses to ATP, the nonselective P$_{2}$ antagonist suramin was used. Exposure to suramin caused a slight increase in resting arteriolar diameter, although the vessels clearly retained their capacity to dilate (Table 1). In the presence of suramin, the local constriction to ATP was attenuated (9.6 ± 1.9 vs. 3.5 ± 1.7 μm, P < 0.05), whereas the local (7.1 ± 1.4 vs. 10.3 ± 0.9 μm, P > 0.05) and upstream (5.7 ± 1.1 vs. 7.2 ± 1.3 μm, P > 0.05) dilations were not (Fig. 4C).

To investigate whether the local (Fig. 5A) and upstream (Fig. 5B) dilator response to ATP is produced via stimulation of P$_{1}$ receptors, a nonselective P$_{1}$ antagonist XAC was used. With XAC, the magnitude of the local constriction to ATP was unchanged (9.5 ± 1.7 vs. 10.2 ± 2.1 μm, P > 0.05), whereas the local (7.9 ± 1.8 vs. 2.5 ± 1.1 μm, P < 0.05) and upstream dilations (11.6 ± 1.2 vs. 3.0 ± 1.0 μm, P < 0.05) were inhibited (Fig. 5C).

Hydrolysis to Ado Facilitates ATP-Induced Dilation

Nucleotidases present on the cell’s extracellular membrane degrade adenine nucleotides to Ado. 5’-Ectonucleotidase is the enzyme implicated in the final step of Ado formation via this pathway, catalyzing the breakdown of AMP to Ado. To test whether ATP hydrolysis to Ado, with subsequent stimulation of P$_{1}$ receptors (vs. direct binding of ATP to P$_{1}$ receptors), was the trigger for the dilation, we used AOPCP, which is a specific inhibitor of 5’-ectonucleotidase (Fig. 6). Treatment with AOPCP caused a slight increase in resting diameter, although the vessels still retained their capacity to dilate (Table 1). AOPCP attenuated the local dilation to AMP (16.0 ± 1.7 vs. 6.1 ± 1.4 μm, P < 0.05) but not Ado (16.1 ± 3.2 vs. 14.7 ± 2.3 μm, P > 0.05). In the presence of AOPCP, the local constriction to ATP was unchanged (8.8 ± 2.5 vs. 9.6 ± 1.8 μm, P > 0.05). The local (12.4 ± 1.6 vs. 5.3 ± 1.9 μm, P < 0.05) and upstream (9.6 ± 1.6 vs. 7.0 ± 1.7 μm, P < 0.05) dilations to ATP were attenuated in the presence of AOPCP.

Initiation of Dilator Signals by Purines Occurs Primarily in ECs

To determine whether ECs or SMCs are primarily responsible for the initiation of vasomotor responses to purines, observations were made during agonist appli-
cation at the local site in vessels with intact ECs (Fig. 7A) or following selective removal of ECs (Fig. 7B). Air embolism resulted in an increase in resting arteriolar tone (Table 1). The average peak responses are shown in Fig. 7C. EC denudation abolished ACh-induced dilations (17.9 ± 2.1 vs. 1.6 ± 0.9 μm, \(P < 0.05\)), establishing that ECs were successfully disrupted with air treatment. In EC-denuded vessels, the magnitude of the local constriction to ATP was unchanged (−18.8 ± 2.5 vs. −21.4 ± 4.8 μm, \(P > 0.05\)), whereas the dilation (8.5 ± 2.2 vs. 0.6 ± 0.4 μm, \(P < 0.05\)) was abolished. The dilation to Ado was significantly reduced in EC-denuded vessels (19.2 ± 2.3 vs. 4.1 ± 0.9 μm, \(P < 0.05\)). As expected, endothelium-denuded vessels maintained their capacity to dilate to SNP (20.5 ± 3.9 μm, \(P < 0.05\) from baseline).

**EC Ca\(^{2+}\) Increases in Response to ATP and Ado**

ATP application increased EC Ca\(^{2+}\) at both the local (Fig. 8A) and upstream (Fig. 8B) site. The increase in the average peak fura ratio was 33 ± 5% locally and 19 ± 3% upstream (\(P < 0.05\) from baseline). The change in diameter was the same as that described earlier, i.e., a biphasic response at the local site and only dilation upstream. Ado application also caused an
increase in EC Ca\(^{2+}\) at both the local (Fig. 8C) and upstream (Fig. 8D) site. In this case, the increase in the average peak fura ratio was 29 ± 4% locally and 17 ± 6% upstream (\(P < 0.05\) from baseline). Again, as described earlier, the vasomotor response to Ado involved only dilations. With ACh, the increase in the average peak fura ratio was 168 ± 32% (\(P < 0.05\) from baseline and all purine responses). This confirmed that our system had the capacity to detect changes in EC Ca\(^{2+}\) greater than those recorded during purinergic stimulation should they have occurred.

Dilation by \(P_1\) Receptor Stimulation Requires an Increase in EC Ca\(^{2+}\)

To assess whether an increase in EC Ca\(^{2+}\) is required for the arteriolar dilations associated with purinergic stimulation, observations were made in con-

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**Fig. 4.** Response of arterioles to 2 min of ATP (10\(^{-4}\) M) application in the absence (control) or presence of the \(P_2\) receptor antagonist suramin (10\(^{-5}\) M). Values are means ± SE (n = 8). A: averaged time course of the local change in diameter. B: averaged time course of the upstream change in diameter. C: peak local and upstream changes in diameter. *Significantly different from control response (\(P < 0.05\)).

**Fig. 5.** Response of arterioles to 2 min of ATP (10\(^{-4}\) M) application in the absence (control) or presence of the \(P_1\) receptor antagonist xanthine amine congener (XAC, 10\(^{-6}\) M). Values are means ± SE (n = 7). A: averaged time course of the local change in diameter. B: averaged time course of the upstream change in diameter. C: peak local and upstream changes in diameter. *Significantly different from control response (\(P < 0.05\)).
control vessels and following buffering of EC Ca\textsuperscript{2+} with the Ca\textsuperscript{2+} chelator BAPTA. Loading of BAPTA resulted in a decrease in resting arteriolar tone. However, the vessels retained their capacity to dilate (Table 1). In the +BAPTA region of the arteriole, ACh-induced dilation was significantly reduced (16.3 ± 3.9 vs. 4.6 ± 2.4 μm, P < 0.05), establishing successful buffering of EC Ca\textsuperscript{2+} (Fig. 9A). The local response to Ado (Fig. 9B) was abolished in the +BAPTA region (15.2 ± 3.0 vs. 2.2 ± 1.1 μm, P < 0.05), whereas in the −BAPTA region it remained intact (12.4 ± 2.4 μm, P > 0.05 compared with control). Buffering EC Ca\textsuperscript{2+} also abolished the manifestation of the upstream dilation (8.7 ± 1.3 vs. 2.4 ± 1.1 μm, P < 0.05, Fig. 9C) despite its initiation at the local site. BAPTA-loaded regions constricted to NE (−24.5 ± 4.2 μm, P < 0.05 from baseline), indicating selective buffering of EC Ca\textsuperscript{2+}, and dilated to SNP (14.5 ± 4.3 μm, P < 0.05 from baseline), indicating ample dilator capacity (Fig. 9D).

**DISCUSSION**

The present study demonstrates that in intact blood-perfused arterioles, stimulation of P\textsubscript{1} and not P\textsubscript{2} purinergic receptors initiates a vasodilator response, which spreads axially along the vessel wall to upstream regions. There is a corresponding increase in local EC Ca\textsuperscript{2+} that is also conducted upstream. Local and upstream increases in EC Ca\textsuperscript{2+} are required for manifestation of the respective responses. Furthermore, we show that intact endothelium is required for the initiation of the dilator signal. In contrast to the dilation, constriction initiated by purines is a result of stimulation of P\textsubscript{2X} purinergic receptors. This constriction remains localized. It is independent of endothelium and occurs via direct stimulation of receptors on SMCs.

ATP produces a dose-dependent constriction followed by dilation at the site of receptor occupation. Only the dilator signal is propagated along the vessel wall and results in an upstream vasomotor response. Ado causes dose-dependent dilations at the site of receptor stimulation and, like ATP, dilation upstream.
The magnitude of the dilator responses to each of these purines is the same, which, based on their dose-response curves, is consistent with both purines acting via the same signaling pathway. Constrictions to Ado [reported by Doyle et al. (12) following stimulation of the A3 receptor subtype in isolated arterioles] were never observed in the in situ vascular preparation of the current study.

Inhibition of P2X receptors with PPADS abolished the local constriction without affecting the local and remote dilations. Consistent with interpretations of studies conducted on isolated vessels (6, 23), our results show that ATP causes constriction via P2X receptors. Additionally, our results show that the dilator response is not linked to the P2X receptor pathway. These findings with PPADS strongly support that at the receptor level the local constriction and dilation do not originate at the same source but rather the biphasic response to extracellular ATP is the result of activation of at least two independent pathways.

Inhibition of P2 receptors with suramin attenuated the local constriction to ATP (confirming effective inhibition of P2 receptors at the concentration used in our study) but surprisingly did not reduce the dilator responses. In several systems, the vasodilator action of ATP has been associated with stimulation of P2Y receptors, but in many cases this link lacks explicit confirmation (13, 35). Our findings show that in an intact system, P2 receptors are not involved in the local and upstream dilations initiated by ATP. We speculate that because the architecture of the vascular and parenchymal cells is intact, highly active nucleotidases present in the extracellular space (discussed below) modulate the ATP response in ways that are distinct from cells in culture.

The finding that the ATP induced local and propagated dilations do not involve P2 receptors led us to the hypothesis that ATP was acting via stimulation of a P1 receptor pathway. Consistent with this hypothesis, direct stimulation of P1 receptors with Ado produces local and upstream dilations that are not different from those initiated by ATP (dose response and EC Ca2+ data). In fact, blocking P1 receptors with XAC resulted in a complete inhibition of the dilations to ATP,
whereas the constriction was unaffected. This further supports that the constriction and dilation are mediated by independent pathways and demonstrates that P1 receptors play a crucial role in the local and upstream dilator response to ATP. A similar outcome was obtained using a different P1 receptor antagonist, 8-phenyltheophylline (n/H110053, data not shown). Little is known about the distribution of P1 receptor subtypes (A1, A2a, A2b, and A3) in microvessels, which is why XAC, an antagonist that is nonselective between subtypes of P1 receptors, was used at a concentration known to inhibit dilations to Ado in small arterioles (27). Identification of the specific P1 receptor involved in the dilator responses to ATP was beyond the scope of the current study.

There are two obvious mechanisms by which ATP could be stimulating P1 receptors. First, even though ATP preferentially binds P2 over P1 receptors, at the concentrations used in this study ATP itself could be binding P1 receptors. Alternatively, ectoenzymes could be degrading ATP to Ado, which in turn stimulates P1 receptors. To distinguish between these two possibilities, AOPCP, a specific inhibitor of 5'-ecto-nucleotidase, was used to block the final step of the conversion of ATP to Ado. This protein presumably represents the major enzyme responsible for the formation of extracellular nucleotide from nucleotide 5'-monophosphates and thus plays an important role in the formation of Ado from AMP (34). However, it should be kept in mind that the pattern of catalytic activities at the cell surface is in actuality much more complicated than the linear hydrolysis chain from ATP to Ado assumed here, and any product of each hydrolysis step is itself likely to contribute to triggering a vasomotor response. As expected, AOPCP dramatically decreased the local dilation to AMP without affecting that to Ado, verifying

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Fig. 9. A: averaged time course of the local response to 2 min of ACh (10^-4 M) in control and applied to the BAPTA-perfused region (+BAPTA). B: averaged time course of the local response to 2 min of Ado (10^-4 M) in control and applied to the BAPTA-perfused region (+BAPTA) and the region that remained blood filled during BAPTA loading (−BAPTA). C: averaged time course of the upstream change in diameter in control and in the BAPTA-perfused region (+BAPTA) in response to 2 min of Ado application. D: peak changes in diameter of control, BAPTA-loaded region (+BAPTA), and region remaining blood filled during BAPTA loading (−BAPTA) in response to ACh, Ado, norepinephrine (NE, 10^-4 M), and sodium nitroprusside (SNP, 10^-3 M). Values are means ± SE (n = 4). *Significantly different from control response (P < 0.05).
its specific inhibition of 5'-ectonucleotidase. Additionally, AOPCP did not affect the local constriction to ATP (supporting that AOPCP was not interfering with P2 receptor-mediated signaling) but decreased both the local and upstream dilations, indicating that Ado produced from the degradation of ATP was ultimately stimulating P1 receptors.

To determine which vascular cell type is primarily responsible for the initiation of the vasomotor responses to purines described above, the EC layer was denuded by air embolism. This approach has been used in isolated blood vessels to distinguish between EC- and SMC-dependent mechanisms (7, 20) but has not previously been applied to small arteries that are intact and blood perfused. The possibility that the endothelium was not denuded but that EC function was only disrupted by air treatment cannot be eliminated. Regardless, the effect of the EC denudation procedure on the dilator response to ATP supports that the dilation is initiated in ECs. In conjunction with observations using purinergic receptor antagonists, the responses of endothelium-denuded arterioles clearly indicate that P2X receptors are present on SMCs, whereas P1 receptors are on ECs. The equal magnitude of constriction evoked in arterioles with or without endothelium supports that SMC functionality was not compromised during EC removal and indicates that ATP-induced constriction is via direct stimulation of receptors on SMCs. The response to Ado was almost abolished in vessels without endothelium, providing evidence that in this tissue bed ECs dominate Ado-mediated dilations. This is in contrast to the well-established P1 receptor-mediated SMC pathway for dilator responses to Ado but is consistent with less frequently encountered reports indicating at least partial involvement of ECs in the response (31).

As described earlier, ATP utilizes Ca2+ as a second messenger molecule regardless of whether P2X or P2Y receptor subtypes are stimulated. The role of Ca2+ subsequent to Ado/P1 receptor stimulation is less well established. Whereas many EC-mediated dilator pathways depend on intracellular Ca2+ as a signaling intermediate (11), a direct link between purinergic agonists and increased EC Ca2+ has not yet been shown in arterioles in situ. We hypothesized that the EC-dependent arteriolar dilations associated with ATP or Ado stimulation involve increases in EC Ca2+. Such an increase in EC Ca2+ was indeed observed at the local site during application of either purine, consistent with ECs being the target for receptor ligand interactions. Importantly, an increase in EC Ca2+ at the upstream site was also observed. Inhibiting the ability of ECs to alter Ca2+ eliminated P1 receptor-mediated local and upstream dilations. Our findings thus demonstrate that the rise in EC Ca2+ is not only required for the local dilation but also for the manifestation of the conducted dilation associated with purinergic stimulation. This is the first report of changes in EC Ca2+ being associated with conducted dilations. Conducted vasodilator responses are believed to result from the electrotonic spread of a hyperpolarizing signal (30, 32). Furthermore, influx of Ca2+ into ECs as a consequence of membrane hyperpolarization has been shown (21). Our findings thus support a model in which the conduction of a hyperpolarizing signal transmitted axially via the endothelium acts as the trigger for the upstream increase in EC Ca2+ and subsequent dilation. An alternative hypothesis is that the local increase in EC Ca2+ caused by P1 receptor stimulation spreads from cell to cell by gap junction channels or via a paracrine pathway as has been established in other systems (8, 33) and underlies the resulting propagated dilations. The time course of the change in EC Ca2+ and associated dilations that we observed suggests that the increase in Ca2+ is related to the initiation of a vasodilator signal while the maintenance of the dilation is achieved by other means. Such mechanisms could involve changes in Ca2+ sensitivity, as demonstrated in SMCs (4), or be independent of changes in endothelial whole cell Ca2+ (24). Whether the rise in EC Ca2+ triggers the release of an endothelium-derived dilator or hyperpolarizes the EC (e.g., via activation of Ca2+-dependent K+ channels), either of which could subsequently act on SMCs via paracrine or myoendothelial mechanisms, is unknown.

In conclusion, it is demonstrated here that stimulation of P1 receptors on ECs produces an increase in EC Ca2+ as well as a decrease in vascular resistance that spreads axially along the vessel wall to remote regions. Collectively, these findings expand the current understanding (9, 10, 19, 22) of how dilator responses to ATP may be initiated and transmitted in an intact blood-perfused system in situ. In addition, our novel identification of the involvement of EC Ca2+ in the conducted response reveals EC Ca2+ signaling as a required signaling intermediate for the manifestation of communicated vasodilator signals throughout the microvasculature. Thus the current study advances the understanding of signaling pathways by which blood flow is regulated, particularly during the complex integrated response of arterioles to local metabolites.

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