P2 receptor-mediated afferent arteriolar vasoconstriction during calcium blockade

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Inscho, Edward W., and Anthony K. Cook. P2 receptor-mediated afferent arteriolar vasoconstriction during calcium blockade. Am J Physiol Renal Physiol 282: F245–F255, 2002. First published August 15, 2001; 10.1152/ajprenal.00038.2001.—Experiments were performed to determine the role of L-type calcium channels on the afferent arteriolar vasoconstrictor response to ATP and UTP. With the use of the blood-perfused juxtamedullary nephrine technique, kidneys were perfused at 110 mmHg and the responses of arterioles to α,β-methylene ATP, ATP, and UTP were determined before and during calcium channel blockade with diltiazem. α,β-Methylene ATP (1.0 μM) decreased arteriolar diameter by 8 ± 1% under control conditions. This response was abolished during calcium channel blockade. In contrast, 10 μM UTP reduced afferent arteriolar diameter to a similar degree before (20 ± 4%) and during (14 ± 4%) diltiazem treatment. Additionally, diltiazem completely prevented the vasoconstriction normally observed with ATP concentrations below 10 μM and attenuated the response obtained with 10 μM ATP. These data demonstrate that L-type calcium channels play a significant role in the vasoconstrictor influence of ATP and ATP but not UTP. The data also suggest that other calcium influx pathways may participate in the vasoconstrictor response evoked by P2 receptor activation. These observations support previous findings that UTP-mediated elevation of intracellular calcium concentration in preglomerular vascular smooth muscle cells relies primarily on calcium release from intracellular pools, whereas ATP-mediated responses involve both voltage-dependent calcium influx, through L-type calcium channels, and the release of calcium from intracellular stores. These results support the argument that P2X and P2Y receptors influence the diameter of afferent arterioles through activation of disparate signal transduction mechanisms.

afferent arterioles; calcium channels; cytosolic calcium; renal microcirculation; P2X receptors; P2Y receptors; adenosine 5'-triphosphate; uridine 5'-triphosphate; α,β-methylene adenosine 5'-triphosphate; cadmium

PREVIOUS STUDIES SUGGEST THAT renal microvascular smooth muscle expresses both P2X and P2Y receptors (2, 8, 10, 13, 16, 25, 34, 39–41). This finding is supported by more recent studies focused on determining the calcium signaling pathways involved in P2X and P2Y receptor activation, using freshly isolated afferent arterioles (16) and vascular smooth muscle cells obtained from rat preglomerular vascular segments (24, 41). Published reports using a collection of experimental approaches have established the presence of specific P2 receptor subtypes in the renal vasculature. Chan and co-workers (7) have shown pronounced expression of P2X1 receptors along the preglomerular microvasculature. These immunohistochemical data are supported by functional studies demonstrating responsiveness of the renal microvasculature to α,β-methylene ATP (13, 19, 22, 24–26, 33, 37). P2X3 receptors, which also respond to α,β-methylene ATP (36), could also be present and could contribute to the renal microvascular response to ATP stimulation.

P2Y2 receptors have also been strongly implicated in influencing the diameter of rat afferent arterioles (13, 19, 22, 24). These studies showed marked renal vasoconstriction in response to treatment with UTP or the UTP analog ATP-γ-S but little or no response to UDP or 2-methylthio ATP. This agonist profile is consistent with the activation of P2Y2 receptors.

Isolated microvascular smooth muscle cell experiments revealed that ATP and selective P2X and P2Y agonists increase intracellular calcium concentration through disparate calcium signaling mechanisms. Selective P2Y receptor activation increases intracellular calcium concentration primarily through mobilization of calcium from intracellular stores (24, 27). P2X receptor activation with α,β-methylene ATP increases intracellular calcium concentration largely through activation of voltage-dependent calcium influx pathways (27, 41). Interestingly, the endogenous ligand ATP, which activates both P2X and P2Y receptors, increases intracellular calcium concentration by stimulating voltage-dependent calcium influx and calcium mobilization from intracellular stores (24, 27, 41).

The purpose of the present study was to extend the calcium signaling observations made with freshly isolated preglomerular smooth muscle cells (24, 41) to functional responses obtained in the intact renal microvasculature from which they were derived. Experiments were performed to determine the role of L-type calcium channels in P2X and P2Y receptor-mediated...
vasoconstriction of afferent arterioles. We determined the effect of calcium channel blockade on the afferent arteriolar vasoconstriction induced by P2X receptor activation with α,β-methylene ATP, by P2Y receptor activation with UTP, and by nonselective P2 receptor activation with the endogenous ligand ATP.

**METHODS**

Studies were approved by the Tulane University Advisory Committee for Animal Resources and by the Committee on Animal Use for Research and Education at the Medical College of Georgia. Experiments were conducted in vitro, using the blood-perfused juxtamedullary nephron technique, as previously described (6, 21, 22). Two male Sprague-Dawley rats (350–400 g) were used for each experiment. Rats were anesthetized with pentobarbital sodium (40 mg/kg ip) and pretreated for 30 min with the converting enzyme inhibitor enalaprilat (2 mg iv) (21, 22). Perfusate blood was collected and prepared, as previously described (21, 22). Briefly, blood was collected from the nephrectomized blood donor rat into a heparinized syringe (500 U). The plasma and erythrocyte fractions were separated, and the leukocyte fraction was discarded. The erythrocytes underwent two saline washes before being combined with the filtered (0.2-μm exclusion) plasma to obtain a hematocrit of ~33%. The reconstituted blood was filtered through 5-μm nylon mesh and saved for later use.

The right renal artery of the kidney donor was cannulated and perfused with a Tyrode buffer solution containing 5.2% bovine serum albumin (Calbiochem, La Jolla, CA) and a complement of L-amino acids (Sigma, St. Louis, MO) (26). The right renal vein was ligated and vented near the inferior vena cava to facilitate renal perfusion and to prevent mixing of the perfusate with circulating blood. The rat was exsanguinated with the use of a heparinized syringe (500 U) via a carotid artery cannula, and blood was processed with blood collected from the blood donor rat. The perfused kidney was removed and sectioned along the longitudinal axis, leaving the papilla intact on the dorsal two-third portion of the kidney (6). The papilla was reflected out of the visual field, and the pelvic mucosa was removed to expose the main arterial branches, renal tubules, glomeruli, and related microvasculature of the juxtamedullary nephrons. Ligation of the terminal ends of the large arteries restored intravascular pressure to the perfused cortical and papillary tissue.

After completion of the microdissection procedures, the cell-free perfusate was replaced with the reconstituted blood. The blood perfusate was stirred continuously in a closed reservoir while being oxygenated with a 95% O2-5% CO2 gas mixture. Perfusion pressure was continuously monitored, using a pressure cannula positioned in the tip of a double-barreled perfusion cannula and connected to a Statham P23Db pressure transducer linked to a polygraph recorder (Grass Instruments, Quincy, MA). Perfusion pressure was fixed at 110 mmHg. The inner cortical surface of the kidney was continuously superfused with warmed (37°C) Tyrode buffer containing 1% bovine serum albumin, and the kidney was allowed to equilibrate for at least 15 min.

The perfusion chamber, containing the prepared kidney, was positioned on the stage of a Nikon Optiphot-2UD microscope (Nikon, Tokyo, Japan) equipped with a Zeiss water-immersion objective (×40). The tissue was transilluminated, and the focused image, obtained with a Newvicon camera (NC-70, Dage-MTI, Michigan City, IN), was passed through an image processor (MFJ-1425, MFJ Enterprises, Starkville, MS) and displayed on a video monitor while being simultaneously recorded on videotape for later analysis. Vascular inside diameters were measured at a single site, using an image-shearing monitor (model 901, Instrumentation for Physiology and Medicine, San Diego, CA). The displacement of the video image on the image-shearing monitor was calibrated with a stage micrometer (smallest division = 2 μm). Microvessels were selected for study on the basis of the clarity of the vascular walls and the adequacy of blood flow through the vessel lumen.

**Experimental Protocols**

P2 receptor-mediated microvascular responses were determined with the use of ATP, UTP, and α,β-methylene ATP. ATP is considered the endogenous ligand for P2 receptors and will activate both P2X and P2Y receptors. UTP potently activates P2Y2 and P2Y4 receptors, whereas the ATP analog α,β-methylene ATP is an agonist for P2X1 and P2X3 receptors (36). Measurements of afferent arteriolar diameters were made at 12-s intervals, and the steady-state arteriolar diameter was calculated from the average of all diameter measurements obtained during the final 2 min of each treatment period. Each protocol consisted of six consecutive 5-min periods, during which arterioles were exposed to the control bathing solution or to a similar solution containing UTP, ATP, α,β-methylene ATP, and/or the calcium channel blocker diltiazem. Previous studies have established that 10 μM diltiazem is the minimum concentration required to block vasoconstriction of juxtamedullary afferent arterioles in response to depolarization with 55 mM KCl (25). Each protocol began with a control period to ensure a stable vessel diameter and was followed by exposure to a P2 agonist to establish the control response to each agent. Subsequently, the vessels were permitted to recover while being bathed in the control solution. After the recovery period, calcium channel blockade was imposed and the agonist-induced response was reassessed.

**Series 1: effect of repeated exposure of juxtamedullary afferent arterioles to P2 agonists.** Prolonged exposure to extracellular calcium can result in desensitization of P2 receptors in some tissues, and high concentrations of α,β-methylene ATP are frequently used to desensitize P2X receptors to ATP-evoked responses (1, 11). Because the experimental protocols require comparison of afferent arteriolar responses obtained during two separate periods of α,β-methylene ATP, ATP, or UTP treatment, desensitization of P2 receptor-mediated responses could be a confounding variable. Therefore, time control experiments were performed to establish the reproducibility of the afferent arteriolar vasoconstrictor response produced by duplicate agonist applications, spaced 10 min apart. After the initial control period, the tissue was exposed to a solution containing 1.0 μM α,β-methylene ATP, 10 μM ATP, or 10 μM UTP, and the change in the afferent arteriolar diameter was determined. After 5 min of exposure, the agonist was removed from the bathing medium, and the vessel was allowed to recover in control buffer for 10 min before the same agonist solution was reintroduced. The time course and magnitude of the afferent arteriolar responses to the P2 agonist were compared between the first and second applications.

**Series 2: effect of calcium channel blockade on the afferent arteriolar response to P2 agonists.** Studies were performed to assess the role of L-type calcium channels in the afferent arteriolar response to selective P2X and P2Y receptor activation. Experiments followed the basic protocol described above and involved a control period followed by a 5-min exposure to 1.0 μM α,β-methylene ATP, 10 μM ATP, or 10 μM UTP, and then following the control period with a 10-min exposure to 10 μM diltiazem, followed by 5 min of recovery in control buffer. The tissue was then exposed to a solution containing 1.0 μM α,β-methylene ATP, 10 μM ATP, 10 μM UTP, or 10 μM diltiazem, and the change in the afferent arteriolar diameter was determined.
The average responses to two applications of 10 \( \mu \)M UTP (Fig. 1; \( n = 6 \)) reduced afferent arteriolar diameter from a baseline diameter of 24.8 \( \pm \) 0.9 to 14.9 \( \pm \) 2.0 \( \mu \)m (59 \% of control; \( P < 0.05 \)). The second treatment reduced afferent diameter to 58 \% of the control diameter. Consistent with previous observations (22), the afferent arteriolar response to UTP was more monophasic than responses to ATP treatments induced a significant decrease in afferent arteriolar diameter (\( P < 0.05 \) vs. control).
induced by α,β-methylene ATP or ATP, such that the initial vasoconstriction was only slightly greater than the steady-state diameter. As with α,β-methylene ATP and ATP, both the time course and the magnitude of the first and second responses to UTP were nearly identical. These data demonstrate that repeat applications of α,β-methylene ATP, ATP, and UTP cause comparable vasoconstrictor responses with no evidence of receptor desensitization or tachyphylaxis.

Previous studies have suggested that preglomerular smooth muscle cells express multiple P2 receptor subtypes that are coupled to different calcium signaling pathways (22, 24). Therefore, studies were performed to assess the effect of calcium channel blockade on the afferent arteriolar response to selective P2X and P2Y receptor activation. The results of experiments examining the effect of calcium channel blockade on the afferent arteriolar response to the P2X agonist α,β-methylene ATP are presented in Fig. 2. α,β-Methylene ATP (1.0 μM) administration induced a sharp reduction in afferent arteriolar caliber of 72 ± 6% from a stable control diameter of 21.7 ± 1.9 μm to a minimum diameter of 6.5 ± 1.9 μm within 30 s. Afferent caliber partially recovered to a stable diameter of 19.9 ± 1.7 μm, representing a sustained vasoconstriction of 8 ± 1% (P < 0.05 vs. control). Removal of α,β-methylene-ATP from the superfusate resulted in complete recovery to a diameter similar to control (21.7 ± 1.8 μm). Exposure to 10 μM diltiazem caused 19 ± 3% vasorelaxation to a stable diameter of 22.5 ± 1.9 μm, which is significantly greater than the control diameter (P < 0.05). Subsequent addition of α,β-methylene ATP to the superfusion solution, in the continued presence of 10 μM diltiazem, evoked a rapid initial vasoconstriction of 41 ± 5%, which was significantly blunted compared with control (P < 0.05). Furthermore, there was no evidence of a sustained vasoconstriction in the presence of diltiazem. Afferent diameter returned to 25.5 ± 1.9 μm, which is similar to the diameter obtained with diltiazem alone. These data are in excellent agreement with the results of an earlier report (25).

Similar experiments were performed to determine the effect of calcium channel blockade on the afferent arteriolar response to P2Y receptor activation with 10 μM UTP. This concentration was selected on the basis of previous work that determined 10 μM UTP to be the lowest concentration to induce a significant reduction in afferent arteriolar diameter (22). As shown in Fig. 3, UTP administration stimulated a monophasic vasoconstriction of afferent arterioles that was rapidly reversible on returning the superfusate to the control medium. UTP reduced the diameter of afferent arterioles by 20 ± 4%, from a control diameter of 21.4 ± 0.7 μm to a stable diameter of 17.1 ± 0.8 μm (P < 0.05 vs. control). When the superfusate was returned to the control solution, the diameter of the afferent arterioles returned to values similar to those of control. Administration of 10 μM diltiazem led to a significant vasorelaxation to a stable diameter of 25.4 ± 0.8 μm (P < 0.05 vs. control). In the continued presence of diltiazem, the vasoconstrictor response to 10 μM UTP averaged 14 ± 4% at a stable arteriolar diameter of 21.6 ± 0.9 μm (P < 0.05 vs. control). The magnitude of the UTP-mediated vasoconstriction during calcium channel blockade tended to be slightly smaller than the control response, but this difference was not statistically significant (P > 0.05; n = 7 arterioles).

Studies suggest that rat juxtamedullary afferent arterioles express functional P2X and P2Y receptors and...
that ATP alters afferent arteriolar caliber by activating both receptor subtypes (22, 23, 25). The calcium signaling mechanisms by which ATP alters afferent arteriolar function have not been thoroughly investigated. Therefore, experiments were performed to determine the effect of calcium channel blockade on the response of afferent arterioles to ATP. Experiments were performed using three ATP concentrations (0.1, 1.0, and 10 μM), which have been shown to elicit minimum, intermediate, and maximum vasoconstriction of afferent arterioles, respectively (Fig. 4) (22, 26). As shown in Fig. 4A, 0.1 μM ATP evoked a modest reduction in afferent arteriolar diameter from 19.7 ± 1.3 μm during the control period, which then reached a stable diameter of 17.8 ± 1.3 μm (P < 0.05 vs. control). Diltiazem treatment increased vessel diameter and completely blocked the response to 0.1 μM ATP. The diameter of afferent arterioles examined averaged 24.2 ± 1.4 and 24.2 ± 1.4 μm during the diltiazem and the diltiazem + ATP periods, respectively.

A similar response was observed in arterioles challenged with 1.0 μM ATP (Fig. 4B). In this group, ATP stimulated a biphasic vasoconstrictor response similar to that observed with α,β-methylene ATP. Afferent arteriolar diameter decreased initially by 30 ± 4%, from a control diameter of 20.2 ± 1.0 to 14.1 ± 0.7 μm (P < 0.05), before stabilizing at a diameter of 18.1 ± 0.9 μm (P < 0.05 vs. control). Subsequent treatment with diltiazem markedly blunted the initial response and completely abolished the sustained response. Afferent diameter decreased transiently from an average of 24.1 ± 1.1 μm (P < 0.05 vs. control) during diltiazem alone to a minimum diameter of 22.8 ± 0.9 μm, before reaching a stable level of 24.1 ± 1.1 μm in response to the second exposure to ATP. The diameter during the ATP + diltiazem period was not significantly different from that during treatment with diltiazem alone. Thus, the magnitude of the second response to 1.0 μM ATP was significantly smaller than the response to the first exposure.

The effect of calcium channel blockade on the afferent arteriolar response to 10 μM ATP is illustrated in Fig. 4C. Control diameter averaged 22.0 ± 1.2 μm and decreased rapidly, but transiently, to a minimum diameter of 9.9 ± 1.5 μm in response to ATP. The diameter stabilized at 14.3 ± 0.9 μm, which is significantly smaller than the control diameter (P < 0.05 vs. control). Diltiazem administration increased afferent diameter to 26.0 ± 1.5 μm (P < 0.05 vs. control), and the second ATP exposure decreased the diameter initially to 16.0 ± 1.5 μm, before a sustained diameter of 25.5 ± 0.9 μm was reached. During both treatment periods, 10 μM ATP produced a significant reduction in afferent arteriolar diameter (P < 0.05 vs. control); however, the magnitude of the first ATP-mediated response was significantly greater than the magnitude of the response obtained during calcium channel blockade (P < 0.05 vs. the second response). These data suggest that vasoconstriction induced by low concentrations of ATP are dependent on activation of L-type calcium channels, whereas higher concentrations of ATP can elicit vasoconstriction through activation of L-type calcium channel-dependent and -independent pathways.

Additional experiments were performed to assess the role of calcium influx on the afferent arteriolar vasoconstriction induced by P2 receptor activation. Calcium influx pathways were not selectively blocked by the addition of 3 mM cadmium chloride (Cd²⁺) to the superfusate solution (Figs. 5 and 6). Under these conditions, the K⁺-induced vasoconstriction of afferent arterioles was blocked (Fig. 6, n = 3). During the control period, 55 mM KCl reduced afferent arteriolar diameter by 33 ± 2%, from 15.3 ± 0.6 to 10.3 ± 0.7 μm (P < 0.05). Addition of 3 mM Cd²⁺ to the superfusate increased arteriolar diameter by 39 ± 0.3%. Subsequent exposure to 55 mM KCl, in the continued presence of Cd²⁺, did not result in a significant vasoconstriction. Afferent diameter averaged 21.3 ± 2 with Cd²⁺ alone and 22.6 ± 1.4 μm in the presence of Cd²⁺ and KCl. These data establish that extracellular Cd²⁺ blocks depolarization-induced afferent arteriolar vasoconstriction.
presence of the Cd$^{2+}$, the response to UTP was attenuated compared with the control response, but UTP still evoked a significant reduction in afferent arteriolar diameter of $8 \pm 2\%$, from $24.3 \pm 1.5$ to $22.3 \pm 1.3$ μm ($P < 0.05$).

The effect of superfusion with Cd$^{2+}$ on the response to ATP administration is summarized in Fig. 6. Treatment with ATP concentrations of 1 (n = 4) or 10 μM (n = 6) reduced afferent diameter by 16 ± 3 and 18 ± 1%, respectively. Cadmium treatment increased afferent diameter by an average of 45 ± 7% and completely blocked the sustained vasoconstrictor response to both ATP concentrations. In the presence of Cd$^{2+}$, afferent arteriolar diameter averaged 22.4 ± 1.8 and 22.5 ± 1.9 μm before and during treatment with 1.0 μM ATP and 23.0 ± 0.4 and 23.1 ± 0.5 μm before and during treatment with 10 μM ATP.

KCl, α,β-methylene ATP, UTP, and both concentrations of ATP produced significant reductions in afferent arteriolar diameter during the control response (Figs. 5 and 6). Also shown in Fig. 6 are the average transient responses observed with each agonist (B). All agonists examined elicited transient vasoconstrictions. These transient responses were statistically significant during the control period and during simultaneous

![Fig. 5. Afferent arteriolar response to α,β-MeATP or UTP before and during superfusion with cadmium. Each data point represents the mean vessel diameter (in μm) measured at 12-s intervals throughout the experimental period. A: responses obtained from arterioles (n = 5 afferent arterioles) superfused with control buffer and switched to an identical solution to which 1 μM α,β-MeATP, 3 mM cadmium, or 1 μM α,β-MeATP + 3 mM cadmium was added. B: results obtained from arterioles (n = 7 afferent arterioles) superfused with control buffer and switched to an identical solution to which 10 μM ATP, 3 mM cadmium, or 10 μM UTP + 3 mM cadmium was added. The periods of exposure to α,β-MeATP or UTP are illustrated by solid horizontal bars; the periods of exposure to cadmium are shown by the open labeled horizontal bar. *Significant reduction in diameter compared with control diameter ($P < 0.05$).](http://ajprenal.physiology.org/)
exposure to Cd$^{2+}$. Nevertheless, the transient responses observed during Cd$^{2+}$ treatment were substantially attenuated compared with the control responses.

P2X receptor-mediated responses involve activation of a nonselective cation channel that allows the influx of extracellular sodium and calcium (1, 11, 12, 36). Mefenamic acid is purported to block nonselective cation channels (14, 15, 28). Excessively high concentrations of mefenamic acid may also block L-type calcium channels (27). Experiments were performed to assess the effect of mefenamic acid on the afferent arteriolar response to P2X receptor stimulation with 100 μM mefenamic acid did not alter the initial vasoconstriction to α,β-methylene ATP but significantly attenuated the magnitude of the sustained vasoconstriction by ~66%. Under control conditions, 1 μM α,β-methylene ATP evoked an initial and sustained reduction in afferent diameter of 55 ± 10 and 18 ± 1%, respectively. In the presence of mefenamic acid, the initial and sustained responses to α,β-methylene ATP averaged 57 ± 11 and 6 ± 1%, respectively.

Fig. 7. Effect of mefenamic acid on KCl-mediated afferent arteriolar vasoconstriction. A: effect of step changes in mefenamic acid concentration on an established KCl-mediated arteriolar response (n = 3 arterioles). Arterioles were superfused with control buffer and switched to a solution containing 55 mM KCl substituted for NaCl. After the KCl-mediated vasoconstriction was established, increasing concentrations of mefenamic acid (1–100 μM) were added. B: effect of 100 μM mefenamic acid on the ability of 55 mM KCl to vasoconstrict of afferent arterioles (n = 4 arterioles). KCl and mefenamic acid administration are shown by solid horizontal bars. Each data point represents the mean vessel diameter (in μm) measured at 12-s intervals throughout the protocol. ∗Significant reduction in diameter compared with the preceding control diameter (P < 0.05).

In a subsequent series of control experiments, we examined the effect of 100 μM mefenamic acid pretreatment on KCl-mediated vasoconstriction of afferent arterioles. This concentration was chosen on the basis of the previous series indicating that mefenamic acid did not attenuate an existing KCl-mediated vasoconstriction. As shown in Fig. 7B, control administration of KCl resulted in a 49 ± 3% reduction in afferent diameter from 21.1 ± 0.8 to 10.8 ± 0.7 μm. This response was completely reversed on removal of KCl from the superfusate solution. Administration of 100 μM mefenamic acid did not alter resting afferent arteriolar diameter nor did it attenuate the vasoconstrictor response elicited by KCl. These data indicate that 100 μM mefenamic acid does not impair voltage-dependent afferent arteriolar vasoconstriction.

Experiments were performed to determine the effect of mefenamic acid on the afferent arteriolar vasoconstriction induced by 1.0 μM α,β-methylene ATP. As shown in Fig. 8, control administration of α,β-methylene ATP elicited a typical biphasic response. After a recovery period, administration of 100 μM mefenamic acid did not alter the initial vasoconstriction to α,β-methylene ATP but significantly attenuated the magnitude of the sustained vasoconstriction by ~66%. Under control conditions, 1 μM α,β-methylene ATP evoked an initial and sustained reduction in afferent diameter of 55 ± 10 and 18 ± 1%, respectively. In the presence of mefenamic acid, the initial and sustained responses to α,β-methylene ATP averaged 57 ± 11 and 6 ± 1%, respectively.
DISCUSSION

P2 receptors are a large category of receptors that are divided into two major families, termed P2X and P2Y (1, 3, 17, 36). Stimulation of P2X receptors induces contraction of vascular smooth muscle by directly activating a ligand-gated, nonselective cation channel (1, 3, 11, 12, 17, 36). Activation of the cation current can lead to depolarization of vascular smooth muscle and activation of voltage-dependent Ca\(^{2+}\) channels. In this report, blockade of L-type calcium channels attenuated the initial rapid vasoconstriction and abolished the sustained vasoconstriction induced by the P2X agonist α,β-methylene ATP. This effect of L-type calcium channel blockade is consistent with an earlier report (25).

These data also support recent observations that calcium channel blockade attenuates the increase in intracellular calcium concentration observed in freshly isolated preglomerular vascular smooth muscle cells stimulated with α,β-methylene ATP (41). Administration of the divalent cation Cd\(^{2+}\) also blocked the sustained vasoconstriction evoked by α,β-methylene ATP and ATP but did not abolish the vasoconstriction elicited by UTP, further supporting the suggestion that α,β-methylene ATP, ATP, and UTP induced vasoconstriction of juxtamedullary afferent arterioles by activating varied signal transduction mechanisms.

P2Y receptors are G protein-regulated receptors coupled to phospholipase C in vascular smooth muscle cells and mesangial cells (1, 3, 17, 18, 35, 36, 38). Activation of P2Y receptors can lead to generation of inositol 1,4,5-trisphosphate and the release of calcium from intracellular stores (18, 35, 38). In the present report, stimulation of P2Y receptors with UTP resulted in a monophasic vasoconstriction that was essentially unaltered by calcium channel blockade. The time course and magnitude of the vasoconstrictor responses were similar before and during calcium channel blockade, and the magnitude was only attenuated in the presence of Cd\(^{2+}\). The finding that UTP-mediated renal microvascular vasoconstriction is retained during calcium channel blockade agrees well with previous reports that calcium channel blockade or removal of Ca\(^{2+}\) from the extracellular medium did not significantly alter UTP-induced increases in intracellular calcium concentration in preglomerular smooth muscle cells (24). In addition, these data lend additional support to the argument that at least two distinct P2 receptors are expressed by preglomerular microvascular smooth muscle (13, 19, 22, 24).

ATP is believed to be the endogenous ligand for P2 receptors. We have postulated that extracellular ATP might function as the extracellular messenger that evokes autoregulatory adjustments in afferent arteriolar resistance (19, 23, 31, 33, 34). For this hypothesis to be correct, ATP-mediated afferent arteriolar vasoconstrictor responses must involve activation of L-type calcium channels (5, 25, 30, 32, 34). In the present report, we have shown that afferent arteriolar vasoconstriction, mediated by low concentrations of ATP, are completely abolished by calcium channel blockade. In contrast, afferent arteriolar vasoconstrictor responses induced by high concentrations of ATP were relatively insensitive to calcium channel blockade. This pattern suggests that activation of P2 receptors with low concentrations of ATP stimulates voltage-dependent calcium influx, perhaps through activation of P2X receptors. It is interesting to note that increases in intracellular calcium concentration, mediated by high concentrations of ATP, are reduced by ~50% in the presence of calcium channel blockers or during removal of extracellular calcium (24). These observations suggest that low concentrations of ATP bind preferentially to P2X receptors, stimulating L-type calcium channel-dependent afferent arteriolar vasoconstriction. It is also possible that higher concentrations of ATP activate both P2X and P2Y receptors, leading to both voltage-dependent and voltage-independent calcium influx as well as mobilization of calcium from intracellular stores. This suggestion is supported by the retention of a significant vasoconstriction with 10 μM ATP despite the continued presence of diltiazem. It is further supported by the observation that a high concentration of ATP increases the intracellular calcium concentration in preglomerular smooth muscle cells incubated in a nominally calcium-free solution with a nearly identical time course and magnitude as those observed during calcium channel blockade (24).

Stimulation of P2X receptors with α,β-methylene ATP, under similar circumstances, essentially abolished the rise in intracellular calcium normally observed under control conditions (41).

Despite being able to block or attenuate the sustained vasoconstriction of afferent arterioles exposed to α,β-methylene ATP and low concentrations of ATP, calcium channel blockade did not eliminate the initial phase of the response. It is presumed that the rapid response arises from stimulation of ligand-gated P2X receptors with the subsequent activation of a nonselective cation channel. To test this hypothesis, we examined the effect of the purported nonselective cation channel blocker mefenamic acid (14, 28) on the response. Pretreatment with mefenamic acid had no discernable effect on the time course or magnitude of the initial vasoconstriction elicited by α,β-methylene ATP, but it attenuated the sustained vasoconstriction. These data suggest that a mefenamic acid-sensitive nonselective cation current contributes to the agonist-induced depolarization of afferent arteriolar smooth muscle and facilitates the opening of L-type calcium channels. However, the mechanism responsible for the rapid initial vasoconstriction appears to completely insensitive to mefenamic acid treatment. It is further noted that the initial vasoconstriction induced by α,β-methylene ATP was attenuated by about the same degree by Cd\(^{2+}\) treatment as it was by diltiazem, whereas the effect on ATP and UTP was more pronounced. For example, Cd\(^{2+}\) treatment reversed or attenuated the vasoconstriction induced by α,β-methylene ATP and to 1.0 μM ATP in a pattern comparable to that observed with diltiazem. In addition, superfusion with Cd\(^{2+}\) attenuated or abolished the transient and sustained
vasoconstrictor responses evoked by 10 μM ATP, respectively, while only attenuating the response to UTP. Taken together, these observations suggest that other signal transduction pathways may be involved in mediating the transient and sustained vasoconstrictor responses observed during P2 receptor activation. These pathways could include nonelective cation channels, non-L-type voltage-dependent calcium channels, or store-operated calcium influx pathways. Further studies will need to be performed to better elucidate the mechanisms of this response.

Curiously, diltiazem markedly reduced the increase in intracellular calcium concentration observed in freshly isolated preglomerular smooth muscle cells in response to α,β-methylene ATP (41), whereas a transient vasoconstriction was still observed in the present studies with the use of intact arterioles. The reasons for this discrepancy are unclear but may arise, in part, from differences in the “activation state” that exist between isolated smooth muscle cells compared with vascular smooth muscle cells in intact, pressurized preglomerular microvessels. Vascular smooth muscle cells in intact vascular segments are stretched and depolarized compared with isolated cells. Tension-generating signal transduction pathways are already activated, whereas isolated cells are not exposed to a workload and thus function at a different level of activation. This notion is supported by the observation that calcium channel blockers induce a prompt vasorelaxation of pressurized afferent arterioles (4, 5, 25), but they do not alter basal intracellular calcium concentration in freshly isolated preglomerular smooth muscle cells (2231, 2564, 3058). This difference may result in altered response kinetics between the two experimental conditions and underscores the importance of examining signal transduction mechanisms in both preparations.

The contribution of P2 receptors to the overall regulation of renal hemodynamics and autoregulatory behavior is still unclear. Activation of L-type calcium channels plays a major role in the regulation of preglomerular resistance in response to both agonist stimulation (4, 5, 29, 30, 34), autoregulation, and tubuloglomerular feedback signals (6, 19, 30, 32, 34). Similarly, the mobilization of calcium from intracellular stores also contributes significantly to agonist-induced and pressure-mediated afferent arteriolar vasoconstriction (9, 21, 29). In the present report, the data establish the central role L-type calcium channels play in mediating the afferent arteriolar response to P2X receptor activation by either the P2X agonist α,β-methylene ATP or low concentrations of the endogenous ligand ATP. Results of this study are also consistent with previous observations implicating a P2Y receptor subtype in evoking afferent arteriolar vasoconstriction through a L-type calcium channel-independent pathway, presumably involving IP3-dependent mobilization of calcium from intracellular stores (24, 36). However, the specific P2 receptor subtypes involved remain to be identified. Previous work supports the postulate that P2X1 and P2Y2 receptors are expressed by the juxtaglomerular vasculature (7, 19, 22, 23). Chan and co-workers (7) provided solid immunohistochemical evidence depicting strong distribution of P2X1 receptors along preglomerular microvascular segments and no detectable staining on postglomerular vascular elements. This observation, coupled with the functional evidence demonstrating potent afferent arteriolar vasoconstriction induced by α,β-methylene ATP (19, 23, 25, 33), strongly supports the hypothesis that P2X1 receptors are major contributors to the renal microvascular responses to P2X receptor agonists. Interestingly, P2X1 receptors are known to desensitize when repeatedly challenged by an agonist (36). Deliberate attempts to desensitize P2X1 receptors on juxtaglomerular afferent arterioles resulted in a marked blunting of the vasoconstriction induced by α,β-methylene ATP and abolition of the pressure-mediated autoregulatory response (23).

The possible involvement of P2X3 receptors is also an intriguing possibility. Although definitive evidence of their presence on the renal microvasculature is lacking, P2X3 receptors can be activated by α,β-methylene ATP, and they do not desensitize as readily as P2X1 receptors (36). These receptors might be better suited to produce the sustained vasoconstriction induced by α,β-methylene ATP on juxtaglomerular afferent arterioles. Another possibility is that P2X1 and P2X3 receptors could be present as a multimeric receptor, thus conferring properties of both receptors on the overall response (36).

Functional evidence for the presence of various P2Y receptors in the renal microcirculation can also be found in the literature and have been demonstrated using the juxtamedullary nephron technique (8, 13, 19, 22, 24, 31). Under the present experimental conditions, a strong argument can be made for the involvement of P2Y2 and/or P2Y4 receptors in the afferent arteriolar response to ATP in the rat kidney (36). This conclusion is based on the observation that UTP and ATP-γ-S produce a strong and sustained afferent arteriolar vasoconstrictor response that cannot be mimicked by other P2Y agonists, such as 2-methylthio ATP (22). UTP is a good agonist for P2Y2 and P2Y4 receptors and a poor one for P2Y1 and P2Y6 receptors (36). The fact that rat juxtamedullary afferent arterioles are nearly unresponsive to 2-methylthio ATP, ADP, and UDP (20, 22) essentially rules out a major role for P2Y1 and P2Y6 receptors in this vascular bed. Rank-order potency data compiled from other tissues suggest that P2Y4 receptors exhibit comparable sensitivity to ATP and ADP; however, ADP is a very poor agonist for juxtamedullary afferent arterioles (20). Therefore, on the basis of the information presently available for this vascular bed, P2Y2 receptors are the most likely P2Y receptor subtype involved in the afferent arteriolar response to ATP. The receptor activation and signaling mechanisms known to be utilized by these receptors are also consistent with the signaling mechanisms known to participate in renal preglomerular autoregulatory behavior (21, 34).
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In summary, L-type calcium channels play a major role in regulating the diameter of afferent arterioles. Blockade of L-type calcium channels induces a prompt increase in arteriolar diameter and reduced responsiveness to P2 agonists. The results of the studies presented here reveal that activation of P2 receptors on afferent arterioles induces vasoconstriction through activation of distinct signal transduction pathways. Activation of P2X receptors relies largely on activation of voltage-dependent L-type calcium channels to evoke afferent arteriolar vasoconstriction. In contrast, P2Y receptor activation stimulates vasoconstriction of afferent arterioles largely through L-type calcium channel-independent mechanisms. P2 receptor activation by low concentrations of ATP induces afferent arteriolar vasoconstriction primarily through L-type calcium channel-dependent mechanisms, whereas higher concentrations of ATP invoke additional L-type calcium channel-independent vasoconstrictor mechanisms. These mechanisms are consistent with those known to participate in autoregulatory vasoconstrictor responses and are supportive of the postulate that activation of afferent arteriolar P2 receptors plays a central role in mediating autoregulatory adjustments in afferent arteriolar resistance.

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