Physiological role for $P2X_1$ receptors in renal microvascular autoregulatory behavior

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This study tests the hypothesis that $P2X_1$ receptors mediate pressure-induced afferent arteriolar autoregulatory responses. Afferent arterioles from rats and $P2X_1$ KO mice were examined using the juxtamedullary nephron technique. Arteriolar diameter was measured in response to step increases in renal perfusion pressure (RPP). Autoregulatory adjustments in diameter were measured before and during P2X receptor blockade with NF279 or A_1 receptor blockade with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Acute papillectomy or furosemide perfusion was performed to interrupt distal tubular fluid flow past the macula densa, thus minimizing tubuloglomerular feedback–dependent influences on afferent arteriolar function. Under control conditions, arteriolar diameter decreased by 17% and 29% at RPP of 130 and 160 mmHg, respectively. Blockade of $P2X_1$ receptors with NF279 blocked pressure-mediated vasoconstriction, reflecting an attenuated autoregulatory response. The A_1 receptor blocker DPCPX did not alter autoregulatory behavior or the response to ATP. Deletion of $P2X_1$ receptors in KO mice significantly blunted autoregulatory responses induced by an increase in RPP, and this response was not further impaired by papillectomy or furosemide. WT control mice exhibited typical RPP-dependent vasoconstriction that was significantly attenuated by papillectomy. These data provide compelling new evidence indicating that tubuloglomerular feedback signals are coupled to autoregulatory preglomerular vasoconstriction through ATP-mediated activation of $P2X_1$ receptors.

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Introduction

Autoregulation of renal blood flow is an intrinsic property of mammalian kidneys that involves the combined influences of the myogenic mechanism inherent to vascular smooth muscle and the tubuloglomerular feedback (TGF) mechanism that is unique to the kidney (1). Alterations in renal perfusion pressure (RPP) evoke metered adjustments in renal vascular resistance, to maintain stable renal blood flow and glomerular filtration rate. While the existence of autoregulatory behavior is well established, the mechanisms responsible for transducing changes in RPP to appropriate adjustments in preglomerular resistance remain unresolved. Recently, the debate over identification of the chemical mediator of autoregulatory adjustments in preglomerular resistance came to the fore in a series of opinion articles (2–4) that postulated adenosine (2) and ATP (3) as candidates. Schnermann and others have postulated that adenosine mediates TGF-dependent adjustments in preglomerular resistance (5, 6). Our laboratory has hypothesized that locally released ATP is the chemical mediator of autoregulatory responses through activation of preglomerular P2X receptors (7–9). This postulate is supported by the recent observation that the macula densa cells responsible for TGF-dependent preglomerular-resistance adjustments respond to osmotic stimuli by releasing ATP (10).

P2 receptors are divided into two families, the ligand-gated P2X receptors (11–13) and the G protein–regulated P2Y receptors (11, 12, 14). Studies performed in numerous vascular beds have established the relationship between $P2X_1$ receptor activation and vasoconstriction (7, 11, 12, 15); however, the physiological role of $P2X_1$ receptors in regulating vascular function remains unclear. Studies suggest that P2 receptors contribute to the regulation of renal microvascular function (1, 7–10, 16–18). P2X and P2Y receptors are expressed by preglomerular microvascular smooth muscle (7, 8, 16, 19–23), and previous studies implicate P2 receptors in mediating autoregulatory behavior (7–9, 17, 24). Unfortunately, due to the lack of subtype-specific P2 receptor antagonists, the specific P2 receptor subtype involved in the autoregulatory response has eluded identification.

Recently, a P2 receptor antagonist, NF279, has been developed that exhibits a high degree of selectivity for P2X over P2Y receptors (25–28). $P2X_1$ receptors are heav-

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Nonstandard abbreviations used: tubuloglomerular feedback (TGF); renal perfusion pressure (RPP); N^6-cyclopentyl adenosine (CPA); 1,3-dipropyl-8-cyclopentylxanthine (DPCPX).

ily expressed along the afferent arteriole, suggesting that they play an important role in regulating preglomerular resistance and autoregulatory responsiveness (22). Therefore, experiments were performed to test the hypothesis that pressure-mediated afferent arteriolar autoregulatory responses are mediated by P2X receptors. Similar experiments were performed, using $P2X_1$ KO mice, to determine whether autoregulatory responsiveness is impaired in the absence of the $P2X_1$ receptor. Experiments were performed to directly assess the effects of A_1 receptor blockade on autoregulatory behavior and afferent arteriolar responses to P1 and P2 receptor activation, as gene-knockout strategies have recently implicated A_1 receptor activation in autoregulatory behavior (29, 30). Finally, studies were performed to determine the effect of interruption of TGF influences on the response of afferent arterioles to an increase in perfusion pressure in kidneys from WT and $P2X_1$ KO mice.

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.

Kidney preparation. Videomicroscopy experiments were conducted in vitro using the blood-perfused juxtamedullary nephron technique, as previously described (19, 23). Ninety-four male Sprague-Dawley rats (350–400 g) and 51 mice (15, 31) were used to complete these studies. For each experiment, two animals were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and prepared for videomicroscopy experiments.

Experimental protocols. Afferent arteriolar responses to changes in RPP, or to administration of vasoactive agonists and antagonists, were determined. Autoregulatory behavior was assessed by measurement of changes in afferent arteriolar diameter in response to acute elevations in RPP. Measurements of afferent arteriolar diameter were made at 12-second intervals, and the sustained afferent arteriolar diameter was calculated from the average of measurements made during the final 2 minutes of each treatment period. Each protocol consisted of 7–13 periods of 5 minutes' duration. Each protocol began with a 5-minute control period to ensure a stable vessel diameter and was followed by either agonist stimulation or an increase in RPP to establish the control response.

Effect of P2X receptor blockade on the afferent arteriolar response to acute increases in RPP. The effect of an increase in RPP on afferent arteriolar diameter was determined before and during selective P2X receptor blockade with NF279 (8,8′-[carbonylbis (imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-napthalenetrisulfonic acid hexasodium salt) (25–28). Afferent arteriolar diameters were measured at perfusion pressures of 100, 130, and 160 mmHg and again at 100 mmHg in successive 5-minute periods. Following recovery, the superfusion solution was changed to a similar solution containing 20 μM NF279, and pressure-induced responses were reassessed.

Effect of P2X receptor blockade on an established afferent arteriolar autoregulatory response. These experiments were performed to determine the effect of P2X receptor blockade on the afferent arteriolar vasoconstriction induced by a 60-mmHg increase in RPP. Control afferent diameter was determined at 100 mmHg and again after RPP was increased to 160 mmHg. While RPP was held at 160 mmHg, the superfusion solution was supplemented with 20 μM NF279. After 5 minutes, RPP was returned to 100 mmHg, in the continued presence of NF279. Finally, the control solution was reapplied, and the afferent arteriolar response to increased RPP was reassessed.

Effect of P2X receptor blockade with NF279 on the afferent arteriolar response to vasoactive agonists. The ability of NF279 to inhibit P2 receptor–dependent responses was assessed using the endogenous ligand ATP (1.0 μM) and the P2X receptor agonist α,β-methylene ATP (1.0 μM). Retention of normal P1 receptor–dependent responses in the presence of NF279 was assessed using the A_1 agonist N^6-cyclopentyl adenosine (CPA; 1.0 μM). The effect of NF279 on non–purinoceptor-mediated vasoconstrictor responses was assessed using Ang II; (1.0 nM) and KCl (55 mM). With RPP maintained at 100 mmHg, the control diameter, the response to agonist, and the recovery diameter were determined. Subsequently, 20 μM NF279 was added to the superfusate, and the sequence was repeated.

Effect of A_1 receptor blockade with 1,3-dipropyl-8-cyclopentylxanthine on the afferent arteriolar autoregulatory response. The effect of an increase in RPP on afferent arteriolar diameter was determined in untreated arterioles and in separate arterioles during A_1 receptor blockade with the highly selective A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Blockade of adenosine-mediated (10 μM) vasoconstriction by 1 nM DPCPX was verified in each arteriole, and then the arteriolar response to increases in RPP and to ATP (10 μM) was determined, in the continued presence of DPCPX.

Autoregulatory responsiveness in $P2X_1$ receptor KO mice. $P2X_1$ KO mice and their WT controls were generously provided by Richard J. Evans (University of Leicester). KO mice have been extensively characterized and shown to be unresponsive to $P2X_1$ receptor–dependent agonists (15, 31). Afferent arteriolar autoregulatory responses, induced by 15-mmHg increases in RPP from 65 to 170 mmHg, were assessed in WT and KO mice. In addition, the arteriolar responses to ATP (10 μM), α,β-methylene ATP (1.0 μM), and CPA (10 μM) were determined.

Effect of papillectomy on autoregulatory responsiveness in $P2X_1$ receptor KO mice. These experiments were designed to determine the role of $P2X_1$ receptors in mediating pressure-dependent vasoconstrictor responses induced by the TGF and myogenic mechanisms. Afferent arteriolar autoregulatory responses were assessed in WT and KO mice before and after papillectomy. Acute papillectomy interrupts the flow of distal tubular fluid past the macula densa and minimizes tubuloglomerular feedback–dependent influences on afferent arteriolar function (32–35). Severing of the loops of Henle by papillectomy has been used in this preparation to differentiate

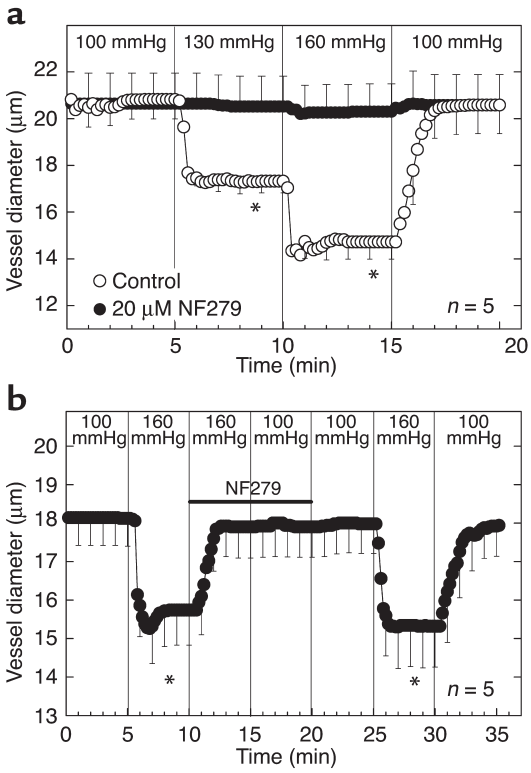

Figure 1
Effect of NF279 on the afferent arteriolar response to increasing RPP. (**a**) The response to an increase in RPP during the control period (open circles) and during exposure to 20 μM NF279 (filled circles). The response during NF279 administration is overlaid on the control response for comparison. (**b**) The afferent arteriolar response to elevations in RPP from 100 to 160 mmHg before, during, and after P2X receptor blockade with NF279. The period of exposure to 20 μM NF279 is illustrated by the black line. *$P < 0.05$ vs. diameter at 100 mmHg.

the relative contributions of the TGF and myogenic mechanisms to overall pressure-mediated autoregulatory responses (32–35). In this series of experiments, perfusion pressure was increased in a single step of 40 mmHg, followed by a recovery period and papillectomy. Ten minutes after papillectomy, the response to perfusion pressure was reassessed. Finally, pressure-mediated autoregulatory responses were examined in some kidneys under passive conditions in which the kidney was perfused and bathed with solutions containing 5 mM EGTA. Time-control studies were performed to verify the reproducibility of autoregulatory responses over the same time frame in mouse kidneys whose papilla remained intact. Diameter measurements were made 103 ± 31 μm from the glomerulus in WT mice and 99 ± 26 μm from the glomerulus in KO mice.

Effect of furosemide on autoregulatory responsiveness in P2X$_1$ receptor KO mice. Furosemide administration has been used by Schnermann and others to inhibit TGF-mediated changes in stop-flow pressure, and autoregulatory behavior (24, 32, 33, 36–45). Accordingly, the role of TGF signaling in afferent arteriolar autoregulatory responses was assessed in WT and KO mice before and during TGF inhibition with furosemide. In this series of experiments, perfusion pressure was increased in a single step of 40 mmHg, followed by a recovery period. After the recovery period, furosemide was added to the perfusate blood (50 μM) and allowed to equilibrate for 10 minutes before the response to perfusion pressure was reassessed. Diameter measurements were made 55 ± 2 μm from the glomerulus in WT mice and 48 ± 3 μm from the glomerulus in KO mice.

Statistical analysis. Data were evaluated using a one-way ANOVA for repeated measures. Differences between group means, within each experimental series, were determined using the Newman-Keuls multiple-range test. P values less than 0.05 were considered to indicate statistically significant differences. All values are reported as the mean ± SE.

Materials. BSA was obtained from Calbiochem-Novabiochem International Inc. (La Jolla, California, USA). CPA was purchased from Research Biochemicals International (Natick, Massachusetts, USA). NF279 was obtained from Tocris Cookson Inc. (Ellisville, Missouri, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Results

Initial experiments determined the effect of P2X receptor blockade, with NF279, on pressure-mediated autoregulatory responses (Figure 1a). Under control conditions, at 100 mmHg, afferent arteriolar diameter averaged 20.8 ± 0.8 μm and decreased by 17% ± 2% ($P < 0.05$ vs. control) and 29% ± 5% ($P < 0.05$ vs. control) when RPP was increased to 130 and 160 mmHg, respectively. Returning RPP to 100 mmHg resulted in a complete recovery to 20.6 ± 1.2 μm. Addition of 20 μM NF279 to the superfusate did not change base-line caliber (20.6 ± 1.2 μm) but markedly attenuated the pressure-mediated vasoconstrictor response ($P < 0.05$). Increasing RPP from 100 mmHg to 130 and 160 mmHg resulted in arteriolar diameters of 20.5 ± 1.3 and 20.3 ± 1.2 μm, respectively.

In separate experiments, we determined the effect of P2X receptor blockade on afferent arteriolar diameter after pressure-mediated autoregulatory adjustments in diameter were already imposed (Figure 1b). Increasing RPP from 100 to 160 mmHg decreased diameter by 13% ± 3% from 18.1 ± 0.7 μm to 15.7 ± 0.9 μm ($n = 5$). With RPP held at 160 mmHg, addition of NF279 to the superfusate reversed the pressure-mediated vasoconstrictor response. Afferent diameter increased from 15.7 ± 0.9 μm to 17.9 ± 0.8 μm, where it remained after RPP was returned to 100 mmHg. Removal of NF279 resulted in complete restoration of a typical pressure-mediated autoregulatory response. During this recovery period, increasing RPP to 160 mmHg decreased afferent diameter by 13% ± 2% from 18.0 ± 0.8 μm to 15.3 ± 1.1 μm. This response is similar to the control response.

ATP is the endogenous ligand for P2X and P2Y receptors (11). α,β-Methylene ATP is a potent agonist for

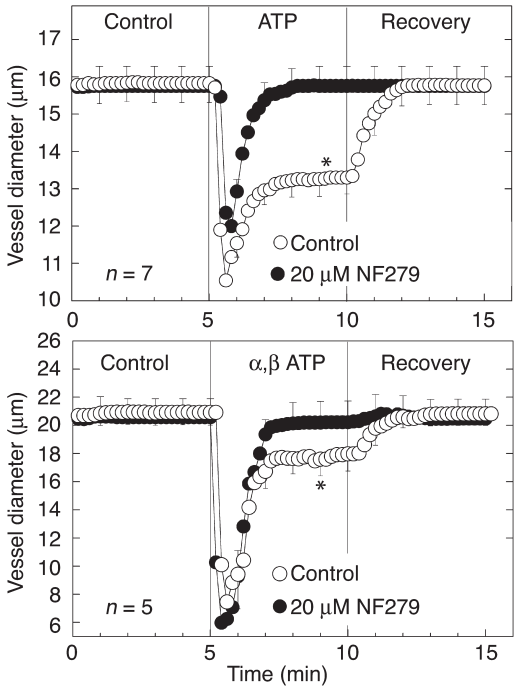

Figure 2
Effect of NF279 on the afferent arteriolar response to ATP (upper panel) and α,β-methylene ATP (lower panel). The arteriolar-diameter response to ATP (1.0 μM) or α,β-methylene ATP (1.0 μM) is shown during the control period (open circles) and during exposure to 20 μM NF279 (filled circles). The response during NF279 administration is overlaid on the control response. *P < 0.05 vs. diameter at 100 mmHg.

$P2X_1$ but a poor agonist for P2Y receptors (11, 12). Figure 2 illustrates the effect of NF279 on the vasoconstriction induced by ATP or α,β-methylene ATP. ATP (1 μM; Figure 2, upper panel) induced a biphasic response composed of a rapid initial vasoconstriction from a control of 15.8 ± 0.5 μm to a minimum of 10.5 ± 0.2 μm before stabilization at a sustained diameter of 13.3 ± 0.4 μm (P < 0.05 vs. control). Diameter returned to the control value during the recovery period and remained unchanged during introduction of NF279. P2X receptor inhibition significantly attenuated the initial vasoconstriction (P < 0.05 vs. control) and abolished the sustained vasoconstriction (P < 0.05 vs. control). Diameter decreased from 15.8 ± 0.5 μm to 12.0 ± 0.2 μm before stabilizing at a diameter identical to that with NF279 alone (15.8 ± 0.5 μm).

A similar profile was observed with the $P2X_1$ agonist α,β-methylene ATP (1.0 μM; Figure 2, lower panel). Afferent diameter decreased from 20.9 ± 0.9 μm (n = 5) to a minimum diameter of 7.4 ± 2.2 μm before stabilizing at 17.8 ± 1.2 μm (P < 0.05 vs. control). Diameter returned to the control value during the recovery period. NF279 treatment did not alter the initial response to α,β-methylene ATP; however, the sustained vasoconstriction was completely abolished. Arteriolar diameter averaged 20.2 ± 1.5 μm, which is significantly different from the control response (P < 0.05).

To determine whether NF279 selectively inhibited autoregulatory responses, we examined the effect of NF279 on arteriolar vasoconstriction induced by KCl, Ang II, and the A_1 agonist CPA (Figure 3). The vasoconstrictor responses to KCl (55 mM; upper panel) were nearly identical in the presence and absence of 20 μM NF279. Afferent diameter decreased by 41% ± 6% and 43% ± 7% in the control and NF279-treated periods, respectively. NF279 also had no significant effect on the response to 1 nM Ang II (middle panel) or the adenosine A_1 agonist CPA (lower panel). With Ang II, arteriolar diameter decreased by 13% ± 1% and 12% ± 2% during control and NF279 periods, respectively. Similarly, CPA decreased diameter by 32% ± 3% during

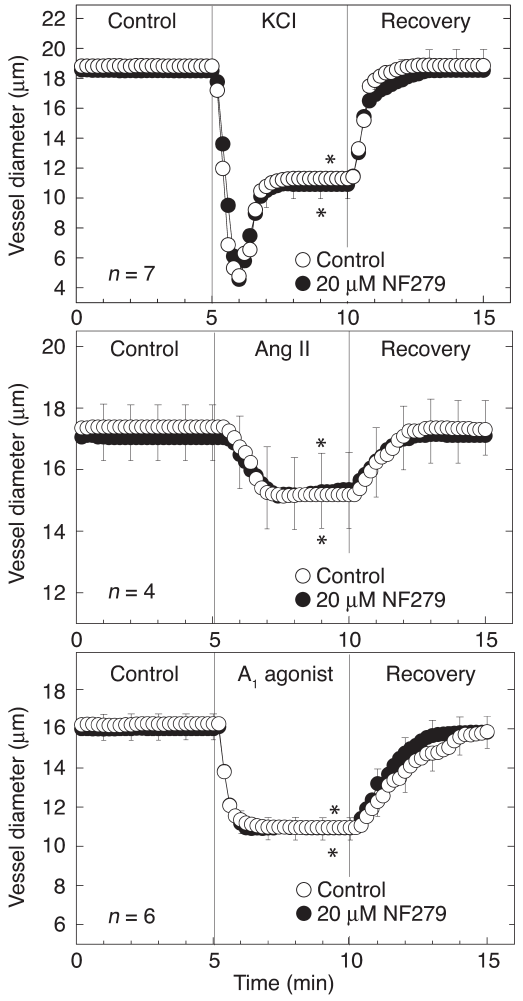

Figure 3
Effect of NF279 on the afferent arteriolar response to vasoconstrictor agonists. The arteriolar-diameter response to 55 mM KCl (upper panel), 1 nM Ang II (middle panel), and 1 μM CPA (lower panel) is shown during the control period (open circles) and during exposure to 20 μM NF279 (filled circles). The response during NF279 administration is overlaid on the control response. *P < 0.05 vs. diameter at 100 mmHg.

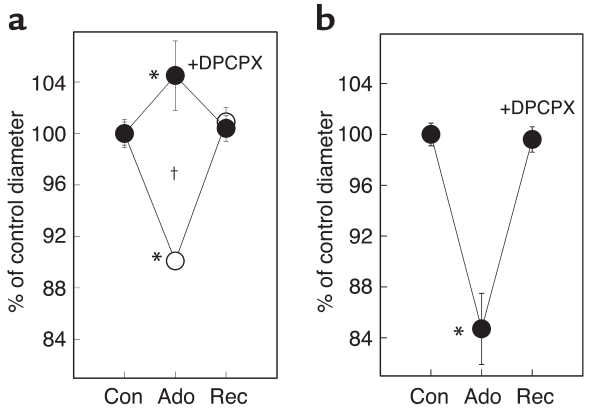

Figure 4
Effect of A_1 receptor blockade on the afferent arteriolar response to adenosine (left panel) and ATP (right panel). Data are expressed as a percentage of the control diameter. The left panel depicts the response to adenosine (Ado; 10 μM; $n = 4$) under control conditions (–DPCPX; open circles) and during exposure to 1.0 nM DPCPX (+DPCPX; filled circles). The right panel depicts the diameter response to ATP (10 μM; $n = 4$) during exposure to 1.0 nM DPCPX (+DPCPX). *$P < 0.05$ vs. diameter at 100 mmHg. Con, control periods; Rec, recovery periods.

both control and NF279 treatment. Therefore, NF279 did not alter the response to any of the vasoconstrictor agonists examined.

Activation of A_1 receptors is postulated to mediate TGF responses (29, 30). Accordingly, experiments were performed to determine the effect of A_1 receptor blockade on pressure-mediated autoregulatory responses and to compare these responses with those obtained with NF279. Blockade of A_1 receptors, with 1 nM DPCPX (Figure 4), prevented adenosine-mediated (10 μM) vasoconstriction but did not block vasoconstriction induced by an equimolar concentration of ATP (10 μM). In the presence of DPCPX, base-line afferent diameter decreased significantly from 16.0 ± 0.8 μm to 15.3 ± 0.8 μm. Subsequent addition of 10 μM adenosine resulted in a 5% ± 3% increase in diameter from 15.3 ± 0.8 μm to 16.0 ± 1.0 μm, in contrast to the 10% ± 2% (15.9 ± 0.8 μm to 14.3 ± 0.8 μm) reduction in diameter evoked by adenosine prior to A_1 blockade. ATP-mediated vasoconstriction was not significantly altered by A_1 receptor blockade. Consistent with control responses presented in Figure 2, arteriolar diameter decreased by 15% ± 3% from 15.3 ± 0.7 μm to 12.9 ± 0.4 μm during ATP administration.

Figure 5 shows the effect of A_1 receptor blockade, with DPCPX, on the pressure-mediated autoregulatory response. Increases in RPP from 100 to 130 and 160 mmHg decreased afferent arteriolar diameter from 15.4 ± 0.7 μm to 13.6 ± 0.6 and 12.0 ± 0.7 μm, respectively. This represents pressure-mediated reductions in afferent arteriolar diameter of 11% ± 2% and 22% ± 2% at RPP of 130 and 160 mmHg, respectively. This response is similar to the control response presented in Figures 1a and 5. Control arterioles decreased in diameter by 17% ± 2% and 29% ± 5% at perfusion pressures of 130 and 160 mmHg, respectively. In each group, the reduction in diameter at 130 and 160 mmHg was statistically significant; however, the magnitudes of the change in the presence and absence of DPCPX were similar across groups.

Pressure-mediated autoregulatory responses were also assessed in similarly prepared $P2X_1$ receptor KO mice and WT controls. In these studies, RPP was increased in 15-mmHg increments from 65 to 170 mmHg. Base-line diameters were similar between the two groups and averaged 14.3 ± 0.5 μm and 13.8 ± 0.4 μm in the KO and WT groups, respectively. As shown in Figure 6, afferent arterioles in kidneys from $P2X_1$ KO mice exhibited static diameters despite stepwise increases in RPP. No significant pressure-mediated vasoconstriction was observed, whereas a tendency toward an increase in diameter was evident at RPP of 155 and 170 mmHg. In contrast, increasing RPP to approximately 140 mmHg produced significant, pressure-dependent vasoconstriction in WT controls. Subsequent RPP increases to 155 and 170 mmHg resulted in a slight increase in diameter.

Current hypotheses suggest that impairment of autoregulatory behavior could reflect either the absence of $P2X_1$ receptors or impaired A_1 receptor–dependent responses. Therefore, experiments were performed to assess the arteriolar response to the A_1 agonist CPA in WT and KO mice. Control diameters were similar and averaged 13.2 ± 0.7 μm in WT and 11.3 ± 0.7 μm in KO mice (Figure 7). Both WT and $P2X_1$ KO mice exhibited similar concentration-dependent declines in response to A_1 receptor activation by CPA up to 1.0 μM. Diameters in WT mice decreased to 11.3 ± 0.8 μm and 10.8 ± 0.9 μm at 0.1 and 1.0 μM CPA, respectively, whereas diameters in KO mice decreased to 9.5 ± 1.0 μm and 8.8 ± 0.9 μm, respectively, at the same agonist

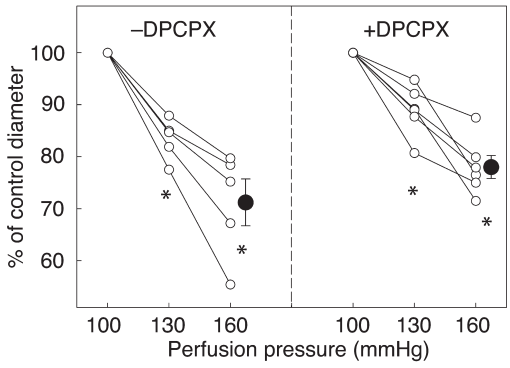

Figure 5
Effect of DPCPX on the afferent arteriolar response to RPP. The diameter response of individual arterioles to increasing RPP is shown in untreated control kidneys (open circles, left panel; $n = 5$) and in kidneys treated with 1.0 nM DPCPX (open circles, right panel; $n = 6$). The mean change in diameter at 160 mmHg is represented by the filled circle bearing SE bars in each panel. Control data are taken from the control period of the arterioles presented in Figure 1a. *$P < 0.05$ vs. diameter at 100 mmHg for mean responses at 130 and 160 mmHg.

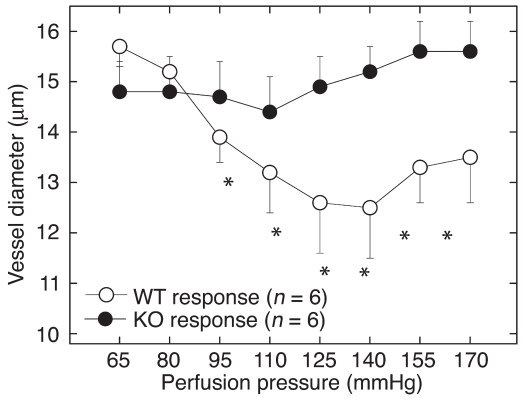

Figure 6
Pressure-mediated autoregulatory responses in $P2X_1$ KO mice (filled circles; $n = 12$) and WT control mice (open circles; $n = 4$). Data are expressed as the mean diameter measured at each pressure. *$P < 0.05$ vs. diameter at 100 mmHg.

concentrations. Furthermore, KO mice exhibited sustained vasoconstriction (9.0 ± 0.9 μm) to 10 μM CPA, whereas arterioles from WT controls responded to this concentration with an increase in diameter to 12.3 ± 0.9 μm.

α,β-Methylene ATP (1.0 μM) reduced arteriolar diameter by approximately 8% in WT controls (Figure 8) from 13.8 ± 0.6 μm to 12.7 ± 0.7 μm, whereas this response was abolished in kidneys from KO mice ($n = 9$). Afferent diameter averaged 12.8 ± 1.1 μm and 12.8 ± 1.1 μm, in the control and agonist periods, respectively. ATP (1.0 μM), which interacts with multiple P2 receptor subtypes, reduced afferent diameter by 12% from 13.1 ± 0.6 μm to 11.5 ± 0.8 μm in kidneys from WT mice and by 8% from 13.2 ± 0.8 μm to 12.4 ± 1.0 μm in kidneys from KO mice.

Papillectomy studies were performed to determine the role of $P2X_1$ receptors in TGF versus myogenic autoregulatory adjustments in afferent arteriolar diameter. As shown in Figure 9, increasing perfusion pressure from 100 to 140 mmHg, in a single step, reduced afferent arteriolar diameter in WT mice by 13% from 12.4 ± 0.7 μm to 10.5 ± 0.9 μm under control conditions with an intact TGF mechanism. Afferent arteriolar diameter did not change significantly following papillectomy, but the autoregulatory response was significantly attenuated. During interruption of distal tubular fluid flow, increasing perfusion pressure from 100 to 140 mmHg reduced afferent arteriolar diameter by just 3% from 11.9 ± 0.5 μm to 11.6 ± 0.5 μm. In KO mice, increasing perfusion pressure from 100 to 140 mmHg had little effect on afferent arteriolar diameter before or after papillectomy. Arteriolar diameter averaged 12.4 ± 0.8 μm and 12.7 ± 0.8 μm at 100 and 140 mmHg, respectively, under control conditions and 12.5 ± 0.6 μm and 12.5 ± 0.8 μm at 100 and 140 mmHg, respectively, after papillectomy. Time controls revealed that repeated increases in perfusion pressure to 140 mmHg reduced afferent diameter by 16% ± 2% and 13% ± 1% for the first and second pressure challenge, respectively, demonstrating that the impairment of responses in the WT mice reflects the effects of TGF interruption by papillectomy.

Arterioles in both groups of mice continued to generate active tension as indicated by the marked relaxation observed upon exposure to EGTA. In WT mice ($n = 4$), 5 mM EGTA increased afferent arteriolar diameter by 33% from 12.0 ± 0.7 μm to 15.9 ± 1.6 μm at 100 mmHg, whereas the diameter of arterioles from KO mice ($n = 3$) increased by 27% from 12.3 ± 0.9 μm to 15.6 ± 1.3 μm. Subsequent elevation of perfusion pressure to 140 mmHg increased diameter further to 17.0 ± 1.7 μm and 16.6 ± 1.0 μm in WT and KO mice, respectively.

In a separate series of experiments, we examined the effect of TGF blockade with furosemide on the autoregulatory response in WT and KO mice. Furosemide (50 μM) was infused into the renal artery to inhibit the TGF response. This approach has been used extensively in the whole kidney and micropuncture models to block TGF-mediated responses (24, 32, 33, 36–45). In this series of experiments, diameter measurements were made 55 ± 2 μm and 48 ± 3 μm from the glomerulus in WT and KO mice, respectively. As shown in Figure 10, elevation of perfusion pressure from 100 to 140 mmHg produced an autoregulatory vasoconstriction of 15% from 13.6 ± 0.5 μm to 11.6 ± 0.6 μm ($n = 5$; $P \leq 0.05$) under control conditions in WT mice. Introduction of furosemide to the perfusate blood did not change base-line diameter significantly but markedly blunted pressure-mediated afferent arteriolar vasoconstriction. During TGF blockade, pressure-mediated afferent arteriolar vasoconstriction was significantly attenuated. Vessel diameter averaged 13.6 ± 0.5 μm and 14.8 ± 0.3 μm at 100 and 140 mmHg, respectively, in the presence of furosemide. These data are in strong agreement with the data in Figure 9 and demonstrate that blockade of TGF-mediated signals by papillectomy or furosemide inhibits pressure-mediated vasoconstriction in WT mice.

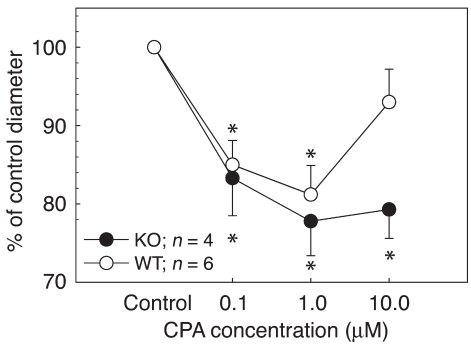

Figure 7
Afferent arteriolar responses to A_1 receptor stimulation with CPA in kidneys from $P2X_1$ KO mice (open circles; $n = 4$) and WT control mice (filled circles; $n = 6$). Data are expressed as a percentage of the control diameter. Each circle represents the mean diameter obtained during the last 2 minutes of each treatment period. *$P < 0.05$ vs. the diameter under the control conditions.

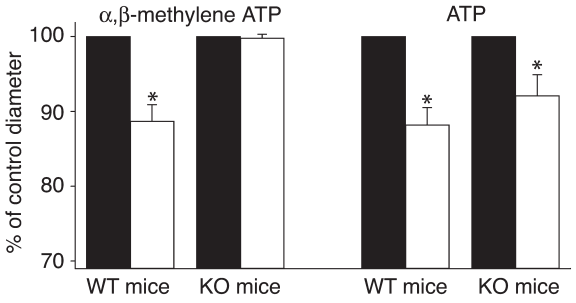

Figure 8
Afferent arteriolar responses to α,β-methylene ATP and ATP in $P2X_1$ KO mice and WT control mice. Data are expressed as a percentage of the control diameter. Each bar represents the mean diameter obtained from seven and thirteen observations under control conditions (black bars) and during exposure to 1.0 μM α,β-methylene ATP or ATP (white bars). *$P < 0.05$ vs. the respective control group.

In contrast, blockade of the TGF mechanism in $P2X_1$ KO mice ($n = 4$) had no effect on the response of afferent arterioles to elevation of perfusion pressure. Under control conditions, arteriolar diameter averaged 14.6 ± 1.2 μm and 15.2 ± 1.4 μm at 100 and 140 mmHg, respectively. During TGF blockade with furosemide, arterial diameter averaged 15.3 ± 1.1 μm and 15.6 ± 1.3 μm, respectively, over the same pressure range. Accordingly, no pressure-mediated autoregulatory responses were observed in mice lacking $P2X_1$ receptors, in marked contrast to the significant TGF-mediated vasoconstriction observed in WT control mice. These data indicate that deletion of the $P2X_1$ receptor results in ablation of the TGF response following elevation of perfusion pressure.

Discussion

The current report establishes a critical role for $P2X_1$ receptors in mediating pressure-dependent autoregulatory adjustments in afferent arteriolar diameter. This role has been detailed using selective $P2X_1$ receptor blockade, and by demonstrating that mice lacking $P2X_1$ receptors also exhibit impaired autoregulatory capability. This impairment of autoregulatory capability occurs despite retention of normal vasoconstrictor responses to Ang II, KCl, or A_1 receptor activation. In contrast, blockade of A_1 receptors eliminated adenosine-mediated vasoconstriction but did not alter afferent arteriolar autoregulatory responses or responsiveness to ATP. Ablation of the $P2X_1$ receptor, in gene-targeted KO mice, selectively eliminated afferent arteriolar vasoconstrictor responses to the $P2X_1$ agonist α,β-methylene ATP and markedly blunted pressure-mediated vasoconstrictor responses while retaining vasoconstrictor responses induced by A_1 receptor activation. Finally, papillectomy markedly attenuated pressure-mediated vasoconstriction in WT mice while having no detectable effect on afferent arterioles from mice lacking $P2X_1$ receptors.

Functional studies from our laboratory (7, 19, 20, 23) and others (16, 46) have established that afferent arterioles express P2X and P2Y receptors. While full characterization of the P2 receptor complement has not been completed, evidence strongly supports the presence of $P2X_1$ and $P2Y_2$ receptors on the preglomerular vascular smooth muscle (7, 19–23, 47, 48). In addition, Chan and coworkers clearly demonstrated the expression of $P2X_1$ receptor protein along the preglomerular vasculature (22). Functional evidence for $P2X_1$ receptor activation shows excellent correlation with immunohistochemical evidence for $P2X_1$ receptor distribution (7, 19, 21, 23, 47). P2X receptor activation and autoregulatory responses involve calcium channel activation (1, 23). Both responses can be blocked in afferent arterioles treated with calcium channel blockers (1, 23, 47). Therefore, the mechanisms known to be responsible for effecting autoregulatory adjustments in afferent arteriolar resistance can be accounted for by $P2X_1$ receptor activation.

As stated above, evidence suggests that preglomerular microvessels express more than just the $P2X_1$ receptor subtype. Previous work from our laboratory suggests that $P2Y_2$ receptors are also expressed, based on functional measures of vasoconstriction (7, 19, 23, 47) or calcium-signaling measurements in freshly isolated preglomerular smooth muscle cells (20). In rat kidney, the $P2X_1$ agonist α,β-methylene ATP is significantly more potent than ATP. ATP concentrations below 10 μM stimulate voltage-dependent calcium influx, whereas higher ATP concentrations are not inhibited by calcium-influx blockade (23). These observations suggest that low ATP concentrations preferentially activate $P2X_1$ receptors and that, as ATP concentrations rise, more P2 receptor subtypes become involved. While this may be true in the rat, there are no data defining the P2 receptor distribution in the mouse renal vasculature. The data in Figure 8 establish that mouse renal microvessels express $P2X_1$ receptors and that ablation of that receptor eliminates vasoconstriction induced by α,β-methylene ATP. These data also suggest that mouse

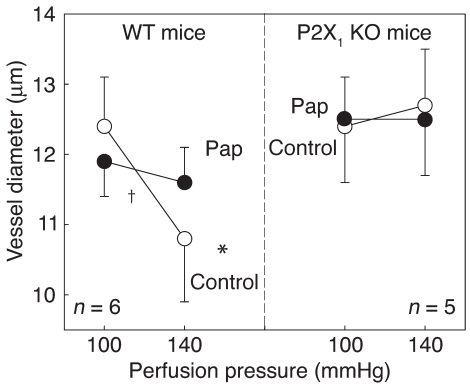

Figure 9
Effect of interruption of TGF by papillectomy on pressure-mediated vasoconstrictor responses in WT mice (left panel) and $P2X_1$ KO mice (right panel). Data are expressed as mean afferent arteriolar diameter before (open circles) and after (filled circles) papillectomy (Pap). *$P < 0.05$ vs. the diameter at 100 mmHg; †$P < 0.05$ vs. the change in diameter before papillectomy.

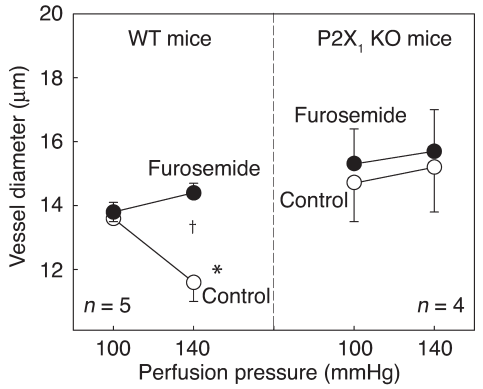

Figure 10
Effect of interruption of TGF with furosemide on pressure-mediated vasoconstrictor responses in WT mice (left panel) and $P2X_1$ KO mice (right panel). Data are expressed as mean afferent arteriolar diameter before (open circles) and during (filled circles) furosemide administration. $^*P < 0.05$ vs. the diameter at 100 mmHg; $^\dagger P < 0.05$ vs. the change in diameter before perfusion with furosemide.

renal microvessels also express another P2 receptor subtype that responds to ATP with a vasoconstriction. This begs the question of what role these receptors play in the regulation of renal microvascular function and what role, if any, they play in the autoregulatory response. Given that microvascular dose-response relationships to P2 receptor–selective agonists, P2 receptor expression profiles, or P2 receptor distribution profiles have not been generated for the mouse preglomerular vasculature, we cannot definitively explain the mechanism by which ATP vasoconstricts afferent arterioles of $P2X_1$ KO mice; but it should be noted that the expression of multiple P2 receptor subtypes by mouse microvessels is consistent with data indicating expression of multiple receptor subtypes in the rat kidney. Indeed, Vial and Evans have shown that multiple receptor subtypes are expressed on mesenteric arteries of $P2X_1$ KO mice (15). Therefore, the involvement of multiple P2 receptor subtypes in the physiological regulation of renal microvascular function may be more complicated than a single receptor subtype.

Earlier attempts to link P2 receptors with activation of autoregulatory responses support the current hypothesis (8, 9). The present studies extend those earlier findings by implicating the P2X receptor family in a causative role. Previous studies have shown that broad measures to inactivate P2X receptor signals all resulted in blockade of pressure-mediated autoregulatory responses (8, 9). P2 receptor desensitization, saturation, or pharmacological blockade with nonselective P2 receptor antagonists eliminated pressure-mediated afferent arteriolar vasoconstriction, without inhibiting arteriolar responses to P2 receptor–independent vasoconstrictor stimuli. P2 receptor saturation also blunts TGF responses (17). While those efforts strongly implicated P2 receptors as effectors of autoregulatory responses, the interventions were too nonspecific to allow identification of the receptor subtype involved.

Recently, NF279 was developed and characterized as a potent and selective antagonist of $P2X_1$ receptors (25–28), and it also may be effective against $P2X_7$ receptors (26). In addition, recent data generated in Xenopus oocytes expressing rat P2X receptors suggest that higher concentrations may inhibit $P2X_2$, $P2X_3$, and $P2X_4$ receptors (28). In in vitro expression systems, NF279 is approximately 90-fold more selective for $P2X_1$ receptors than for $P2X_3$ receptors and is ineffective against adenosine-sensitive A_1 receptors (27, 28). Therefore, experiments were performed to determine the effect of P2X receptor blockade on autoregulatory behavior. NF279 did not alter base-line arteriolar diameter but blocked the pressure-mediated vasoconstrictor responses and the sustained vasoconstriction induced by ATP and α,β-methylene ATP at concentrations consistent with P2X receptor activation (21, 23, 47). Importantly, NF279 did not alter the time course or the magnitude of the response to Ang II, CPA, or KCl. Furthermore, NF279 prevents the elevation of cytosolic calcium concentration by the $P2X_1$ agonist α,β-methylene ATP in freshly isolated preglomerular VSMCs (21). These data support the postulate that $P2X_1$ receptor activation leads to voltage-dependent calcium influx, resulting in agonist-induced afferent arteriolar vasoconstriction and autoregulatory adjustments in afferent arteriolar diameter. Furthermore, the data support the argument that autoregulatory adjustments in afferent arteriolar diameter are mediated through activation of NF279-sensitive $P2X_1$ receptors.

The ability of NF279 to inhibit the vasoconstrictor influence of ATP and α,β-methylene ATP was incomplete. As previously described, activation of P2 receptors with these agonists evoked a biphasic vasoconstriction characterized by a rapid initial response followed by a smaller, sustained response. NF279 completely abolished the sustained response while having little or no effect on the initial phase. This pattern of inhibition exhibits some similarity to that obtained with P2 receptor activation during calcium channel blockade (23). During blockade of L-type calcium channels with diltiazem, the sustained phase is also abolished whereas the initial phase is only attenuated (23). Therefore, experiments were performed to determine whether or not NF279 interfered with the activity of calcium channels. As noted in Figure 3, the afferent arteriolar vasoconstriction elicited by KCl-induced depolarization was unaltered during NF279 treatment. Therefore, blockade of the sustained phase of the vasoconstriction induced by ATP, α,β-methylene ATP, or increases in RPP cannot be attributed to impairment of calcium channel function. Alternatively, these data, combined with the results with Ang II and CPA, strengthen the argument that the ability of NF279 to inhibit autoregulatory responses results from selective blockade of $P2X_1$ receptor activation.

Provocative new data implicating A_1 receptors in the mediation of TGF responses have recently been published by two different groups (29, 30). TGF responses

represent one of the mechanisms involved in mediating autoregulatory behavior. In those studies, TGF responses were measured using micropuncture techniques in anesthetized mice lacking the A_1 receptor (29, 30). Both groups reported that TGF responses were abolished in the A_1 receptor–deficient mice, and either abolished (29) or unchanged (30) in heterozygote mice. These data make a strong case for an essential role for the A_1 receptor in TGF-dependent adjustments in preglomerular resistance.

To address the issue of A_1 receptor involvement in autoregulatory responses, experiments were performed to directly determine the effect of pharmacological blockade of A_1 receptors on pressure-mediated autoregulatory adjustments in afferent arteriolar diameter and responsiveness to ATP. In those experiments, adenosine-mediated vasoconstriction was abolished, but pressure-mediated autoregulatory responses and responsiveness to ATP or the $P2X_1$ receptor agonist α,β-methylene ATP remained intact. These data argue against a causative role for A_1 receptor activation in autoregulatory responses.

Using a similar approach, we assessed the effect of $P2X_1$ receptor deletion on the pressure-mediated autoregulatory responses of KO and WT mice. As shown in Figure 6, $P2X_1$ receptor deletion eliminated pressure-induced afferent arteriolar autoregulatory responses and responsiveness to the $P2X_1$ agonist α,β-methylene ATP and reduced the response to ATP. Pressure-mediated autoregulatory capability was absent, despite the fact that A_1 receptor activation remained a potent mechanism to induce vasoconstriction of afferent arterioles in kidneys from $P2X_1$ receptor KO mice. This absence argues against the necessity for functional A_1 receptors to be present to produce autoregulatory adjustments in preglomerular resistance. The responsiveness of afferent arterioles from A_1 KO mice to ATP is unclear. Therefore, the data presented in the current report reveal clear impairment of pressure-dependent autoregulatory behavior while verifying retention of normal responsiveness to adenosine and A_1 receptor activation, all in the KO mouse model. These important observations strongly support the postulate that acute increases in renal arterial pressure stimulate pressure-dependent autoregulatory responses through activation of preglomerular $P2X_1$ receptors.

KO mice responded to all concentrations of CPA with a vasoconstriction, whereas WT mice responded with vasoconstriction to concentrations up to 1 μM. At 10 μM, the diameter of afferent arterioles from WT mice began to increase. We do not have a reliable explanation for this dilatory response to a high concentration of CPA. Previous studies using the same preparation in the rat indicate that A_2 receptors are expressed by afferent arterioles (49). In addition, high concentrations of adenosine or 2-chloroadenosine have been shown to produce a similar biphasic concentration response profile in rat afferent arterioles (49–51). CPA has been reported to interact with A_2 receptors in cultured renal epithelial cells (52). Thus, it is possible that 10 μM CPA is interacting with A_2 receptors expressed by the mouse microvasculature; however, currently there are no data documenting the expression profiles for adenosine receptors in mouse kidney. It is also possible that the dilation reflects activation of other vasodilatory mechanisms such as stimulation of K channels (53–55).

TGF-dependent control of preglomerular resistance involves communication between the macula densa of the distal nephron and the afferent arteriole. There is general agreement that this mechanism is an important contributor to the regulation of renal hemodynamics and glomerular filtration pressure, but identification of the signaling molecule remains an elusive target. The TGF mechanism adjusts afferent arteriolar diameter in response to inappropriate changes in distal tubular fluid composition or flow. Modulation of vasoconstrictor tone is used as a means of regulating glomerular filtration pressure and thus regulates tubular fluid flow and composition. In the current report, efforts were made to determine the potential role of the $P2X_1$ receptor in TGF-dependent control of afferent arteriolar diameter in WT and $P2X_1$ receptor KO mice. In these studies, acute papillectomy was performed to interrupt distal tubular fluid flow and thus minimize the contribution of TGF-dependent influences on afferent arteriolar resistance. This approach has been used successfully in the rat kidney but has not yet been applied to the mouse kidney (32–35). Afferent arteriolar diameter did not change significantly in either group following acute papillectomy, suggesting that TGF may not exert a significant vasoconstrictor influence on juxtamedullary afferent arterioles at a renal arterial pressure of 100 mmHg. Following papillectomy, the autoregulatory response of kidneys from WT mice was significantly attenuated by approximately 77% compared with paired control responses, whereas no further attenuation in the response to increased perfusion pressure was noted in kidneys from $P2X_1$ KO mice.

We obtained nearly identical results using an alternative means of inhibiting TGF-dependent signaling between the macula densa and the afferent arteriole. Inhibition of TGF signals with furosemide has been used by Schnermann et al., in a micropuncture setting, to investigate TGF influences on stop-flow pressure (36, 37, 40, 41, 45). Furosemide treatment has also been used by other investigators to assess TGF-dependent effects in whole kidney and juxtamedullary nephron preparations (24, 32, 33, 38, 39, 42–44). In the current report, furosemide treatment significantly attenuated pressure-mediated reductions in afferent arteriolar diameter in WT mice and had no effect on afferent arteriolar responses in $P2X_1$ KO mice. The pattern of attenuation in WT mice was nearly identical to the furosemide-mediated attenuation in rat-kidney autoregulatory responses reported by Moore and Casellas (43). In that report, pressure-mediated autoregulatory responses were attenuated by approximately 50% during furosemide treatment, consistent

with the effects of papillectomy and furosemide shown in Figures 9 and 10 for WT mice. These observations suggest that TGF-dependent influences are already absent in $P2X_1$ KO mice.

These data provide several exciting insights. TGF contributes a significant component of vasoconstrictor tone to afferent arterioles from WT mice but has no detectable vasoconstrictor influence in $P2X_1$ KO mice. This difference does not reflect an absence of active tension, given that removal of extracellular calcium, by the introduction of EGTA, produces a comparable 25–27% increase in diameter in both WT and $P2X_1$ receptor KO mice. Increasing perfusion pressure results in an additional 6–7% increase in diameter, consistent with passive arteriolar behavior. Indeed, the papillectomy data and the furosemide data imply that juxtamedullary afferent arterioles of $P2X_1$ KO mice are not subject to detectable TGF-dependent vasoconstrictor influences. Accordingly, these data are consistent with the postulate that $P2X_1$ receptors are necessary for macula densa signals to produce TGF-dependent vasoconstriction.

Several issues must be considered in trying to reconcile observations implicating A_1 receptors versus $P2X_1$ receptors in autoregulatory behavior. The present studies were conducted in vitro in isolated perfused rat and mouse kidneys, focused on juxtamedullary nephrons, and viewed adjustments in arteriolar diameter as the endpoint of autoregulatory behavior. The A_1 KO studies were performed in vivo in mice with micropuncture of superficial nephrons and used stop-flow pressure to represent TGF-dependent adjustments of preglomerular resistance. Therefore, two distinctly different nephron populations were studied, using markedly different experimental approaches to examine different aspects of the overall response. Developmental issues must also be considered. The roles played by adenosine and Ang II in renal development are not well characterized. Genetic knockout of selected genes could have important effects on tissue development and/or organ function. Accordingly, it is important to note that separate ablation of either AT_{1A} receptor expression or A_1 receptor expression abolished TGF responses and responsiveness to adenosine (56, 57). These data could signify that a critical interaction between Ang II and adenosine occurs in the development or operation of TGF signaling pathways, or that local, nonspecific abnormalities occur as a result of the deletion of genes important for kidney development and renal function. Similarly, $P2X_1$ receptor deletion eliminates TGF-dependent autoregulatory responses. This could also reflect as-yet unappreciated developmental issues. This caveat applies to all KO models where the gene is absent throughout development and life.

To our knowledge, the data in the current report are the only data that bear on the renal physiological behavior of the $P2X_1$ KO mouse. Previous studies have focused on the impact of $P2X_1$ receptor deletion on aspects of $P2X_1$ receptor function in vas deferens, mesenteric arteries, urinary bladder, reproductive tissues, and thrombogenic stimuli (15, 31, 58–60). The effects on vas deferens are probably the best documented. The $P2X_1$ receptor is very important for neurogenic contractile responses of the vas deferens during ejaculation. Accordingly, $P2X_1$ receptor deletion markedly reduces male fertility by preventing delivery or sperm in the ejaculate (31). Nerve-stimulated urinary bladder contraction is attenuated in $P2X_1$ KO mice compared with WT controls, consistent with $P2X_1$ receptors playing an important role in neurogenic regulation of bladder function (12, 59). Considerable work needs to be done to more fully explain the impact of $P2X_1$ receptor deletion on renal hemodynamic and excretory function.

In summary, this study provides compelling new evidence for $P2X_1$ receptor involvement in mediating pressure-dependent autoregulatory adjustments in afferent arteriolar diameter. This conclusion is reached through studies performed in two species using normal rat kidneys, as well as through $P2X_1$ receptor KO studies using mouse kidneys. Blockade of P2X receptors with NF279 selectively abolished autoregulatory responses to increases in RPP and the afferent arteriolar vasoconstriction induced by ATP or α,β-methylene ATP. In contrast, the afferent arteriolar vasoconstriction induced by Ang II, KCl, and the A_1 agonist CPA were completely unaltered by $P2X_1$ receptor blockade or $P2X_1$ receptor deletion. A_1 receptor blockade had no detectable effect on autoregulatory responses or ATP-mediated afferent arteriolar vasoconstriction. Selective deletion of $P2X_1$ receptors in KO mice eliminates pressure-dependent autoregulatory behavior and responsiveness to the $P2X_1$ agonist α,β-methylene ATP, while retaining responsiveness to A_1 receptor activation. Therefore, the data presented here strongly support the hypothesis that $P2X_1$ receptor activation plays a critical role in mediating autoregulatory adjustments in resistance and implicate endogenously released ATP as the chemical mediator responsible for autoregulatory behavior and perhaps TGF-mediated adjustments in preglomerular resistance.

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