Calcium signaling pathways utilized by P2X receptors in freshly isolated preglomerular MVSMC

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Received 20 June 2000; accepted in final form 19 February 2001

White, Steven M., John D. Imig, Thu-Thuy Kim, Benjamin C. Hauschild, and Edward W. Inscho. Calcium signaling pathways utilized by P2X receptors in freshly isolated preglomerular MVSMC. Am J Physiol Renal Physiol 280: F1054–F1061, 2001.—This study tested the hypothesis that P2X receptor activation increases intracellular Ca2⁺ concentration ([(Ca²⁺)]ₐ) in preglomerular microvascular smooth muscle cells (MVSMC) by evoking voltage-dependent calcium influx. MVSMC were obtained and loaded with the calcium-sensitive dye fura 2 and studied by using single-cell fluorescence microscopy. The effect of P2X receptor activation on [(Ca²⁺)]ₐ was assessed by using the P2X receptor-selective agonist α,β-methylene-ATP and was compared with responses elicited by the endogenous P2 receptor agonist ATP. α,β-Methylene-ATP induced a transient increase in [(Ca²⁺)]ₐ, dose dependently. Peak increases in [(Ca²⁺)]ₐ averaged 37 ± 11, 73 ± 15, and 103 ± 21 nM at agonist concentrations of 0.1, 1, and 10 μM, respectively. The average peak response elicited by 10 μM α,β-methylene-ATP was ~34% of the response obtained with 10 μM ATP. α,β-Methylene-ATP induced a transient increase in [(Ca²⁺)]ₐ before [(Ca²⁺)]ₐ returned to baseline, whereas ATP induced a biphasic response including a peak response followed by a sustained plateau. In Ca²⁺+-free medium, ATP induced a sharp transient increase in [(Ca²⁺)]ₐ, whereas the response to α,β-methylene-ATP was abolished. Ca²⁺⁺ channel blockade with 10 μM diltiazem or nifedipine attenuated the response to α,β-methylene-ATP, whereas nonspecific blockade of Ca²⁺⁺ influx pathways with 5 mM Ni²⁺ abolished the response. Blockade of P2X receptors with the novel P2X receptor antagonist NF-279 completely but reversibly abolished the response to α,β-methylene-ATP. These results indicate that P2X receptor activation by α,β-methylene-ATP [(Ca²⁺)]ₐ, in preglomerular MVSMC, in part, by stimulating voltage-dependent Ca²⁺⁺ influx through L-type Ca²⁺⁺ channels.

Microvascular smooth muscle cells; adenosine 5’-triphosphate; afferent arteriole; renal microvasculature; 8,8’-[(carbonylimino)bis(1,3,5-napthalenetrisulfonic acid hexasodium salt); α,β-methylene-adenosine 5’-triphosphate; nifedipine; diltiazem; nickel; calcium channels; P2X receptors

EXTRACELLULAR ATP HAS BEEN SHOWN to be an important paracrine regulator of renal epithelial and preglomerular microvascular function (21, 24, 26, 30–33). ATP induces vasoconstriction by activating P2 receptors on preglomerular microvascular smooth muscle cells (MVSMC) (14, 20, 26). This family of P2 receptors is divided into two major groups, classified as P2X and P2Y receptor subtypes (1, 16, 35). Previous studies from our laboratory have shown that inactivation of P2 receptors on preglomerular microvessels inhibits autoregulatory behavior (21, 30, 32). Activation of P2X and P2Y receptors on MVSMC stimulates an increase in intracellular calcium concentration ([(Ca²⁺)]ₐ), by distinct calcium signaling pathways (22, 27). P2X receptors function as ligand-gated, transmembrane cation channels that allow influx of extracellular cations, including calcium (1, 12, 13, 15, 16, 35). In contrast, P2Y receptors are coupled to G proteins and increase [(Ca²⁺)]ₐ, in part, by stimulating mobilization of calcium from intracellular stores (1, 12, 13, 16, 35).

Although the capacity of the kidney to autoregulate renal blood flow has been recognized for many years, the mechanisms by which renal autoregulation occurs remain unclear. Certainly, autoregulatory responses are accomplished through myogenic and tubuloglomerular feedback (TGF)-mediated adjustments in preglomerular resistance (3). TGF is believed to be a major regulatory system coupling changes in distal tubular flow with preglomerular resistance through the actions of the macula densa. We have proposed that ATP, released from the macula densa, serves as the chemical messenger linking the macula densa with regulation of afferent arteriolar resistance through ATP-dependent activation of P2X receptors that are heavily expressed along the preglomerular but not the postglomerular microvasculature (7, 21, 32, 33). This hypothesis is derived from the striking similarities between ATP-mediated afferent arteriolar vasoconstriction and pressure-mediated autoregulatory adjustments in afferent arteriolar diameter. Both stimuli alter afferent arteriolar diameter with similar temporal profiles (21). Both stimuli rely on calcium influx through voltage-gated calcium channels (22, 25, 33). Finally, inactivation of P2 receptors inhibits autoregulatory adjustments in

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afferent arteriolar diameter in response to increasing renal perfusion pressure (21, 30) or increasing distal tubular perfusion (32).

The purpose of this study was to evaluate the calcium signaling pathways involved in the preglomerular smooth muscle response to P2X receptor activation. Freshly isolated MVSMC were exposed to the selective P2X agonist \(\alpha,\beta\)-methylene-ATP. Studies were designed to establish the calcium signaling pathways used by P2X receptors by evaluating the role of extra-cellular calcium and L-type calcium channels in the response to P2X receptor activation. Additional studies were performed to determine the effect of P2X receptor blockade on the response to \(\alpha,\beta\)-methylene-ATP. Responses elicited by the P2X receptor agonist were compared with responses evoked by the endogenous ligand ATP, which activates both P2X and P2Y receptors.

**METHODS**

**Tissue Preparation and Renal MVSMC Isolation**

All studies were performed in compliance with the guidelines and practices dictated by the Tulane University Advisory Committee for Animal Resources. Suspensions of MVSMC were prepared as previously described (22). For each suspension of MVSMC \((n = 33)\), one male Sprague-Dawley CD-VAF rat \((250 \text{ to } 375 \text{ g}; \text{Charles River Laboratories}; \text{Wilmington, MA})\) was anesthetized with pentobarbital sodium \((40 \text{ mg/kg ip})\), and the abdominal aorta was cannulated via the superior mesenteric artery. Ligatures were placed around the abdominal aorta at sites proximal and distal to the renal arteries. The kidneys were cleared of blood and distal to the renal arteries. The kidneys were removed and decapsulated, and the cortical tissue was removed. The renal medullary tissue was removed. The cortical tissue was sieved \((180-\mu m \text{ mesh})\), and the retenate was washed with ice-cold low-calcium PSS. The vascular tissue remaining on the sieve was transferred to an enzyme solution containing 0.075% collagenase \((\text{Boehringer Mannheim, Indianapolis, IN})\), 0.02% dithiothreitol \((\text{Sigma, St. Louis, MO})\), 0.2% soybean trypsin inhibitor \((\text{type 1-S, Sigma})\), and 0.1% BSA dissolved in low-calcium PSS and incubated for 30 min at 37°C. The vascular tissue was transferred to a nylon mesh \((70-\mu m \text{ mesh})\) and washed with ice-cold low-calcium PSS. The retained vascular tissue was transferred to a petri dish containing ice-cold low-calcium PSS for collection of interlobular arteries with attached afferent arterioles. The vascular segments were placed in a solution containing 0.075% papain \((\text{Sigma})\) and 0.02% dithiothreitol in low-calcium PSS. The tissue was incubated at 37°C for 15 min and centrifuged \((2,000 \text{ g for 50 s})\), and the tissue pellet was transferred to a solution containing 0.3% collagenase and 0.2% soybean trypsin inhibitor in low-calcium PSS at 37°C. After 15 min, the mixture was triturated and centrifuged \((500 \text{ g for 5 min})\). The cell pellet was resuspended in 1 ml Dulbecco’s minimum essential medium \((\text{Sigma})\) supplemented with 20% fetal calf serum \((\text{Whittaker Bioproducts, Walkerville, MD})\), 100 U penicillin, and 200 \(\mu g \text{ streptomycin} \,(\text{Sigma})\). Cell suspensions were stored on ice until used.

**Fluorescence Measurements in Single MVSMC**

Experiments were performed by using standard microscope-based fluorescence spectrophotometry techniques \((\text{Photon Technology, Lawrenceville, NJ})\) as previously described (22, 23). The excitation wavelengths were set at 340 and 380 nm, and the emitted light was collected at 510 ± 20 nm. Fluorescence intensity was collected \((5 \text{ data points/s})\) and analyzed with the aid of Photon Technology software. Calibration of the fluorescence data was accomplished in vitro according to the method used by Grynkiewicz et al. (19).

Measurement of \([Ca^{2+}]_i\), in single MVSMC was performed as described previously (22, 23, 27). Suspensions of MVSMC were loaded with fura 2-acetoxymethyl ester \((\text{fura 2-AM}; 10 \mu M; \text{Molecular Probes, Eugene, OR})\), and an aliquot of cell suspension was transferred to the perfusion chamber \((\text{Warner Instrument, Hamden, CT})\), and the chamber was mounted to the stage of a Nikon Diaphot inverted microscope. The cells were superfused at 35°C with a control PSS solution of the following composition \((\text{mM})\): 125 NaCl, 5 KCl, 1 NaH2PO4, 20 HEPES, 1.8 CaCl2, and 0.1 g/l BSA.

For each experiment, fluorescence data were collected from a single cell after background subtraction. A new coverslip was used for each experiment.

**Experimental Approach**

**Series 1.** MVSMC were exposed to \(\alpha,\beta\)-methylene-ATP to determine the effect of P2X receptor activation on \([Ca^{2+}]_i\). At the concentrations used here, \(\alpha,\beta\)-methylene-ATP is selective for the P2X1 and P2X2 purinoceptor subtypes (35). Concentration-response data were obtained by exposing MVSMC to PSS solutions containing \(\alpha,\beta\)-methylene-ATP concentrations of 0.1, 1, and 10 \(\mu M\). Fura 2 fluorescence was monitored in these cells under control conditions \((0-100 \text{ s})\) during exposure to \(\alpha,\beta\)-methylene-ATP \((100-300 \text{ s})\) and during the recovery period, during which \(\alpha,\beta\)-methylene-ATP was removed from the bathing solution \((300-600 \text{ s})\). Agonist-mediated responses were evaluated by determining the magnitude of the peak and late-phase \([Ca^{2+}]_i\) achieved. Peak responses were defined as the maximum \([Ca^{2+}]_i\) attained in the first 150 s of agonist exposure. Sustained responses were calculated by averaging \([Ca^{2+}]_i\) over the final 50 s of agonist exposure. Similar experiments were performed with 10 \(\mu M\) ATP to obtain control data for the endogenous ligand.

**Series 2.** Studies were performed to determine the role of extracellular calcium on the increase in \([Ca^{2+}]_i\), induced by \(\alpha,\beta\)-methylene-ATP. The contribution of calcium influx to the response was determined by exposing single cells to 10 \(\mu M\) \(\alpha,\beta\)-methylene-ATP while they were being bathed in nominally calcium-free PSS (22, 23). Previous studies have shown that \([Ca^{2+}]_i\) remains unchanged when MVSMC are subjected to strong depolarizing conditions while being bathed in calcium-free PSS (23). Fura 2 fluorescence was monitored in these cells under control conditions \((0-100 \text{ s})\), during exposure to calcium-free PSS \((100-150 \text{ s})\), and during subsequent exposure to \(\alpha,\beta\)-methylene-ATP \((150-350 \text{ s})\). These responses were compared with responses obtained from similar cells challenged in normal-calcium PSS. Additional control cells were studied under identical conditions, except that these cells were challenged with 10 \(\mu M\) ATP, and the responses were compared with those obtained with \(\alpha,\beta\)-methylene-ATP.

**Series 3.** The contribution of calcium influx to the MVSMC response to \(\alpha,\beta\)-methylene-ATP was further evaluated under conditions in which the extracellular calcium concentration remained within the physiological range. For these experiments, cells were challenged with \(\alpha,\beta\)-methylene-ATP while
response was further suppressed to 20 nM (a calcium channel blockade. from similar cells challenged in normal-calcium PSS without These responses were compared with responses obtained between the calcium channel antagonists and P2X receptors. 

10 mM nifedipine. Fura 2 fluorescence was monitored in these being bathed in a PSS solution containing 5 mM Ni2+. Ni2+ was used as a nonselective calcium channel antagonist (2). Fura 2 fluorescence was monitored in these cells under control conditions (0–100 s), during exposure to 5 mM Ni2+ in the presence of 1.8 mM Ca2+ (100–150 s) and during subsequent exposure to α,β-methylene-ATP in combination with Ni2+ and normal Ca2+ (150–350 s). These responses were compared with responses obtained from similar cells challenged in normal-calcium PSS without added Ni2+.

Series 4. Additional experiments were performed to assess the role of L-type calcium channels in the MVSMC response to α,β-methylene-ATP. For these experiments, cells were challenged with α,β-methylene-ATP while being bathed in a PSS solution containing the L-type calcium channel antagonists diltiazem or nifedipine. Previous studies have established that diltiazem is an effective inhibitor of ATP- and KCl-mediated increases in [Ca2+], in these cells (22, 23). Control studies were performed to verify the ability of 10 μM nifedipine to block the increase in [Ca2+]i induced by KCl. Exposure of cells to 90 mM KCl resulted in a peak change in [Ca2+]i in microvascular smooth muscle cells (MVSMC). Characteristic responses to 0.1 (A), 1 (B), and 10 μM (C) α,β-methylene-ATP are shown.

Series 5. Previous studies suggest that pregglomerular vasoconstrictor responses to α,β-methylene-ATP may be mediated through activation of P2X receptors (10, 14, 18, 32). Therefore, we employed a novel, selective P2X receptor antagonist [8,8′-carbonylbis[(iminio-4,1-phenylencarbonylimino-4,1-phenylencarbonylimino)bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt; NF-279; Tocris Cookson] to assess the involvement of P2X receptors in the MVSMC response to α,β-methylene-ATP (11, 29). For these experiments (n = 23), fura 2 fluorescence was monitored under control conditions (0–100 s), during exposure to 20 μM NF-279 (100–300 s), and during subsequent exposure to α,β-methylene-ATP in combination with NF-279 (300–400 s).

Five cells were subjected to a washout period of 300 s to remove NF-279 and α,β-methylene-ATP from the bath. After the washout period, cells were exposed to 10 μM α,β-methylene-ATP a second time.

Statistical Analysis

Data are presented as means ± SE. Within-group comparisons of peak [Ca2+]i with baseline [Ca2+]i were analyzed using ANOVA for repeated measures. Differences in baseline [Ca2+]i, and steady-state [Ca2+]i, between treatment groups were analyzed by ANOVA. Post hoc tests were performed using Tukey’s test. Statistical probabilities of <0.05 (P < 0.05) are considered significantly different.

RESULTS

Experiments were performed to establish the concentration-response profile for α,β-methylene-ATP. Figure 1 presents representative traces depicting the changes in [Ca2+]i elicited by increasing concentrations (0.1, 1, and 10 μM) of α,β-methylene-ATP. Exposure of MVSMC to α,β-methylene-ATP evoked a concentration-dependent increase in [Ca2+]i, that typically included a rapid peak response followed by a gradual return to steady-state levels similar to baseline. Figure 2 presents the average responses in series 1 experiments. Baseline [Ca2+]i was similar across all three treatment groups. The peak [Ca2+]i elicited by each concentration of α,β-methylene-ATP was significantly different from baseline and averaged 37 ± 11, 73 ± 15, and 103 ± 21 nM, respectively. In contrast, the steady-state [Ca2+]i was not significantly different from the respective baseline [Ca2+]i at each α,β-methylene-ATP concentration tested.

Figure 3 shows typical traces for cells treated with 10 μM α,β-methylene-ATP (A) and 10 μM ATP (B).
Resting \([Ca^{2+}]_i\), averaged 99 ± 6 nM for cells treated with \(\alpha,\beta\)-methylene-ATP (n = 49 cells) and 77 ± 4 nM for the ATP-treated cells (n = 31 cells). The peak \([Ca^{2+}]_i\), achieved by cells treated with \(\alpha,\beta\)-methylene-ATP averaged 180 ± 17 nM, which was significantly lower than the peak \([Ca^{2+}]_i\), attained in cells treated with ATP (315 ± 39 nM). The temporal pattern of the response to \(\alpha,\beta\)-methylene-ATP is different from responses elicited by ATP. The average response elicited by \(\alpha,\beta\)-methylene-ATP includes a rapid rise in \([Ca^{2+}]_i\), but the peak response is followed by a more rapid decline in \([Ca^{2+}]_i\). The typical ATP response also includes a rapid increase in \([Ca^{2+}]_i\), to a peak value, followed by a sustained plateau phase sometimes exhibiting periods of \([Ca^{2+}]_i\) oscillation (Fig. 3B). \([Ca^{2+}]_i\) returns to baseline after ATP is removed from the bathing medium. The magnitude of the steady-state \([Ca^{2+}]_i\), averaged 10 ± 2 nM above the baseline (P < 0.05) for cells treated with \(\alpha,\beta\)-methylene-ATP and 32 ± 6 nM for cells treated with ATP. The magnitude of the steady-state \([Ca^{2+}]_i\) in \(\alpha,\beta\)-methylene-ATP-treated cells is significantly smaller (P < 0.05) than for cells treated with ATP.

Previous studies have shown that ATP increases \([Ca^{2+}]_i\) in MVSMC by stimulating \(Ca^{2+}\) influx from the extracellular medium and by mobilization of \(Ca^{2+}\) from intracellular stores (22, 27). Experiments were performed to compare the contribution of extracellular \(Ca^{2+}\) to the increase in \([Ca^{2+}]_i\), stimulated by \(\alpha,\beta\)-methylene-ATP and ATP. Typical responses to \(\alpha,\beta\)-methylene-ATP and ATP are presented in Fig. 4. Figure 4A shows the response of a single cell to 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP while the cell was being bathed in \(Ca^{2+}\)-free medium. Removal of \(Ca^{2+}\) from the extracellular medium abolished the response to \(\alpha,\beta\)-methylene-ATP. \([Ca^{2+}]_i\) averaged 108 ± 15 nM in normal-calcium buffer and 108 ± 15 nM during exposure to calcium-free conditions (n = 12 cells). During exposure to 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP, peak and steady-state \([Ca^{2+}]_i\), averaged 124 ± 15 and 91 ± 11 nM, respectively. These \([Ca^{2+}]_i\) values are not significantly different from those for control or calcium-free \([Ca^{2+}]_i\). Control cells (n = 5) challenged with 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP in normal-calcium conditions exhibited typical increases in \([Ca^{2+}]_i\) from a baseline of 101 ± 12 to peak and steady-state \([Ca^{2+}]_i\), of 183 ± 40 and 97 ± 10 nM, respectively. Figure 4B shows the response of a single cell to 10 \(\mu\)M ATP while the cell was being bathed in calcium-free medium. In contrast to the effect of calcium removal on the response elicited by \(\alpha,\beta\)-methylene-ATP, cells treated with ATP exhibited significant increases in \([Ca^{2+}]_i\). For the cells in this treatment group (n = 13), the baseline \([Ca^{2+}]_i\) averaged 90 ± 5 nM under control conditions and 91 ± 5 nM when cells were exposed to calcium-free medium. Subsequent exposure to 10 \(\mu\)M ATP induced a sharp increase in \([Ca^{2+}]_i\) to a peak value of 348 ± 47 nM before a rapid decline to 89 ± 5 nM. In contrast, paired control cells (n = 13) exposed to 10 \(\mu\)M ATP in normal-calcium medium exhibited an increase in \([Ca^{2+}]_i\), from a baseline of 76 ± 5 nM to a peak of 455 ± 95 nM, before the level stabilized to a concentration of 88 ± 5 nM.

P2X receptor activation involves opening a ligand gated cation channel that directly increases \([Ca^{2+}]_i\), and causes membrane depolarization (15, 35). Therefore, studies were performed to assess the \(Ca^{2+}\) influx pathways involved in the MVSMC response to \(\alpha,\beta\)-methylene-ATP. Cells were exposed to \(\alpha,\beta\)-methylene-ATP while being bathed in control buffer containing 1.8 mM \(Ca^{2+}\) plus either \(Ni^{2+}\), diltiazem, or nifedipine. As shown in the representative traces presented in Fig. 5, 5 mM \(Ni^{2+}\) (A) and 10 \(\mu\)M diltiazem (B) blocked attenuated the response of these cells to 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP, respectively. In \(Ni^{2+}\)-treated cells (n = 11), \([Ca^{2+}]_i\), averaged 119 ± 5 and 117 ± 5 nM during exposure to control and \(Ni^{2+}\)-containing solutions, respectively, and remained unchanged when challenged with 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP. Similarly, in diltiazem-treated cells (n = 16), \([Ca^{2+}]_i\), averaged 122 ± 9 and 117 ± 11 nM during exposure to control

Fig. 3. Response of \([Ca^{2+}]_i\) to \(\alpha,\beta\)-methylene-ATP and ATP in the presence of 1.8 mM extracellular calcium. A: a typical response of a MVSMC exposed to 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP while the cell was being bathed in normal-calcium medium. B: a typical response of a similar cell to exposure to 10 \(\mu\)M ATP. Solid horizontal bars, periods of exposure to \(\alpha,\beta\)-methylene-ATP or ATP administration.

Fig. 4. Response of \([Ca^{2+}]_i\) to \(\alpha,\beta\)-methylene-ATP and ATP in the absence of extracellular calcium. A: a typical response of a MVSMC to 10 \(\mu\)M \(\alpha,\beta\)-methylene-ATP while the cell was being bathed in calcium-free medium. B: a typical response of a MVSMC to 10 \(\mu\)M ATP while the cell was being bathed in calcium-free medium. Solid horizontal bars, periods of exposure to calcium-free medium and to \(\alpha,\beta\)-methylene-ATP or ATP administration.
and diltiazem-containing solutions, respectively. Subsequent exposure to 10 μM α,β-methylene-ATP increased [Ca\(_{2+}\)]\(_i\) by only 27 ± 9 nM. Similarly, in nifedipine-treated cells (n = 15), exposure to α,β-methylene-ATP increased [Ca\(_{2+}\)]\(_i\) by only 41 ± 7 nM. The peak responses observed in the presence of diltiazem and nifedipine are significantly smaller than control responses (P < 0.05).

Presently, there are approximately seven P2X receptor subtypes that have been cloned and expressed (15, 16, 35). Evidence has shown that the P2X\(_1\) receptor subtype is heavily expressed along the preglomerular microvasculature (7), and α,β-methylene-ATP is known to be a potent agonist of P2X\(_1\) receptors (15, 16, 35). Therefore, we tested the hypothesis that α,β-methylene-ATP elevates [Ca\(_{2+}\)]\(_i\) in MVSMC by activation of P2X receptors. In these experiments, cells were pre-treated with the novel P2X receptor antagonist NF-279 before and during exposure to α,β-methylene-ATP. In five experiments, cells were challenged with α,β-methylene-ATP in the presence of NF-279; the bath solution was then switched to the control buffer, and the cells were washed for 300 s. The washed cells were exposed a second time to α,β-methylene-ATP but this time without the P2X receptor blocker. A typical example of one of these experiments is shown in Fig. 6. Preincubation with NF-279 abolished the increase in [Ca\(_{2+}\)]\(_i\) normally observed in response to α,β-methylene-ATP. In contrast, removal of NF-279 from the bathing medium resulted in an increase in [Ca\(_{2+}\)]\(_i\) when the cell was challenged a second time with α,β-methylene-ATP. This observation verifies the responsiveness of the cells and confirms the effectiveness of the P2X receptor blockade with NF-279. Analysis of data collected from 23 cells demonstrates that [Ca\(_{2+}\)]\(_i\) averaged 65 ± 3 and 66 ± 3 nM during the control and NF-279 period, respectively. Consistent with the example shown in Fig. 6, the [Ca\(_{2+}\)]\(_i\) averaged 74 ± 3 and 68 ± 3 nM during the peak and steady-state periods of exposure to α,β-methylene-ATP. These [Ca\(_{2+}\)]\(_i\) values are not significantly different from those for the control and NF-279 periods.

**DISCUSSION**

Renal hemodynamic control is accomplished by local adjustments in intrarenal vascular resistance (3). The majority of these resistance adjustments are preglomerular and occur at the level of the afferent arterioles (3). Numerous neural, humoral, and paracrine agents have been shown to exert some influence on renal vascular resistance (3, 33). Recently, interest has turned to the potential involvement of extracellular nucleotides as physiological regulators of renal vascular resistance (8, 24, 30, 33, 38). We and others have shown that exposure of the renal vasculature to extracellular nucleotides results in rapid and reversible alterations in renal vascular resistance, renal microvascular diameter, and renal perfusion (9, 14, 24–26, 30, 38, 39). Other studies have begun to investigate the intracellular signaling pathways involved in the preglomerular smooth muscle response to P2X receptors known to be expressed by MVSMC (7).

P2 receptors were first defined by Burnstock (5) in 1978. Since then, P2X receptors have grown into a large family of receptors divided into two basic categories (1, 6, 16, 17, 35). P2X receptors are described as ligand-gated channels whereas P2Y receptors are G protein-regulated receptors (1, 35). Previous studies have shown that renal microvascular and MVSMC responses to P2 receptor stimulation with ATP involve the activation of both P2X and P2Y receptor subtypes (20, 22). In addition, evidence suggests that each receptor type activates different calcium signaling pathways (22). The present report focuses on the calcium signaling pathways involved in the MVSMC response.

![Fig. 5. Response of [Ca\(_{2+}\)]\(_i\) to α,β-methylene-ATP during nonselective blockade of calcium influx pathways with Ni\(_{2+}\) or to blockade of L-type calcium channels with diltiazem. A: a typical response of a cell to 10 μM α,β-methylene-ATP in the presence of 1.8 mM extracellular calcium and 5 mM Ni\(_{2+}\); B: a typical response of a cell to 10 μM α,β-methylene-ATP in the presence of 1.8 mM extracellular calcium and 10 μM diltiazem. Solid horizontal bars: periods of exposure to Ni\(_{2+}\) and diltiazem (100–500 s) and to α,β-methylene-ATP in combination with Ni\(_{2+}\) or diltiazem (200–400 s).](image)

![Fig. 6. Effect of P2X receptor blockade on the [Ca\(_{2+}\)]\(_i\) response to α,β-methylene-ATP. A typical response of a MVSMC to α,β-methylene-ATP is shown during treatment with the P2X\(_1\)-selective receptor antagonist NF-279 (20 μM). Solid horizontal bars: periods of exposure to NF-279 (100–500 s), NF-279 + α,β-methylene-ATP (300 to 500 s), and α,β-methylene-ATP alone (800–1,000 s).](image)
to P2X receptor activation with the P2 agonist α,β-methylene-ATP. This stable ATP analog is reported to be selective for P2X, P2X3, P2X5, and P2X6 receptors (15, 35) and is either inactive or requires concentrations in excess of 100 μM to activate different splice variants of the P2X2 receptor (4, 37). α,β-Methylene-ATP is also a very poor agonist of P2Y receptors (35). Immunohistochemical studies have shown the P2X1 receptor to be highly expressed along the preglomerular microvasculature (7) but not the postglomerular microvasculature. Interestingly, the microvascular segments that stain positively for P2X1 receptors also vasoconstrict when exposed to ATP or α,β-methylene-ATP (26).

Stimulation of P2X receptors activates an inwardly directed nonselective cation current, which can contribute to the elevation of [Ca2+]i (1, 12, 13, 15, 34, 35). The present studies were performed to test the hypothesis that exposure of MVSMC to α,β-methylene-ATP would result in an elevation of [Ca2+]i, through activation of calcium influx pathways. The data demonstrate that P2X receptor activation with α,β-methylene-ATP results in a concentration-dependent elevation of [Ca2+]i. Furthermore, the magnitude and time course of the response to α,β-methylene-ATP are markedly different from those evoked by an equimolar concentration of ATP. α,β-Methylene-ATP increased [Ca2+]i by ~84% whereas an identical concentration of ATP increased [Ca2+]i by 309%. The response to α,β-methylene-ATP was transient whereas the response to ATP exhibited a sustained elevation of [Ca2+]i. These data demonstrate that both ATP and α,β-methylene-ATP increase [Ca2+]i, but suggest that the responses occur by activation of different calcium signaling mechanisms and/or different receptor subtypes.

Reliance on calcium influx for the increase in [Ca2+]i is confirmed by exposing cells to α,β-methylene-ATP while they are being bathed in nominally calcium-free medium or by blocking endogenous calcium influx pathways. The data in Fig. 4 clearly demonstrate that removal of calcium from the extracellular medium completely abolishes the calcium response evoked by α,β-methylene-ATP. Similarly, nonspecific blockade of calcium influx pathways by the addition of Ni2+ to the extracellular medium while a physiological concentration of extracellular calcium is maintained completely eliminated the increase in [Ca2+]i in response to α,β-methylene-ATP exposure. Therefore, the increase in [Ca2+]i found to occur under control conditions involves activation of a nickel-sensitive calcium influx pathway rather than the release of calcium from intracellular stores.

These data are consistent with previous observations that the afferent arteriolar vasoconstrictor response elicited by α,β-methylene-ATP could be totally blocked when calcium was removed from the extracellular medium (25). In those studies, EGTA was added to the bathing medium and the blood perfusate to reduce the concentration of free calcium in the extracellular environment. Exposure of afferent arterioles to 1 μM α,β-methylene-ATP during low-calcium conditions eliminated the vasoconstrictor response normally observed. Returning the extracellular calcium concentration to physiological levels, by the addition of excess calcium, restored the afferent arteriolar vasoconstrictor response on subsequent exposure of these arterioles to α,β-methylene-ATP. These data support the argument that renal microvascular responses to α,β-methylene-ATP require the influx of calcium from the extracellular environment.

Although the studies described above establish the requisite role of calcium influx in the calcium signaling response to α,β-methylene-ATP, they do not address the specific nature of the influx pathway responsible for the response. Previous studies have shown that the sustained phase of the afferent arteriolar vasoconstric- tion elicited by α,β-methylene-ATP can be blocked with the L-type calcium channel antagonists diltiazem or felodipine, whereas the initial vasoconstriction was significantly attenuated (25). Furthermore, ATP-mediated elevation of [Ca2+]i is markedly attenuated by the calcium channel blocker diltiazem (22, 27). Therefore, we investigated the possibility that L-type calcium channels might be involved in the calcium response to α,β-methylene-ATP-mediated P2X receptor activation. Calcium channel blockade with nifedipine or diltiazem attenuated the increase in [Ca2+]i, induced by α,β-methylene-ATP. Despite the presence of calcium channel blockers, α,β-methylene-ATP still induced a small transient calcium response that was not observed under calcium-free conditions or in the presence of extracellular Ni2+. Therefore, the calcium channel blocker data suggest that P2X receptor activation by α,β-methylene-ATP stimulates a nickel-sensitive calcium influx pathway that is partially dependent on the activation of L-type calcium channels to effect the elevation of [Ca2+]i. These data are consistent with the hypothesis that α,β-methylene-ATP is activating the ligand-gated, nonselective cation channel that is structurally integrated into the P2X receptor (15, 35). Activation of this cation channel could lead to membrane depolarization and activate voltage-operated calcium channels. It is interesting to note that the residual increase in [Ca2+]i observed during calcium channel blockade corresponds with the residual transient vasoconstriction that is observed when afferent arterioles are challenged with α,β-methylene-ATP during calcium channel blockade but is absent when calcium is removed from the bathing medium.

An alternative explanation could be that Ni2+, diltiazem, and nifedipine all interfere with the binding of α,β-methylene-ATP to the receptor and thus impair the response to agonist stimulation. This possibility seems unlikely given the broad disparity in the structures of the agents concerned and the net impact each had on α,β-methylene-ATP-mediated responses. Ni2+ has been used to examine membrane currents evoked by many different agonists in many different cell types. There have not been any implications from those studies that Ni2+ directly interferes with agonist binding.
The calcium channel blockers used in the present study are well-established agents, whose selectivity for L-type calcium channels has been well characterized. In the present report, small residual responses were observed in response to \( \alpha,\beta \)-methylene-ATP exposure. The magnitude of the response was qualitatively larger in the nifedipine-treated cells compared with the diltiazem-treated cells, but this difference was not statistically significant. The overriding observation in these studies is that removal of calcium from the extracellular medium or general blockade of calcium influx pathways with a high concentration of \( \text{Ni}^{2+} \) resulted in complete blockade of the \( \alpha,\beta \)-methylene-ATP-mediated increase in calcium. Selective blockade of L-type calcium channels with two structurally dissimilar calcium channel blockers resulted in partial inhibition of the calcium response. These observations indicate that stimulation of P2X receptors with \( \alpha,\beta \)-methylene-ATP activates a calcium influx pathway that relies, in part, on the opening of L-type calcium channels as well as one or more additional influx pathways.

Interestingly, ATP, which activates both P2X and P2Y receptors, increases \( [\text{Ca}^{2+}]_i \) by stimulating both calcium influx through voltage-gated L-type calcium channels and calcium mobilization (22, 27). These findings are confirmed in the present study by the demonstration that \( [\text{Ca}^{2+}]_i \) increases transiently in cells treated with ATP while they are being bathed in calcium-free medium. Thus experimental evidence supports the expression of multiple P2 receptor subtypes and isoforms by preglomerular MVSMC.

Immunohistochemical evidence has demonstrated that the preglomerular microvasculature of the rat stained heavily for expression of P2X1 receptors whereas no evidence of staining was observed on the postglomerular efferent arteriole (7). Autoradiographic data also support the existence of binding sites for \(^{3}H\)-labeled \( \alpha,\beta \)-methylene-ATP along the interlobular arteries and afferent arterioles but not postglomerular efferent arterioles (7). Interestingly, this immunohistochemical distribution and autoradiographic profile mirrors the functional assessment of the preglomerular and postglomerular responsiveness to P2 receptor stimulation (26). In those studies, only the preglomerular microvascular segments (arcuate and interlobular arteries and afferent arterioles) responded to ATP with rapid, biphasic vasoconstrictor responses (26). The postglomerular efferent arteriole was unaffected by ATP treatment (26). In addition, pharmacological assessment of afferent arteriolar responsiveness to a variety of P2 agonists revealed that the P2X1 agonist \( \alpha,\beta \)-methylene-ATP was the most potent agonist tested (20). These observations support the involvement of P2X1 receptors in the renal microvascular response to extracellular ATP.

In the present report, we evaluated a newly developed receptor antagonist, NF-279, which is purported to be a highly potent antagonist at human P2X1 receptors (11, 28, 29, 36) and may also be effective against P2X2 receptors (28). In addition, recent data generated in *Xenopus laevis* oocytes expressing rat P2X receptors suggest that higher concentrations may have some inhibitory properties at P2X2, P2X3, and P2X4 receptors (36). However, electrophysiological studies indicate that P2X2 and P2X4 receptors are unresponsive to 10 \( \mu \text{M} \) \( \alpha,\beta \)-methylene-ATP (11, 29). Exposure of freshly isolated MVSMC to NF-279 had no effect on baseline \( [\text{Ca}^{2+}]_i \), but completely eliminated the increase in \( [\text{Ca}^{2+}]_i \) associated with exposure to \( \alpha,\beta \)-methylene-ATP. In addition, when NF-279 was removed from the bathing medium and \( \alpha,\beta \)-methylene-ATP was reapplied, cells that were previously unresponsive to \( \alpha,\beta \)-methylene-ATP now responded with an increase in \( [\text{Ca}^{2+}]_i \), although the response was noticeably broader. We cannot be certain as to the reason for the broader response, but several possibilities can be considered. The simplest explanation would be that the NF-279 was not completely washed from the bathing solution or dissociated from the receptors during the washout period. Alternatively, the reversibility of NF-279 blockade of P2X receptors on preglomerular smooth muscle may be incomplete, resulting in a partial retention of P2X receptor blockade under the conditions used here. Finally, the cellular P2 receptors could have undergone partial desensitization during the first exposure to \( \alpha,\beta \)-methylene-ATP. This would lead to a blunted, and perhaps slower, response during the subsequent exposure. Nevertheless, restoration of responsiveness to \( \alpha,\beta \)-methylene-ATP after washout of the NF-279 confirms that these cells are responsive to \( \alpha,\beta \)-methylene-ATP but that blockade of P2X receptors with NF-279 prevented \( \alpha,\beta \)-methylene-ATP from stimulating a response. This observation strongly supports the contention that increases in \( [\text{Ca}^{2+}]_i \) induced by \( \alpha,\beta \)-methylene-ATP occur through the selective activation of P2X receptors.

In summary, the data presented here provide in vitro evidence that exposure of freshly isolated preglomerular MVSMC to \( \alpha,\beta \)-methylene-ATP results in a prompt, concentration-dependent elevation of intracellular calcium concentration. \( \alpha,\beta \)-Methylene-ATP elevates calcium by stimulating the influx of extracellular calcium through a nickel-sensitive, voltage-dependent pathway involving L-type calcium channels. The response elicited by \( \alpha,\beta \)-methylene-ATP is markedly different from the responses elicited by ATP alone and is completely and reversibly blocked using a selective P2X receptor antagonist. The results of these studies are in agreement with the hypothesis that P2X receptor activation vasoconstricts preglomerular microvessels by stimulating L-type calcium channel-dependent elevation in \( [\text{Ca}^{2+}]_i \).

The authors thank Elizabeth LeBlanc for excellent technical assistance in these studies.

This work was supported by grants from the American Heart Association (AHA 95001370) and the National Institute of Diabetes and Digestive and Kidney Diseases (DK-44628 and DK-38226). E. W. Inscho is an Established Investigator of the American Heart Association.
REFERENCES