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Unusual absence of endothelium-dependent or -independent vasodilatation to purines or pyrimidines in the rat renal artery

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Unusual absence of endothelium-dependent or -independent vasodilatation to purines or pyrimidines in the rat renal artery.

Background. Adenosine triphosphate (ATP) is a cotransmitter with noradrenaline (NA) in sympathetic perivascular nerves. It has a dual role in the maintenance of vascular tone as ATP, released from endothelial cells during shear stress or hypoxia, induces vasodilatation via endothelial P2Y receptors or by direct action on smooth muscle. The role and distribution of P2 receptors is well characterized for many blood vessels but not for the rat renal artery. This study aims to determine whether ATP is a vasoconstrictor cotransmitter with NA and whether ATP induces vasodilatation via the endothelium or smooth muscle.

Methods. On isolated rat renal arteries, electrical field stimulation (EFS) in the absence and presence of antagonists to P2X receptors and α_1-adrenoceptors was examined. Concentration-response curves were constructed to NA, ATP, α,β-methylene ATP (α,β-meATP), uridine triphosphate (UTP), and 2-methylthio ADP (2-MeSADP) on low tone. Curves to acetylcholine (ACh), 2-MeSADP, and UTP were constructed on raised tone. Immunofluorescent localization of P2X and P2Y receptor subtypes was performed.

Results. Electrical field stimulation induced vasoconstriction, partially inhibited by the P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid, and predominantly by prazosin. Exogenous NA and ATP mimicked EFS; immunostaining for $P2X_1$ and $P2X_2$ receptors was expressed on vascular smooth muscle. Unusually, ATP, 2-MeSADP, and UTP failed to induce vasodilatation. Acetylcholine induced vasodilatation. α,β-meATP, 2-MeSADP, and UTP induced vasoconstriction via $P2X_1$, $P2Y_1$, and $P2Y_2$ receptors, respectively. Immunostaining for $P2X_1$, $P2Y_1$, and $P2Y_2$ receptors was expressed on the vascular smooth muscle.

Conclusion. Adenosine triphosphate and NA are cotransmitters in sympathetic nerves supplying the rat renal artery, NA being the dominant partner. The novel feature of this vessel is that purines and pyrimidines do not produce either endothelium-dependent or -independent vasodilatation; $P2X_1$, $P2Y_1$, and $P2Y_2$ receptors on the smooth muscle all mediate vasoconstriction.

Key words: ATP, constriction, dilatation, rat, renal artery, P2X, P2Y.

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In 1929, Drury and Szent-Györgyi [1] published data describing the physiologic activities of extracellular adenine compounds. Many studies followed, confirming the role of purine nucleotides on the cardiovascular system [2]. In 1972, Burnstock [3] proposed adenosine triphosphate (ATP) as a transmitter involved in non-adrenergic, non-cholinergic (NANC), nerve-mediated responses of smooth muscle in the gastrointestinal tract and bladder, the term "purinergic" was coined, and the ATP receptors termed purinoceptors. In 1978, Burnstock [4] proposed that receptors selective for adenosine and adenosine monophosphate (AMP) be designated P1-purinoceptors and those selective for ATP and adenosine diphosphate (ADP) designated as P2-purinoceptors. Further subdivisions followed: P1 receptors into A_1, A_{2A}, A_{2B}, and A_3 and P2 receptors into P2X and P2Y subtypes based on pharmacologic profiles [5]. Later, on the basis of cloning and signal transduction studies, P2 receptors were divided into two families: a P2X ligand–gated ion channel family and a P2Y G protein–coupled family [6]. Currently, seven subtypes of both P2X and P2Y families are recognized [7–9].

There is dual purinergic control of vascular tone. Adenosine triphosphate released from perivascular nerves acts on P2X receptors on smooth muscle to mediate vasoconstriction. Adenosine triphosphate released from endothelial cells during changes in flow and hypoxia acts on P2Y receptors producing vasodilatation [see 10, 11]. The relative amounts of ATP and NA vary significantly in sympathetic nerves in different blood vessels, for instance, sympathetic transmission in guinea pig submucous arterioles and rabbit mesenteric artery jejunal branches are totally purinergic [12, 13], sympathetic transmission of the rabbit splenic artery is largely purinergic [14], but in the rabbit pulmonary artery it is almost entirely noradrenergic [15]. In other blood vessels, such as the rabbit saphenous vein, both ATP and NA contribute significantly to sympathetic stimulation [16]. Exogenously applied ATP induces vasodilatation by an action at $P2Y_1$ or $P2Y_2$ receptors, located either on vascular endothelial cells, leading to the release of nitric oxide (NO) and con-

sequently, vasodilatation [7], or on P2Y receptors present on vascular smooth muscle, for example, as with the rabbit hepatic artery and portal vein [17, 18]. Uridine triphosphate released from endothelial cells by shear stress [19] stimulates endothelial $P2Y_2$ receptors mediating vasodilatation via the production of NO [20]. $P2Y_2$ receptors mediating vasoconstriction have been identified in some vessels, for example, in the rat mesenteric arterial bed [21] and in the golden hamster mesenteric arterial bed [22].

While considerable attention has been paid to the effects of purines on the renal vascular bed [23], there are few studies of the isolated renal artery. In isolated pig renal arteries, NA causes vasoconstriction of the artery and prazosin partially inhibits electrically stimulated vasoconstrictions [24]. Similarly, human extra- and intrarenal arteries constrict in response to NA [25]. In other studies on the rabbit and human renal arteries, ATP was found to induce vasodilatation sensitive to inhibition by the P1 receptor antagonist 8-p-(sulphophenyl) theophylline, suggesting that in both humans and rabbits, the vasodilatation is in part due to adenosine acting on smooth muscle P1 purinoceptors after enzymatic breakdown of ATP [26]. In the rabbit renal artery, adenosine and the P2Y receptor agonists ADPβS and 2-methylthio ATP (2-MeSATP) induced vasodilatation, attenuated by the antagonist, reactive blue 2. The presence of a nitric oxide synthase (NOS) inhibitor had a strong inhibitory effect on responses to 2-MeSATP, indicating that this agonist was inducing endothelium-dependent vasodilatation via the production of NO [26].

In this study of rat renal arteries, the aim was to investigate whether ATP acts as a vasoconstrictor cotransmitter with NA and whether ATP can induce vasodilatation either via the endothelium or by direct action on smooth muscle. To answer these questions, organ bath pharmacologic and immunohistochemical techniques were employed.

METHODS

Animals

Male Sprague-Dawley rats (250 to 300 g) were killed by exposure to an increasing CO_2 concentration and death was ensured by cervical dislocation. This was in accordance with the Home Office (UK) regulations (The Humane Killing of Animals under Schedule One of the Animals Scientific Procedures Act 1986) and principles of good laboratory care were followed. After the abdominal cavity was opened, the kidneys and renal arteries were removed and placed in physiologic saline. The renal arteries were then isolated from the kidneys and aorta and cleared of surrounding fatty tissues under a dissecting microscope.

The isolated renal arteries were cut into rings of approximately 4 mm in length (this was almost the full length of each artery) and suspended horizontally in 10 mL organ baths by inserting a tungsten wire through the lumen of the vessel. Care was taken not to damage the endothelium. A second tungsten wire was then inserted into the lumen; one end was attached to a rigid support, the other to a Grass (Quincy, MA, USA) FT03C force-displacement transducer. The organ baths contained continuously gassed (95% O_2/5% CO_2) Kreb's solution of the following composition (mmol/L): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8, $CaCl_2$ 2.52, pH 7.2, and maintained at 37 ± 1°C. The ring preparations were placed under an initial tension of 1 g and allowed to equilibrate for 1 hour prior to the start of the experiments. Mechanical activity was recorded using the software PowerLab Chart for Windows (Version 4; ADInstruments, New South Wales, Australia).

Transmural electrical field stimulation (EFS) was delivered to the vessel via platinum electrodes situated on either side of the renal artery. The vessels were stimulated (75 V, 0.1 millisecond, 1 to 32 Hz) for 10 seconds at 5 minute intervals and a frequency-response curve was constructed in the absence of any blocking agents. On some preparations, a second frequency-response curve was constructed in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; 30 μmol/L), a non-selective P2 receptor antagonist, and then a third frequency-response curve was constructed in the presence of both PPADS (30 μmol/L) and prazosin (3 μmol/L), an α_1-adrenoceptor antagonist. On other preparations, following a control frequency-response curve, a second curve was constructed in the presence of prazosin (3 μmol/L), followed by a third curve in the presence of both prazosin (3 μmol/L) and PPADS (30 μmol/L). Finally, in both types of experiments, a fourth curve was constructed in the presence of tetrodotoxin (TTX; 1 μmol/L) to isolate any part of the response that was due to direct stimulation of the smooth muscle. In other experiments, the control frequency-response curve was repeated in the presence of guanethidine (3 μmol/L) alone or TTX (1 μmol/L) alone. All antagonists were allowed to equilibrate for 20 minutes before the frequency-response curves were constructed.

On separate preparations, concentration-response curves were constructed both on low tone (vessels that had not been pre-constricted with NA) or raised tone (vessels that had been pre-constricted with NA).

At low tone, a cumulative concentration-response curve was constructed (i.e., consecutive doses added without wash-out of the previous dose) for NA (10 nmol/L to 1 mmol/L). Non-cumulative concentration response curves (i.e., consecutive doses added individually following wash-out of the previous dose) were constructed to ATP (1 μmol/L to 1 mmol/L), α,β-methylene ATP (α,β-meATP;

0.3 μmol/L to 0.1 mmol/L), 2-MeSADP (0.1 μmol/L to 0.1 mmol/L) and UTP (1 μmol/L to 1 mmol/L).

The concentration inducing half-maximal exposure (EC_{50}) concentration for NA was calculated for each preparation and this concentration was used to pre-constrict the vessel. On NA pre-constricted vessels, cumulative concentration-response curves were constructed for acetylcholine (ACh; 10 nmol/L to 1 mmol/L), ATP (0.1 μmol/L to 1 mmol/L), 2-MeSADP (10 nmol/L to 0.3 mmol/L), and UTP (0.1 μmol/L to 1 mmol/L).

Since alteration in extracellular pH is known to affect the response to ATP at $P2X_2$ receptors [27], in some experiments the effect of changing the pH of the Kreb's to 6.5 was examined. In these experiments, the response to a single concentration of ATP (0.1 mmol/L) and α,β-meATP (3 μmol/L) was repeated twice in Kreb's at pH 7.2. The pH of the Kreb's was then adjusted to pH 6.5 and added to the organ bath for 5 minutes, then the ATP and α,β-meATP responses were repeated.

A single concentration of KCl (120 mmol/L) was added to each vessel at the end of each experiment to give a standard contraction.

Immunohistochemistry

Isolated rat renal arteries were blocked in OCT and frozen down in isopentane, pre-cooled in liquid nitrogen. Using a Reichert Jung CM1800 cryostat (Leica Instruments, Nubloch, Germany), 12 μm sections of renal artery were cut and mounted on gelatine-coated slides ready for immuno-staining.

The immunofluorescent localization of purinoceptors was carried out using the revised protocol described by Llewellyn-Smith and co-workers [28, 29]. The sections were air-dried for 30 minutes at room temperature and then fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer for 2 minutes at room temperature before being washed in phosphate-buffered saline (PBS) at room temperature 3 times for 5 minutes each time. Non-specific binding sites were blocked by a 20 minute incubation with 10% normal horse serum (NHS) in PBS containing 0.05% merthiolate (thimerosal). Primary antibodies for the $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ receptors were diluted to 1.25 μg/mL to 5 μg/mL and the $P2Y_1$ and $P2Y_2$ receptor primary antibodies diluted to 1.5 μg/mL to 6 μg/mL with 10% NHS-PBS-merthiolate. The $P2X_3$ receptor primary antibody was diluted with 10% NHS containing 0.2% Triton X-100 to 1.25 μg/mL to 5 μg/mL. The specimens were then incubated overnight at room temperature with the primary antibodies. The secondary antibody, biotinylated donkey anti-rabbit immunoglobulin G (IgG) diluted 1:500 in 1% NHS-PBS-merthiolate, was applied and incubated for 1 hour. After washing, the sites of the antigen-antibody binding were revealed by using streptavidin-fluorescein [fluoroscein isothiocyanate (FITC)-green fluorophore] diluted 1:200 in PBS-merthiolate. The sections were again washed and then incubated for 5 minutes with Pontamine Sky Blue [0.1% in 1% dimethyl sulfoxide (DMSO)/PBS] to reduce background autofluorescence staining. The specimens were mounted with Citifluor and coverslipped.

As a control for non-specific immunoreaction, one slide was included in each experiment on which the primary antibody had been omitted.

The sections were viewed under a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany), and selected areas were photographed with a digital Leica DC200 (Leica Instruments) camera. The pictures were processed using Adobe Photoshop 5.0 software (San Jose, CA, USA) on an Apple Power Macintosh G3 (Apple, Cupertino, CA, USA).

Drugs used

ACh, ATP, guanethidine, α,β-meATP (a selective agonist for $P2X_1$ and $P2X_3$ receptors [7]), merthiolate, 2-MeSADP (a selective agonist for $P2Y_1$ receptors [8]), NA, NHS, PPADS (a non-selective P2 antagonist [7]), prazosin, streptavidin-fluorescein, TTX, and UTP were purchased from Sigma Chemical Co. (Poole, UK); formaldehyde stabilized with 10% methanol was obtained from AnalaR, (BDH) (Poole, UK); biotinylated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Primary P2X receptor antibodies were kindly donated by Roche Bioscience (Palo Alto, CA, USA). Primary P2Y antibodies were obtained from Alomone Labs (Jerusalem, Israel).

Statistical analysis

Responses to EFS: Contractile responses in the absence and presence of PPADS, prazosin, or both, are expressed as mean % contraction of the maximum control response ± SE (N) animals. Individual frequency-response curves are compared using a two-way analysis of variance (ANOVA) followed by a post hoc (Tukey) test since analysis of the data revealed it to be normally distributed and therefore the use of this test was appropriate.

Low tone concentration-response curves: Concentration-response curves to NA are expressed as mean % of the maximum contraction ± SE (N). The EC_{50} values for NA in the absence and presence of the endothelium were compared using an unpaired Student t test. The EC_{50} concentration was used to pre-contract the vessel. Contraction-response curves to ATP, α,β-meATP, 2-MeSADP, and UTP are expressed as mean % of the KCl-induced contraction ± SE (N). Individual concentration-response curves are compared using a two-way analysis of variance (ANOVA) followed by a post hoc test.

Raised-tone concentration-response curves: Relaxant responses to ACh are expressed as mean % reduction of the NA-induced contraction ± SE (N).

All statistical analyses were performed by means of the software Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). A probability level of $P < 0.05$ was taken as significant for both the ANOVA and t tests.

RESULTS

Organ bath pharmacology

Electrical field stimulation. Electrical field stimulation (75 V, 0.1 milliseconds, 1 to 32 Hz, 10 second stimulation every 5 minutes) of the renal artery induced frequency-dependent contractions that were guanethidine (3 μmol/L) and TTX (1 μmol/L) sensitive indicating that the responses were sympathetic and nerve-mediated in origin (Fig. 1A).

Frequency-response curves in the absence of any blocking agents were constructed. The control curves were repeated in the presence of either PPADS (30 μmol/L) or prazosin (3 μmol/L) alone, and finally in the presence of both antagonists. PPADS (30 μmol/L) alone caused a significant ($P < 0.0001$) inhibition of the control frequency-response curve (Fig. 1B). In the presence of PPADS, the addition of prazosin (3 μmol/L) resulted in the almost total inhibition ($P < 0.0001$) of the response to EFS (Fig. 1B).

Prazosin (3 μmol/L) alone caused a large, significant ($P < 0.0001$) inhibition of the control frequency-response curve (Fig. 1C). In the presence of prazosin, the addition of PPADS (30 μmol/L) significantly ($P < 0.001$) blocked the residual response to EFS (Fig. 1C).

Exogenously applied agonists. On low-tone preparations of renal artery, NA (10 nmol/L to 1 mmol/L) induced concentration-dependent, sustained vasoconstrictions and concentration-response curves were constructed (Fig. 2A). From the individual concentration-response curves the EC_{50} concentration was calculated. This concentration was then used to pre-constrict the vessels.

Concentration response curves to NA from vessels with an intact endothelium were compared to those with a disrupted endothelium. The concentration response curves to NA from renal arteries with a disrupted endothelium were found to be significantly different ($P < 0.001$) from those vessels whose endothelium was intact (Fig. 2A), indicating that in the absence of the endothelium NA was inducing larger constrictions than in the presence of the endothelium.

The mean pD_2 value ($-\log EC_{50}$ concentration) for NA was calculated in the absence and presence of the endothelium. These were found to be 5.38 ± 0.10 ($N = 11$) in the absence of the endothelium and 5.20 ± 0.14 ($N = 10$) in the presence of the endothelium. These values were compared using an unpaired Student t test

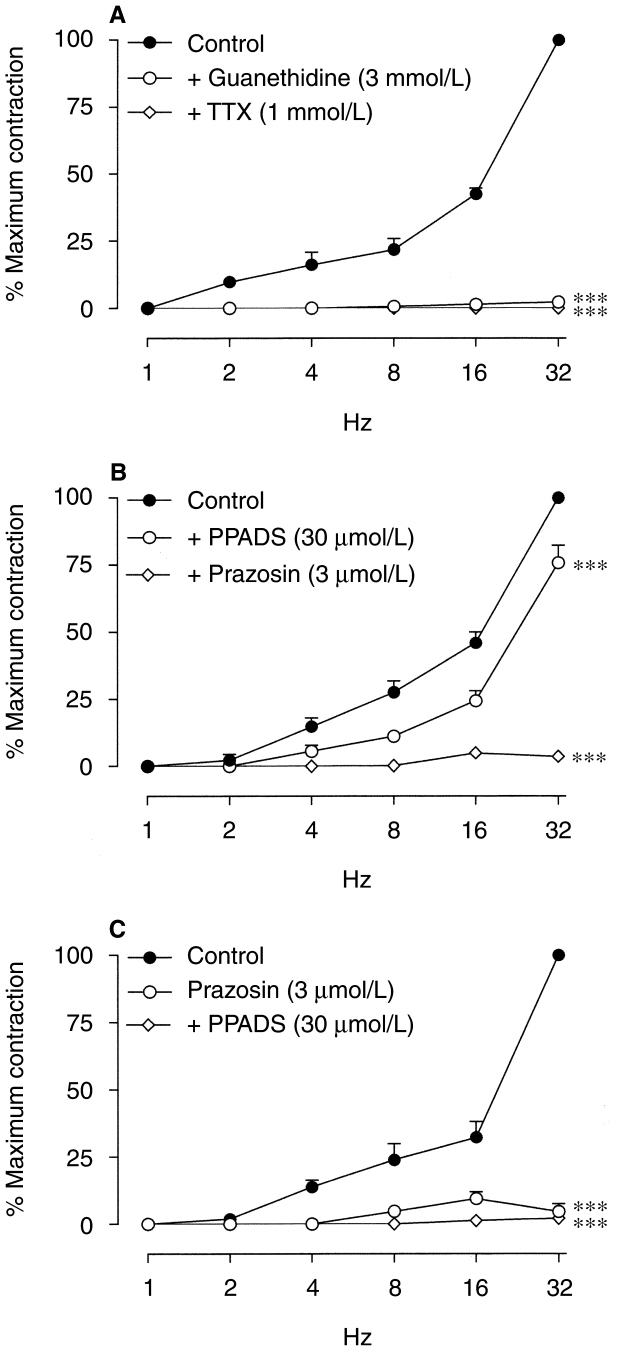


Fig. 1. Effect of antagonists on electrical field stimulation (EFS) of the rat renal artery (75 V, 0.1 milliseconds, 1 to 32 Hz, 10 second stimulation every 5 minutes). (*A*) Frequency-response curves in the absence (control; $N = 4$) and presence of either guanethidine (3 μmol/L; $N = 4$) or TTX (1 μmol/L; $N = 4$). (*B*) Frequency-response curves in the absence (control; $N = 6$) and presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (30 μmol/L; $N = 6$). In the presence of both PPADS (30 μmol/L) and prazosin (3 μmol/L; $N = 6$). (*C*) Frequency-response curves in the absence (control; $N = 6$) and presence of prazosin (3 μmol/L; $N = 6$). Finally, in the presence of both prazosin (3 μmol/L) and PPADS (30 μmol/L; $N = 6$). All symbols are mean ± SE (unless error bars masked by the symbol), expressed as % of the maximum control response. Statistical significance was tested by two-way analysis of variance (ANOVA). ***$P < 0.0001$ (applicable to the whole curve).

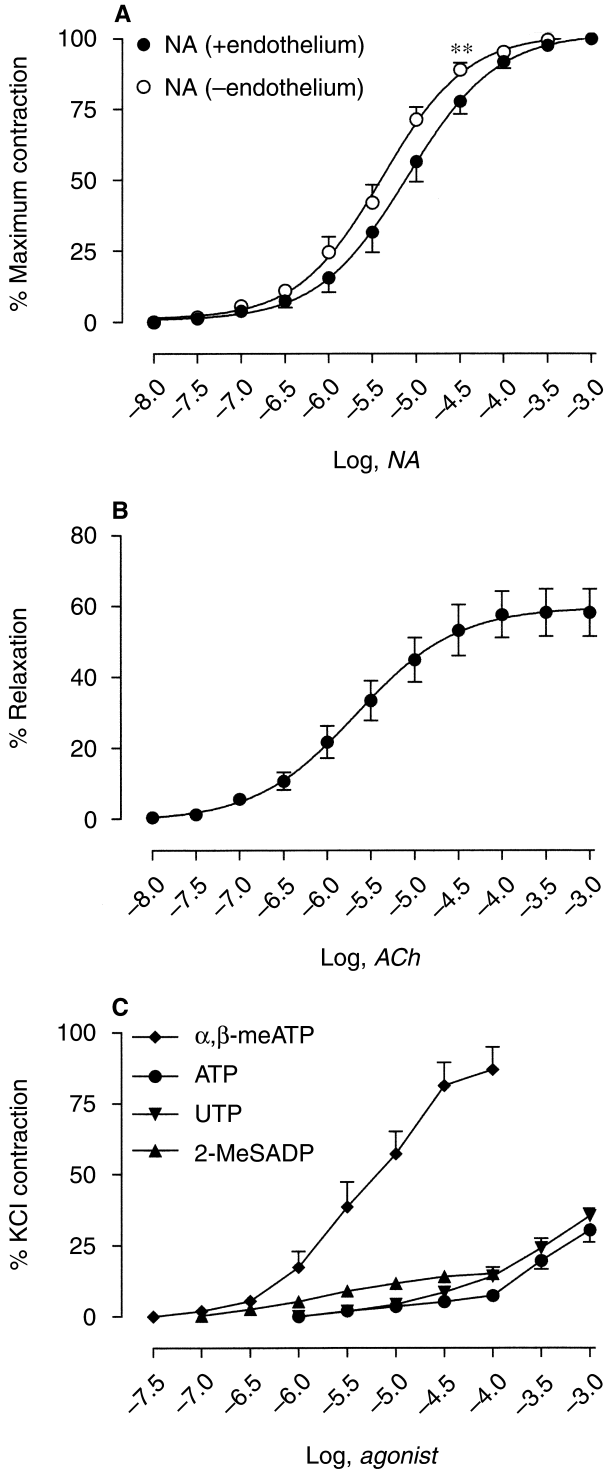


Fig. 2. Effects of exogenously applied agonists on the rat renal artery. (*A*) Cumulative concentration-response curves for noradrenaline (NA) (10 nmol/L to 1 mmol/L) expressed as % of the maximum response, in the absence ($N = 11$) and presence ($N = 10$) of the endothelium. (*B*) Cumulative concentration-response curves for acetylcholine (Ach) (10 nmol/L to 1 mmol/L; $N = 13$) expressed as % relaxation of the NA (EC_{50} concentration) contraction. (*C*) Non-cumulative concentration-response curves for α,β-meATP (0.3 μmol/L to 0.1 mmol/L; $N = 6$), 2-MeSADP (0.1 μmol/L to 0.1 mmol/L; $N = 5$), uridine triphosphate (UTP) (1 μmol/L to 1 mmol/L; $N = 5$) and adenosine triphosphate (ATP) (1 μmol/L to 1 mmol/L; $N = 5$) expressed as % of the KCl (120 mmol/L) contraction. All symbols are mean ± SE (unless error bars masked by the symbol). Statistical significance was tested by two-way analysis of variance (ANOVA). $^{**}P < 0.001$ (applicable to the whole curve).

and were not found to be statistically significantly different ($P = 0.2820$).

On NA pre-constricted preparations of renal artery, ACh (10 nmol/L to 1 mmol/L; $N = 13$) induced concentration-dependent vasodilatation and concentration-response curves were constructed (Fig. 2B). The ability of an individual preparation of renal artery to dilate in the presence of ACh was taken as an indication of the integrity of the endothelium. In those vessels where the endothelium had been disrupted, ACh failed to induce vasodilatation.

On low-tone preparations in the presence of the endothelium, α,β-meATP (0.3 μmol/L to 0.1 mmol/L), 2-MeSADP (0.1 μmol/L to 0.1 mmol/L), ATP (1 μmol/L to 1 mmol/L), and UTP (1 μmol/L to 1 mmol/L), all induced concentration-dependent contractions that were not sustained, and non-cumulative concentration-response curves were constructed for each agonist (Fig. 2C). The order of potency for these agonists was: α,β-meATP > 2MeSADP > UTP > ATP. The curves to UTP and ATP were compared using a two-way ANOVA test, and the curves were seen to be statistically different from each other ($P < 0.05$), with UTP being slightly more potent than ATP.

As none of the P2 agonists reached a maximum response, pD_2 values could not be calculated and the results were expressed as a % of the KCl contraction.

On NA pre-constricted preparations of renal artery neither ATP (0.1 μmol/L to 1 mmol/L; Fig. 3A), 2-MeSADP (10 nmol/L to 0.3 mmol/L; Fig. 3B), nor UTP (0.1 μmol/L to 1 mmol/L; Fig. 3C) induced vasodilatation, in the presence of either an intact or disrupted endothelium; indeed, UTP increased the tone of the vessel still further.

Altering the pH of the Kreb's from 7.2 to 6.5 had no potentiating effect on the responses to either ATP or α,β-meATP. Control constrictions to ATP (0.1 mmol/L) at pH 7.2 (taken as 100%), were found to be 52.5 ± 18.7% ($N = 3$) in the presence of pH 6.5, which was not significantly different from the control ($P = 0.1263$), whereas control constrictions to α,β-meATP (3 μmol/L) (taken as 100%), were 45.9 ± 5.5% ($N = 3$) in the presence of pH 6.5, which was significantly different from the control ($P < 0.01$). The alteration in pH was found to generally inhibit the vessel from constricting.

Immunohistochemistry

P2X receptor staining. $P2X_1$ receptor immunoreactivity was clearly present on the smooth muscle of the renal

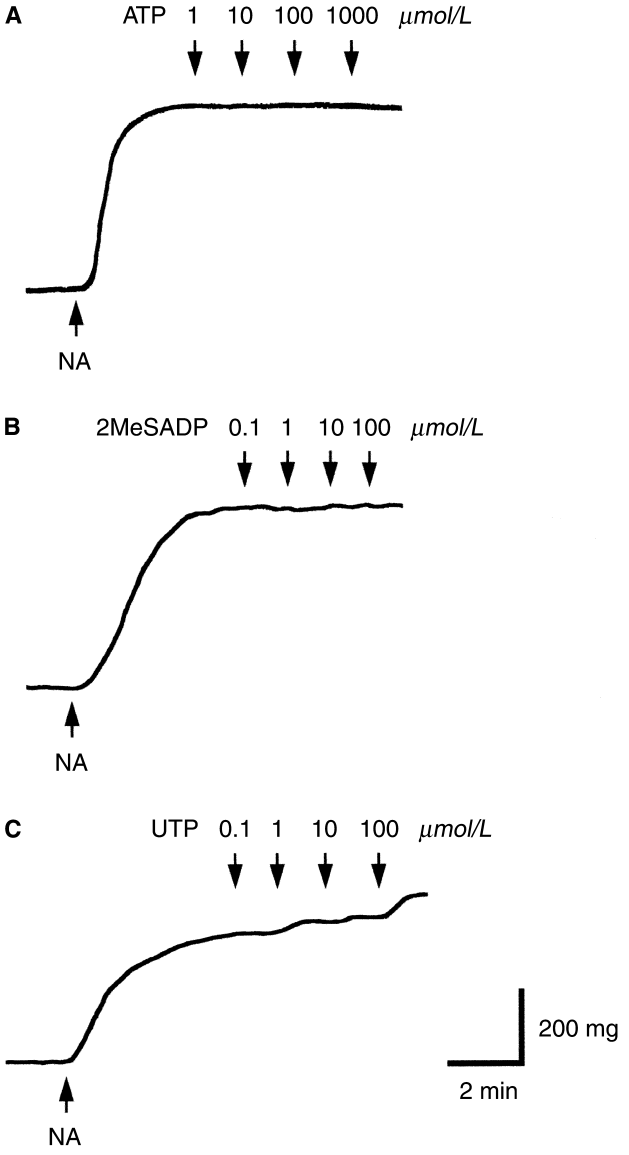


Fig. 3. Examples of original traces of the effect of exogenously applied agonists on the rat renal artery. (*A*) Cumulative concentration-response curves to adenosine triphosphate (ATP) (1 to 1000 μmol/L) on a noradrenaline (NA) (EC_{50} concentration) pre-constricted renal artery in the absence of the endothelium. (*B*) Cumulative concentration-response curve to 2-MeSADP (0.1 to 100 μmol/L) on a NA (EC_{50} concentration) pre-constricted renal artery in the presence of the endothelium. (*C*) Cumulative concentration-response curves to uridine triphosphate (UTP) (0.1 to 100 μmol/L) on a NA (EC_{50} concentration) pre-constricted renal artery in the presence of the endothelium.

artery ($N = 3$; Fig. 4a). $P2X_2$ receptor immunoreactivity was also observed on the smooth muscle of the renal artery ($N = 3$; Fig. 4b). No $P2X_1$ or $P2X_2$ immunoreactivity was observed on the vascular endothelium in sections of the renal artery. No immunostaining for $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, or $P2X_7$ receptors was observed for the renal artery. To ensure that the immunostaining observed was specific, controls were included from which the primary antibodies had been omitted ($N = 3$; Figure 4 C and D). Some low-level background fluorescence remained.

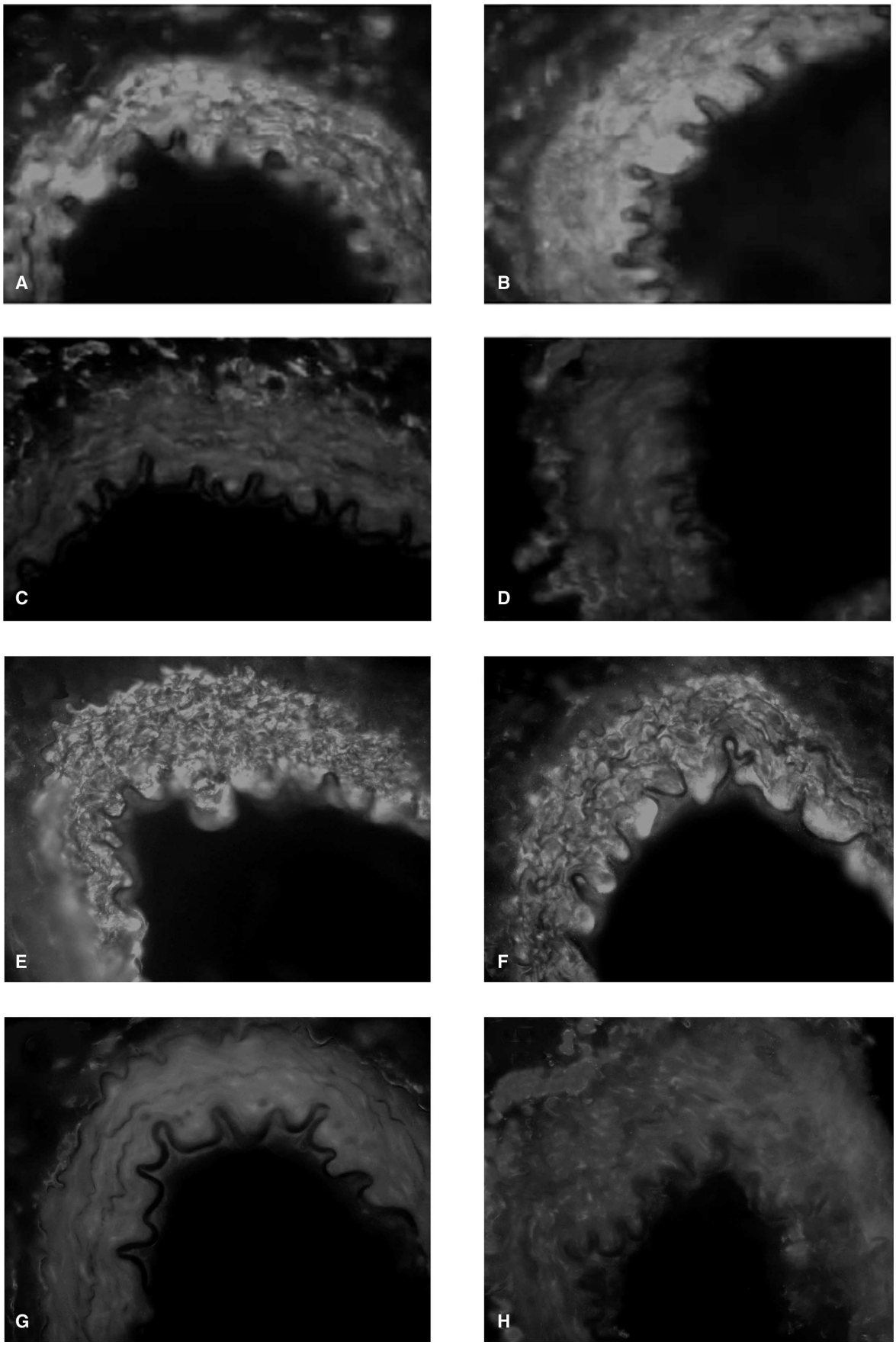
P2Y receptor staining. $P2Y_1$ receptor immunoreactivity was observed on the smooth muscle of the renal artery ($N = 3$; Fig. 4E). $P2Y_2$ receptor immunoreactivity was also observed on the smooth muscle of the renal artery ($N = 3$; Fig. 4F), although usually at a lower intensity than that observed for $P2Y_1$ receptors. To ensure all staining observed was specific, controls were included from which the primary antibodies had been omitted ($N = 3$; Figure 4 G and H).

DISCUSSION

One aim of this study of the rat renal artery was to investigate whether ATP is a cotransmitter with NA in sympathetic nerves to produce vasoconstriction, as occurs in many other blood vessels [7, 30].

Evidence is presented that ATP is indeed a cotransmitter with NA and induces vasoconstriction. The fact that guanethidine and TTX inhibit the response to EFS indicates that sympathetic nerves are being stimulated. Further evidence obtained from the use of antagonists shows that both ATP and NA are being released from these nerves. The α_1-adrenoceptor antagonist, prazosin, inhibited a large proportion (over 90%) of the response to EFS, while the non-selective P2X receptor antagonist, PPADS, only produced about 5 to 10% inhibition at most stimulation frequencies. PPADS was used as the inhibitor of $P2X_1$-mediated vasoconstrictions in this instance, since the more recently developed selective $P2X_1$ antagonist 2′,3′-O-(2,4,6-trinitrophenyl) ATP (TNP-ATP) is known to breakdown rapidly when used in whole tissue studies such as this study [9]. In previous studies of the rat isolated kidney vascular bed, the vasoconstrictor response due to low frequencies of stimulation (2 Hz) was entirely due to the release of ATP [31, 32]. In addition to the results from perivascular nerve stimulation, the effect of exogenously applied agonists supports the con-

Fig. 4. $P2X_1$, $P2X_2$, $P2Y_1$, and $P2Y_2$ immunostaining of the rat renal artery. (*A*) Specific immunoreactivity for $P2X_1$ receptors was clearly present in the smooth muscle of the renal artery. (*B*) $P2X_2$ receptor immunoreactivity was observed in the smooth muscle of the renal artery. (*C* and *D*) Controls consisting of the omission of the primary antibody to $P2X_1$ and $P2X_2$, respectively. (*E*) $P2Y_1$ receptor immunoreactivity was present in the smooth muscle of the renal artery. (*F*) $P2Y_2$ receptor immunoreactivity was expressed in the smooth muscle of the renal artery. (*G* and *H*) Controls consisting of the omission of the primary antibody to $P2Y_1$ and $P2Y_2$, respectively. Magnification of all plates, ×400.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cept of cotransmission. Exogenously applied NA and ATP mimicked the responses to nerve stimulation. NA acted on α_1-adrenoceptors to produce sustained contractions, while ATP, and its stable analog α,β-meATP, act on $P2X_1$ receptors to produce a faster transient contraction.

In common with other blood vessels such as the rat mesenteric, pulmonary, basilar, and coronary arteries where $P2X_1$ receptors have been visualized immunohistochemically on vascular smooth muscle cells [33–35], the presence of $P2X_1$ receptors on the vascular smooth muscle of the rat renal artery was demonstrated by immunostaining. $P2X_2$ receptor immunostaining was also shown to be present in the rat renal artery. The question as to which P2X receptor subtype is responsible for the vasoconstriction was partly answered by the contractile activity of α,β-meATP. α,β-meATP is a selective agonist for $P2X_1$ and $P2X_3$ receptors, but is inactive at $P2X_2$ receptors. Since $P2X_3$ receptor immunostaining has not been demonstrated in the renal vascular smooth muscle, it can be assumed that the vasoconstriction responses seen to α,β-meATP are a consequence of the activation of $P2X_1$ receptors on the smooth muscle of the renal artery. The presence of $P2X_2$ receptors on the smooth muscle of the rat renal artery is by no means unusual. Other vessels also exhibit immunoreactivity to $P2X_2$ receptors, including the rat mesenteric and pulmonary arteries, aorta, and urinary bladder veins [33], and mRNA for $P2X_2$ receptors was found in the smooth muscle of rat coronary vessels and aorta [36]. A reduction in pH is known to potentiate responses to ATP at recombinant $P2X_2$ receptors [27], and in the rat tail circulation, purinergic sympathetically-mediated vasoconstriction was found to be sensitive to changes in pH via a P2X receptor [37]. However, $P2X_2$ receptors are unlikely to be a major contributor to the contractile responses of ATP in the rat renal artery, since alteration in the pH of the Kreb's solution, from 7.2 to 6.5, had no potentiating effect on responses to ATP.

No endothelium-dependent vasodilatation was produced in response to either ATP or UTP in the rat renal artery. However, we have shown, as have others, that ACh acts via the renal artery endothelium to induce relaxation in an NO-independent manner [38, 39]. Similarly, an inhibitory adenosine A2b receptor located on rat renal endothelial cells and stimulating the release of NO has been identified [40]. Agonists for P2 receptors on raised tone preparations of rat renal artery do not induce vasodilatation either in the presence or absence of the endothelium. On the contrary, 2-MeSADP and UTP, agonists for $P2Y_1$ and $P2Y_2$ receptors, respectively [8, 41], both induced vasoconstriction of this vessel. Since both agonists were active it would imply that both receptors are present, and since constriction occurred in either the absence or presence of the vascular endothelium, their location can be assumed to be on the smooth muscle. This is confirmed by immunohistochemical staining of this vessel for P2Y receptors, where both $P2Y_1$ and $P2Y_2$ receptor fluorescence was observed, concentrated on the smooth muscle of the renal artery. Other blood vessels exhibit constriction in response to P2Y agonists. For example, in the rat mesenteric artery, UTP induces constriction acting via a $P2Y_2$ receptor [34], and the rat aorta, tail artery, and saphenous vein constrict in response to UTP [42–44]. Why this vessel expresses two different P2Y receptors, both mediating vasoconstriction, and why purine and pyrimidine nucleotides do not elicit vasodilatation is not clearly understood. The rat renal artery is particularly short (approximately 4 to 5 mm in length) compared to the renal arteries of humans and rabbits, which are considerably longer. Both human and rabbit renal arteries possess endothelial P2Y receptors which induce endothelium-dependent vasodilatation via NO [26]; this species difference may be related to the difference in length of the vessels. It is possible that stimulation of either of these P2 receptors may lead to long-term trophic actions on cell growth, proliferation, and cell death [45]; it is perhaps significant that the response of the isolated rat renal artery to these agonists is the opposite of their effects upon the renal vascular bed. Perfused rat kidneys, when exposed to 2-MeSATP, respond with vasodilatation, this response being blocked by the NOS inhibitor N^{ω} nitro-L-arginine methyl ester [46], indicating the presence of $P2Y_1$ receptors on the vascular endothelium. Release of an agent that markedly dilates the vascular bed but acts to constrict the artery feeding the bed may act as a feedback safety mechanism to minimize rapid dilatation of the vascular bed.

CONCLUSION

This study has shown cotransmission of ATP and NA in the rat renal artery and in common with a minority of other blood vessels, the presence on the vascular smooth muscle of $P2X_1$, $P2Y_1$, and $P2Y_2$ receptors, all of which mediate vasoconstriction of the vessel. This vessel appears to be devoid of a nucleotide receptor inducing vasodilatation.

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