Localization of P2X₁ purinoceptors by autoradiography and immunohistochemistry in rat kidneys


Localization of P2X₁ purinoceptors by autoradiography and immunohistochemistry in rat kidneys. Am. J. Physiol. 274 (Renal Physiol. 43): F799–F804, 1998.—P2 receptors have been identified in rat kidney by autoradiography, using the radioligand [³H]α,β-methylene ATP, and by immunohistochemistry, using a polyclonal antibody to the P2X₁ purinoceptor. They have been localized to the vascular smooth muscle of intrarenal arteries, including arcuate and interlobular arteries, and afferent arterioles, but not glomeruli, postglomerular efferent arterioles, or renal tubules. We conclude that at least some of the P2 receptors present on vascular smooth muscle are of the P2X₁ subtype. The functional significance of these findings in the vascular control of the kidney is discussed.

EXOGENOUS ADENOSINE 5′-TRIPHOSPHATE

Adenosine 5′-triphosphate (ATP) and adenosine were first recognized to have important biological actions over 70 years ago (14), but the concept that these extracellular purines may act as mediators and regulators of cell function was not proposed until over 30 years later (3). In 1978, Burnstock (8) put forward an early classification of purine receptors distinguishing between adenosine, P1, and ATP, P2 purinoceptors. Originally, the renal effects of intrarenal infusion of ATP were reported to be an increase in renal blood flow (RBF) and a decrease in glomerular filtration rate (GFR) (26). However, more recent studies have shown that extracellular ATP can have variable effects on RBF and GFR, depending on which of the P2 receptor subtypes are stimulated predominantly (12, 22, 23). In the renal microcirculation there are two major subtypes of P2 receptors, P2X and P2Y, which until recently have been characterized pharmacologically according to their respective rank order of responses to selected purine and pyrimidine agonists (18, 20–22). These receptors have now been further divided into P2X₁-7 and P2Y₁-8 subtypes according to their molecular identity and their intracellular signal transduction pathways (1, 10).

More detailed studies of the effects of extracellular ATP on the renal microvasculature have demonstrated vasoconstriction of arcuate and interlobular arteries (20, 22) and glomerular afferent arterioles (18, 20–22, 27), mediated directly by P2X purinoceptors; ATP has no effect on the efferent arteriole (20, 22). In contrast, activation of P2Y receptors causes large preglomerular arcuate artery vasodilatation mediated indirectly by nitric oxide and prostacyclin release from endothelial cells (16, 20, 25). Thus the vascular effects of ATP depend on its route of administration, with intrarenal infusion stimulating mainly endothelial P2Y receptors and causing vasodilatation and increased RBF, and extravascular exposure activating P2X purinoceptors on vascular smooth muscle and producing vasoconstriction and decreased RBF (20, 23); it is the balance between stimulation of these P2 receptor subtypes that determines the biological effect of extracellular ATP on renal blood vessels.

In the present study, [³H]α,β-methylene ATP, which has been used as a radioligand for the P2X₁ purinoceptor in both visceral and vascular smooth muscle (4–6), was used to detect P2X₁ purinoceptor binding sites in rat kidney. Localization of the P2X₁ receptor subtype, which from in situ hybridization studies is thought to be the P2X₁ receptor subtype present on smooth muscle (7), has been investigated by immunohistochemistry using a polyclonal antibody specific against the P2X₁ receptor.

MATERIALS AND METHODS

All experiments were conducted on male Sprague-Dawley rats weighing 160–180 g.

Microdissection. The rat was anesthetized with an intraperitoneal injection of intralav (120 mg/kg body wt). A midline abdominal incision was made, and the aorta and left kidney were exposed. The aorta was cannulated, and the left kidney was isolated and perfused in situ with Hanks' buffer solution (Life Technologies) containing 2% collagenase (Worthington). The kidney was then removed, and small corticomedullary wedges of tissue were cut from a thin slice of kidney. These wedges were incubated in 1% collagenase Hanks' buffer solution at 37°C for 20 min, gassed with a mixture of 95% O₂:5% CO₂. The tissue sections were exposed. The aorta was cannulated, and the left kidney was isolated and perfused in situ with Hanks' buffer solution containing 2% collagenase (Worthington). The kidney was then removed, and small corticomedullary wedges of tissue were cut from a thin slice of kidney. These wedges were incubated in 1% collagenase Hanks' buffer solution at 37°C for 20 min, gassed with a mixture of 95% O₂:5% CO₂. The tissue sections were exposed. The aorta was cannulated, and the left kidney was isolated and perfused in situ with Hanks' buffer solution containing 2% collagenase (Worthington).
overnight at 4°C. Segments for immunohistochemistry were kept at −20°C. The slides were fixed in 4% buffered paraformaldehyde (in PBS) in preparation for radioligand binding studies, using [3H]β,β-methylene ATP (DuPont) for P2X purinoreceptors (5, 6).

Radioligand binding. The fixed microdissected vascular segments were preincubated at 30°C for 10 min in 50 mM Tris-HCl buffer (pH 7.4), followed by incubation in 50 mM Tris-HCl buffer containing 10 nM [3H]β,β-methylene ATP for 15 min. Competitive binding was performed in the presence of 100 µM β,γ-methylene ATP (Sigma). The segments were washed in ice-cold Tris-HCl twice for 2 min each time and finally rinsed in ice-cold distilled water for 1 min. After air drying at room temperature, the slides were stored overnight in a desiccator at 4°C.

 Autoradiography. The photographic emulsion (Ilford K5 diluted 1:1 in deionized water) was melted in a glass vessel at about 40°C in a water bath in a dark room. The microdissected vascular segments were coated by dipping the slides into the emulsion. The slides were held vertically to dry in air and then kept in a light-tight box at 4°C for 10–14 days. After exposure, the coated slides were developed in Phenasil (Ilford, 1:4 in deionized water, at room temperature for 4 min) and fixed in 0.3 M sodium thiosulfate (Sigma) for another 4 min. Finally, they were washed thoroughly with distilled water, air dried, and then mounted using Aqua Poly/Mount (Polysciences). The autoradiograms were visualized and examined under a Zeiss Axiophot microscope in dark and bright fields. The photographic emulsion (Ilford K5 diluted 1:1 in deionized water) was melted in a glass vessel at about 40°C in a water bath in a dark room. The microdissected vascular segments were coated by dipping the slides into the emulsion. The slides were held vertically to dry in air and then kept in a light-tight box at 4°C for 10–14 days. After exposure, the coated slides were developed in Phenasil (Ilford, 1:4 in deionized water, at room temperature for 4 min) and fixed in 0.3 M sodium thiosulfate (Sigma) for another 4 min. Finally, they were washed thoroughly with distilled water, air dried, and then mounted using Aqua Poly/Mount (Polysciences). The autoradiograms were visualized and examined under a Zeiss Axiophot microscope in dark and bright fields. The photographic emulsion (Ilford K5 diluted 1:1 in deionized water) was melted in a glass vessel at about 40°C in a water bath in a dark room. The microdissected vascular segments were coated by dipping the slides into the emulsion. The slides were held vertically to dry in air and then kept in a light-tight box at 4°C for 10–14 days. After exposure, the coated slides were developed in Phenasil (Ilford, 1:4 in deionized water, at room temperature for 4 min) and fixed in 0.3 M sodium thiosulfate (Sigma) for another 4 min. Finally, they were washed thoroughly with distilled water, air dried, and then mounted using Aqua Poly/Mount (Polysciences). The autoradiograms were visualized and examined under a Zeiss Axiophot microscope in dark and bright fields. The photographic emulsion (Ilford K5 diluted 1:1 in deionized water) was melted in a glass vessel at about 40°C in a water bath in a dark room. The microdissected vascular segments were coated by dipping the slides into the emulsion. The slides were held vertically to dry in air and then kept in a light-tight box at 4°C for 10–14 days. After exposure, the coated slides were developed in Phenasil (Ilford, 1:4 in deionized water, at room temperature for 4 min) and fixed in 0.3 M sodium thiosulfate (Sigma) for another 4 min. Finally, they were washed thoroughly with distilled water, air dried, and then mounted using Aqua Poly/Mount (Polysciences). The autoradiograms were visualized and examined under a Zeiss Axiophot microscope in dark and bright fields.

Generation of the P2X1 antibody. Although there is a significant conservation of sequence identity within the transmembrane domains of the seven P2X receptor subtypes, we have used sequence analysis to identify which regions are the least conserved among members of this ligand-gated ion channel family to generate subtype-selective antibodies. One such region, the COOH terminus, has been used to generate a P2X1-selective polyvalent antiserum. The COOH-terminal 15 amino acids of the rat and human P2X1 receptor proteins are identical to one another but different from the sequences present in the other six P2X receptor subtypes. A synthetic peptide comprising amino acid residues 85–39 of the P2X1 receptor (NH2-ATSSTLGLQENMRTS-COOH; GenBank accession no. X80477) was covalently linked to keyhole limpet hemocyanin, and rabbits were immunized with the conjugated peptide in multiple monthly injections (performed by Research Genetics, Huntsville, AL). IgG fractions were isolated from the immune sera and the preimmune controls, using chromatography on DEAE Affi-Gel Blue (Bio-Rad) or following the method of Harboe and Ingild (17).

The specificity of the antisera was verified by immunoblotting with membrane preparations from cloned P2X1 receptor-expressing CHO-K1 cells. Cell pellets of CHO-K1 cells expressing the human P2X1 receptor were homogenized in 10× (wt/vol) ice-cold homogenization buffer (50 mM Tris, 1 mM EDTA, pH 7.4, supplemented with 2 µg/ml soybean trypsin inhibitor and 10 µg/ml bacitracin). The homogenate was then centrifuged at 45,000 g for 20 min. The resulting pellet was resuspended in 1× (original wt/vol) homogenization buffer using three 30-s full-setting bursts with a Polytron homogenizer. The homogenate was centrifuged at 3,000 g for 10 min, and the supernatant was reserved on ice. The pellet was resuspended in the same volume of homogenization buffer and rehomogenized. This homogenate was centrifuged as before, and the supernatant was combined with the first. The supernatant was then centrifuged at 45,000 g for 20 min. The resulting pellet was resuspended in 1× (original wt/vol) homogenization buffer. Membrane preparations were separated on 10% SDS-polyacrylamide gels. Proteins were then electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 3 h at 4°C in 50 mM Tris, pH 8.0, with 3% nonfat dry milk (Sigma). The blots were then probed overnight with either preimmune sera, immune sera, or immune sera preabsorbed for 2 h at 4°C, with an excess of the peptide used to raise the antisera. The membranes were washed three times for 20 min each time in 50 mM Tris and 1 mM EDTA, pH 7.4, and incubated with horseradish peroxidase-conjugated Protein A (Bio-Rad, Richmond, CA). The blots were washed as before and visualized using the Renaissance chemiluminescence system from DuPont-NEN (Boston, MA) and X-OmatAR film (Kodak, Rochester, NY).

 Immunohistochemistry. Kidneys obtained from the male Sprague-Dawley rats (as above) were embedded in OCT compound (BDH) and frozen in isopentane precooled in liquid nitrogen. The tissues were sectioned at a thickness of 10 µm in a cryostat, collected on gelatin-coated slide, and air dried at room temperature. The slides were then stored at −20°C ready for immunohistochemistry.

The frozen kidney sections and microdissected vascular structures were allowed to equilibrate at room temperature for at least 10 min and then fixed in 4% formaldehyde-0.03% picric acid in 0.1 M phosphate buffer (pH 7.4) for 2 min. After the endogenous peroxidase activity was blocked in 50% methanol-0.3% hydrogen peroxide (H2O2) for 10 min, the sections were incubated for 3 days at 4°C in the primary P2X1 antibody, diluted in 1:100 (optimal dilution determined after previous titration experiments) in 10% normal horse serum/phosphate-buffered solution (PBS) to block the nonspecific binding sites. The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch), and the avidin-biotin complex technique (Vectastain Elite ABC kit) was used to increase the sensitivity of antigen localization. Diaminobenzidine tetrahydrochloride and H2O2 were used as the enzyme substrate that produced a reddish brown precipitate in the sections.

Three different sets of control experiments were performed to establish a specific immunoreaction: sections were incubated with P2X1 antibody pretreated with an excess of the homologous peptide antigen, the primary antibody was replaced with nonimmune rabbit antisera, or the primary P2X1 antibody was omitted.

**RESULTS**

The antibody raised against P2X1 purinoreceptor labeled the intrarenal vasculature from the branch renal artery to the afferent arterioles but not the efferent arterioles or glomeruli (Figs. 1 and 2). The P2X1 immunoreactivity was found predominantly on the vascular smooth muscle cells, and no immunoreactivity was detected in the tubules. The control preparations showed no P2X1 immunoreactivity. The immunoblotting experiments revealed that the P2X1 antisera recognized the recombinant P2X1 receptor expressed in CHO-K1 cells (60-kDa band) and that preabsorption with an excess of the synthetic peptide used for the generation of the antibody eliminated this immunoreactivity (Fig. 3). No signal was observed with the preimmune serum. The [3H]β,β-methylene ATP radioligand
Fig. 1. Immunohistochemical staining (reddish brown) for P2X1 purinoceptors along the microdissected intrarenal vasculature. a: Low magnification (×35) showing arcuate artery (ArA), interlobular artery (IA), and afferent arteriole (AA). b: Higher magnification (×280) of the area B of a showing P2X1 immunoreactivity on the vascular smooth muscle cells on IA and AA. c: High-magnification (×280) light micrograph showing positive P2X1 immunoreactivity on vascular smooth cells of the microdissected IA and AA. No significant immunostaining was detected in the glomerulus (G).
binding study also demonstrated the distribution of P2 receptors along the intrarenal vascular tree. The silver grains were seen more clearly using a confocal microscope in reflection mode (Fig. 4). The autoradiographic findings are consistent with the presence of P2X₁ receptors on interlobular arteries and afferent arterioles but not on efferent arterioles (Fig. 5).

**DISCUSSION**

Sources of extracellular ATP in the kidney include perivascular nerves (co-released with other neurotransmitters such as norepinephrine from sympathetic nerve terminals), erythrocytes, platelets, mast cells, and endothelium (9, 16). The concentration of ATP in cells is in the millimolar range, and a significant proportion of a cell's ATP can be released without compromising viability. Extracellular ATP is labile, and the actual concentrations achieved adjacent to potential target cells are probably in the millimolar range, whereas circulating concentrations are <1 µM (24). Extracellular ATP is rapidly hydrolyzed by membrane-bound ectoenzymes to adenosine, which can then bind to P1 purinoceptors (A₁/A₂) (16).

The immunohistochemical findings of specific labeling of interlobular arteries and afferent arterioles with the P2X₁ antibody were consistent with our radioligand binding results using the selective radioligand [³H]α,β-methylene ATP, but to what is this ligand binding? It was originally claimed to bind to P2X purinoceptors...
We now know that only two of the P2X receptor subtypes, P2X1 and P2X3, are highly sensitive to \( \alpha, \beta \)-methylene ATP; the P2X3 is found mainly on sensory neurons (11). Thus, when \( ^{3}H \alpha, \beta \)-methylene ATP binds to smooth muscle cells, it is a specific marker of the P2X1 purinoceptor subtype. Moreover, our own additional immunolocalization studies using specific polyclonal antibodies to P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7, developed by our collaborators in Roche Biosciences, only detected immunoreactivity for P2X2 and P2X6 in the renal vasculature (unpublished results).

The P2X1 purinoceptor subtype is present on vascular smooth cells of arcuate arteries, interlobular arteries, and afferent arterioles. That this purinoceptor is localized to vascular smooth muscle is confirmed by a matching pattern of immunostaining using anti-chicken gizzard smooth muscle myosin (courtesy of Prof. U. G. Stewart, Technical University Darmstadt). No P2X1 immunoreactivity was detected in efferent arterioles, glomeruli or renal tubules.

Our findings support the functional evidence that extracellular ATP has a paracrine or neurocrine role in controlling the renal microvasculature. Recent studies have indicated that extracellular ATP can influence renal blood vessels directly by binding to P2X (vasoconstriction) and P2Y (vasodilatation) receptors (12, 15) and not as a result of enzymatic breakdown to adenosine. Studies using isolated blood-perfused juxtamedullary nephrons have shown that the afferent arteriole is very sensitive to ATP (22). In the afferent arteriole of this preparation, ATP induces a rapid and sustained vasoconstriction. In contrast, studies of the arcuate and interlobular arteries show only transient vasoconstriction and require higher concentrations of ATP (22). Extracellular ATP does not cause vasoconstriction of the efferent arteriole (22). P2Y receptor stimulation on endothelial cells induces release of nitric oxide and prostacyclin from these cells and causes large preglomerular arcuate artery vasodilatation (20). As yet, there is no functional evidence for the presence of P2Y receptors on smaller intrarenal vessels (interlobular arteries, afferent and efferent arterioles).

In vivo micropuncture experiments on the influence of extracellular ATP on the tubuloglomerular feedback (TGF) mechanism have reported that ATP, like adenosine, causes vasoconstriction of the afferent arteriole (pharmacologically P2X dependent); if generated locally, it could affect the TGF response (23). The TGF mechanism is a negative feedback system in which changes in a luminal signal (tubular fluid flow rate and sodium chloride delivery and transport) at the macula densa region of the loop of Henle (thick ascending limb) are sensed and then transduced to increases or decreases in afferent arteriolar tone. This mechanism is important in maintaining normal sodium balance and extracellular fluid volume through changes in GFR. Unlike adenosine, ATP is more likely to have a modulatory role in TGF. As well as its direct vasoconstrictor effect, ATP can also reduce TGF responsiveness to increases in distal tubular flow rate and sodium chloride delivery (23). More recent studies have shown that P2X receptors are also involved in renal autoregulation and control of afferent arteriole resistance in response to increased renal perfusion pressure (19). Extracellular ATP could also have an indirect effect on the efferent arteriole via the local release of renin from P2Y receptor activation and subsequent generation of angiotensin II (2, 13).

In summary, we have demonstrated that intrarenal P2X1 purinoceptors are confined to the vasculature but are not found on the efferent arterioles and glomeruli. These findings are consistent with previously published functional evidence that P2X purinoceptors may have an important role in controlling intrarenal hemodynamics.

Address for reprint requests: G. Burnstock, Autonomic Neuroscience Institute, Royal Free Hospital School of Medicine, Rowland Hill St., London NW3 2PF, UK.

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