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P2Y Receptors Present in the Native and Isolated Rat Glomerulus

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P2 receptors, rat glomerulus · P2Y receptors · Purinergic · Extracellular nucleotides · ATP

Abstract
Extracellular ATP can mobilize intracellular calcium in rat glomeruli by interacting with P2Y receptors. However, the identity of the receptor subtypes involved is not known. In the present study, we have used RT-PCR to identify mRNAs for specific P2Y receptor subtypes expressed in the rat glomerulus: mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was detected. Functional expression of $P2Y_1$ and $P2Y_2$/$P2Y_4$, but not $P2Y_6$, receptors in intact glomeruli was confirmed by measuring the relative stimulation of the inositol phosphate pathway induced by selective agonists of a particular receptor subtype. Finally, we have used available polyclonal antibodies to confirm the expression of $P2Y_1$ and $P2Y_2$ in the glomerulus, in mesangial cells and glomerular epithelial cells (podocytes), respectively; but we could not demonstrate $P2Y_4$ or $P2Y_6$ receptor expression by this means. In a separate series of experiments, we have examined the possibility that intra-renal sympathetic nerve terminals are a source of extracellular ATP and that this would be supported, though not excluded, by supersensitivity to ATP following denervation. Nucleotide-induced stimulation of the inositol phosphate pathway was measured in both control rats and rats that had been sympathectomized by intraperitoneal injection of 6-hydroxydopamine. The response to norepinephrine was measured as a positive control. In the sympathectomized rats, the effect of norepinephrine was significantly enhanced, whereas ATP-induced inositol phosphate production was unaffected, being similar in both groups of animals.

Introduction

Several groups have clearly demonstrated that extracellular ATP mobilizes intracellular free calcium in renal epithelia [1, 2], an action mediated by P2Y receptors that are generally coupled via phospholipase C (PLC) to the activation of the inositol phosphate (IP) pathway [3].

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Functional evidence for the presence of P2Y receptors in the intact mammalian glomerulus has been found previously by measuring ATP-induced rises in intracellular calcium ($[Ca^{2+}]_i$) [1] and changes in glomerular size [4], but a search of the literature reveals only limited information on the nature and role of these receptors.

RT-PCR studies have shown the presence of $P2Y_1$ [5], $P2Y_2$ [6, 7], $P2Y_4$ [6] and $P2Y_6$ [5, 7] mRNA in the intact glomerulus, although at the protein level only functional and/or immunochemical expression of the $P2Y_1$ [4, 8] and $P2Y_2$ receptors [7], respectively, has been reported. Moreover, the expression pattern of $P2Y_1$ and $P2Y_2$ receptors in the different cell types comprising the glomerulus has not been investigated, probably because of the complex structural architecture of the glomerulus, which consists of a central glomerular tuft of endothelial (~20%) and mesangial (~25%) cells, encapsulated by a double layer of visceral (podocytes) and parietal epithelial (~55%) cells [9].

Currently, most of the detailed information on glomerular P2Y receptors comes from studies of cultured cells and suggests the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes in glomerular mesangial cells [6, 10, 11], $P2Y_1$, $P2Y_2$ and $P2Y_6$ in glomerular epithelial cells (podocytes) [5], and the $P2Y_2$ receptor in glomerular endothelial cells [12, 13]; all are based on mRNA detection and/or agonist profiling, rather than direct detection of protein expression. Moreover, findings in cultured cells can be confounded by the tendency for these cells to de-differentiate and to acquire properties that are different from their native source [14].

Therefore, the main aim of the present study was to try and identify in native intact glomeruli which P2Y receptor subtypes are present, in which cell type, and how functional they might be, by using a combination of experimental techniques. Firstly, RT-PCR was used to characterize the different P2Y receptor mRNAs expressed in isolated glomeruli ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$). Secondly, available antibodies against $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were used to detect and localize expression of receptor proteins by immunohistochemistry. Thirdly, measurements of nucleotide-induced phosphoinositide production and increases in intracellular calcium ($[Ca^{2+}]_i$) were used to identify which of these receptors are functionally active.

Another aim of this study was to explore (indirectly) a potential source of extracellular ATP (which is still speculative [15]) that could activate these P2 receptors. Studies have suggested that for some nephron structures the renal sympathetic nerves might be a source of ATP. Indeed, it is well established that ATP is a co-transmitter with norepinephrine in renal nerves and that at low frequency electrical stimulation ATP is the main transmitter released by these fibres [16], and renal nerve stimulation can affect glomerular size [17]. To investigate further the potential role of renal sympathetic nerves in supplying extracellular ATP to the glomerulus, we measured nucleotide-stimulated IP production in chemically sympathectomized animals to determine if there is an increase in sensitivity to purines – denervation supersensitivity – as has been reported for catecholamines (though not yet for ATP) in arteries following sympathectomy [18].

Methods

Isolation of Rat Glomeruli and Parietal Sheets

All experiments were performed on glomeruli isolated from male Sprague-Dawley rats (180–220 g) following anaesthesia with sodium pentobarbital (Nembutal; 50 mg/kg body weight, i.p.). In one group of animals, regression of sympathetic nerve terminals was induced by injection of 6-hydroxydopamine (6-OHDA). Briefly, sympathectomy was performed by injection of 6-OHDA on 2 consecutive days. On the first day, 6-OHDA was injected intraperitoneally at a concentration of 100 mg/kg, and on the second day it was injected at a concentration of 250 mg/kg. On the third day, the kidney was removed under anaesthesia, as described.

The left kidney was perfused via the renal artery with 5 ml of Hepes-buffered saline solution (HBSS; containing (in m*M*): 140 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 NaH_2PO_4, 1.0 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 2 NaOH, 5 glucose, 3% dextran and 0.1% bovine serum albumin, pH 7.4) to remove blood, and was then rapidly removed. The renal cortex was chopped finely and then filtered through nylon mesh (Precision Textiles Ltd, Lancs., UK) of decreasing size: 150, 100 and 80 μm. Glomeruli were retained on the last of these meshes and re-suspended in a few millilitres of HBSS. Glomeruli were then selected under stereomicroscopic observation to be intact (with parietal sheet), of uniform size and free of either tubular or microvasculature fragments. All procedures were performed at 4°C. In some experiments, parietal sheets were microdissected from isolated glomeruli as previously described [19, 20]. Most of the parietal sheets used were attached to the glomerulus and appeared as a thin, lid-like flap adjacent to the glomerulus.

Extraction of mRNA and RT-PCR

RNA was extracted from isolated glomeruli using a micro-method adapted from the guanidium thiocyanate phenol/chloroform method, as described previously [5, 21]. Briefly, pools of isolated glomeruli were transferred with 5–10 μl of microdissection medium into 400 μl of denaturing solution (4 *M* guanidium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 m*M* β-mercaptoethanol and 20 μg of yeast tRNA). After phenol/chloroform extraction and isopropyl alcohol precipitation, the final pellet was vacuum-dried and re-suspended in a RNA dilution buffer (10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA, 2 m*M* dithiothreitol, 40 units/ml ribonuclease inhibitor (RNasin; Promega Corp., Madison, Wisc., USA)). It was determined earlier that the yield of this extraction procedure was in

excess of 90% [21]. Specific primers were selected from the sequence of the rat $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and β-actin receptor cDNA using Oligo Primer analysis software (MedProbe, Oslo, Norway). The sequence of the P2Y primers was as already reported [2, 22]. The intron-spanning β-actin primer sequence was: sense ACCTTCAACACCCCAGCCATGTACG and anti-sense CTGATCCACATCTGCTGGAAGGTGG. RNAs extracted from these glomeruli (~2.5 glomeruli per reaction) were reverse transcribed for 50 min at 42°C with 0.5 μg Oligo(dT) 12–18 Primer, using a first-strand cDNA synthesis kit for RT-PCR (Gibco BRL Superscript II RNase H- reverse transcriptase). After denaturation at 95°C for 3 min, 5% of the resulting product was used as a template with PCR Core System I (Promega). For each receptor, 35 PCR cycles were carried out under the following conditions: 95°C, 30 s; 62°C, 1 min (annealing); 72°C, 1 min (extension), 5 cycles; 95°C, 30 s; 56°C, 1 min; 72°C, 1 min, 30 cycles plus a last additional cycle with a 10-min extension stage. For β-actin, only 25 cycles were carried out as follows: 95°C, 30 s; 65°C, 45 s; 72°C, 1 min, 25 cycles plus a last cycle with a 10-min extension stage. The resulting PCR products were resolved on a 2% (wt/vol) agarose gel containing 10 μg/ml ethidium bromide and visualized under UV illumination. The nature of the PCR product was confirmed by sequencing (Oswel DNA Sequencing Laboratories, Southampton, Hants., UK). In all experiments, the presence of possible contaminants was checked by control RT-PCR reactions on samples in which either mRNA was excluded (blank) or in which reverse transcriptase had been excluded from the reverse transcription mixture (RTase–). All experiments were carried out on the same PCR machine; each P2Y subtype was tested for at least twice in extracts from each of 5 animals.

Measurement of Phosphoinositide Metabolism in Intact Glomeruli

The method has been described in detail before [23]. Briefly, glomeruli were labelled for 2 h in a humid atmosphere (at 37°C) in HBSS (2 m*M* Ca^{2+}) containing ~ 300 μCi/ml *myo*-[^{3}H]inositol (Amersham, Berks., UK). After labelling, glomeruli were extensively rinsed at room temperature to remove extracellular *myo*-[^{3}H]inositol and then batches of 10 were transferred in 2 μl HBSS to glass test tubes containing 48 μl of HBSS. Incubation of glomeruli with agonist was initiated by addition of 50 μl of HBSS containing the agent (at double concentration) and LiCl (final concentration of 10 m*M*).

Reactions were terminated after 15 min by adding 940 μl chloroform:methanol (1:2, vol:vol) and 200 μl of 5 m*M* EDTA-tris(hydroxymethyl)aminomethane (5 m*M*; pH 7.0). Water and chloroform (300 μl of each) were added to the reaction tube, which was then centrifuged for 5 min at 3,000 *g* at 4°C to separate phases. For each sample, the upper hydrophilic phase was removed for chromatography, while 500 μl of water and 500 μl of methanol were added to the remaining, hydrophobic phase. After vortexing and a repeat centrifugation, the lower phase containing phosphoinositides was recovered and evaporated to dryness in counting vials before the radioactivity was measured.

Radioactivity associated with free inositol, glycerophosphoinositol (GPI) and with IPs was separated by chromatography as described previously [23]. Samples were applied to columns containing 0.25 g Dowex AG1-X8 resin and free inositol, GPI and IP were successively and respectively eluted with the following: (1) Hepes-NaOH (3 m*M*), pH 7.0; (2) ammonium formate (30 m*M*), and (3) ammonium formate (1 *M*) and formic acid (0.1 *M*). The radioactivity contained in each eluate and in the phosphoinositide extract was counted by β-emission spectrometry following addition of 15 ml Aquasol-2 scintillation cocktail (Canberra Packard, UK) to each vial. Five replicates were performed for each condition in each experiment and used to give an average value per rat. These data were obtained following conversion of the counts in each fraction to dpm in order to account for variable quenching. The results from these assays are expressed as the percentage of the total radioactivity present in the IPs fraction as this has previously been shown to correlate well with agonist-stimulated IP production [23].

Measurement of Intracellular Free Calcium

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured radiometrically either on slit-open glomeruli or on isolated parietal sheets, deposited onto thin glass coverslips and embedded in 1% agarose (type IX), as previously described [20]. After loading with fura 2-AM (5 μ*M* in microdissection medium; Molecular Probes, USA) for 1 h at room temperature, these structures were superfused with HBSS at a rate of ~ 1 ml/min (corresponding to an exchange rate of the fluid covering the tubule of 8–10 times/min). Following a 5-min equilibration period, fura 2 fluorescence was measured using a standard photometric set up (PTI Photoscan II System, Kontron) [20]. All solutions were stored in individual reservoirs at room temperature until use; the temperature was raised to 37°C just prior to entry to the perfusion chamber. Following subtraction of autofluorescence from the fluorescence intensities of fura 2 at 340 and 380 nm, $[Ca^{2+}]_i$ was calculated from the standard equation [24] using a dissociation constant of fura 2 for calcium of 224 nm.

Immunohistochemistry

Sprague-Dawley rats (250 g) were killed by intraperitoneal injection of 90 mg/kg Sagatal and the left kidneys were removed. The tissue was embedded in OCT compound (BDH/Merck, Leicester, UK) and frozen in iso-pentane pre-cooled in liquid nitrogen. The tissues were sectioned at 8 μm using a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. The slides were stored at –20°C and allowed to return to room temperature for at least 10 min prior to use. Rabbit polyclonal antibodies to P2Y receptor subtypes were obtained from Alomone Laboratories (Jerusalem, Israel), with the exception of $P2Y_6$, which was a gift from Prof. Jens Leipziger [25]. The specificity of P2 subtype-specific antibodies was immunohistochemically determined by replacement of primary antibody with non-immune rabbit serum. Pre-absorption of P2Y subtype antibodies with excess of the appropriate synthetic peptide used for generation of the antibodies eliminated immunoreactivity. For experiments to detect $P2Y_4$ immunoreactivity, the avidin-biotin (ABC) technique was used as previously described [26]. For dual staining experiments, an immunofluorescent technique was employed. The slide-mounted sections were fixed in 4% formaldehyde in phosphate-buffered saline for 2 min. Non-specific protein binding sites were blocked by a 20-min incubation in 10% NHS. P2 subtype antibodies were diluted to 1.25–5 μg/ml in 10% NHS and the sections were incubated overnight with antibody at room temperature. Marker antibody for glomerular epithelial cells (podocytes), rabbit anti-WT-1 [27], was obtained from Santa Cruz Biotechnology (Calif., USA) and marker antibody for mesangial cells, mouse anti-Thy-1 [19], was obtained from Abcam Ltd (Cambridge, UK). The secondary antibody for $P2Y_1$ was donkey anti-rabbit Cy3 (Jackson Immunoresearch, Luton, UK) and for anti-Thy-1 was donkey anti-mouse Oregon green. Antibodies for $P2Y_2$ and the podocyte marker WT-1 were raised in the same species, therefore further steps

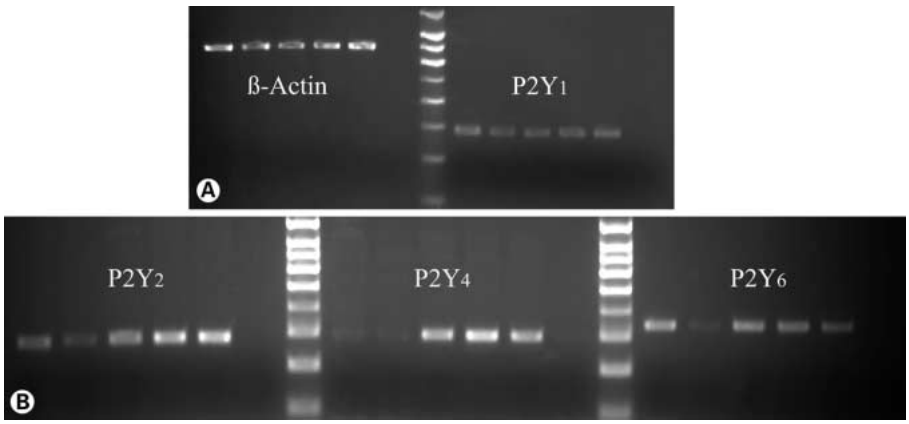

Fig. 1. Sample RT-PCR gels for (**A**) β-actin (699 base-pairs) and $P2Y_1$ (289 base-pairs) and (**B**) $P2Y_2$ (279 base-pairs), $P2Y_4$ (294 base-pairs) and $P2Y_6$ (339 base-pairs) mRNA in glomeruli isolated from 5 rats. Lane 6 (blank) is the negative control (PR mix plus primers, but not RNA).

were required to ensure elimination of cross-reactivity. $P2Y_2$ receptors were detected by tyramide signal amplification (Renaissance, TSA indirect, NEN, USA) using antibody concentrations below the detection limit of a fluorophore-coupled secondary antibody. After incubation overnight with anti-$P2Y_2$, the layers of secondary antibody were biotinylated with donkey anti-rabbit IgG (Jackson Immunoresearch), ExtrAvidin peroxidase (Sigma, Poole, UK), the tyramide signal amplification solution was applied for 8 min, and the final layer was streptavidin fluorescein (Amersham Lifescience, Bucks., UK). Finally, sections were incubated overnight with anti-WT-1 and then detected with donkey anti-rabbit Cy3. The results were analysed and recorded using a Zeiss Axioplan light/fluorescent microscope (Jena, Germany) and images were captured using a Leica DC200 digital camera (Leica, Germany).

Statistics

Data are presented as mean ± SE. Where appropriate, statistical significance was determined by unpaired t-tests for two-group comparison or by ANOVA for multiple comparisons using GraphPad Instat software and curve fitting was done using GraphPad Prism software (GraphPad Software Inc., San Diego, Calif., USA). A difference with $p < 0.05$ was considered to be statistically significant.

Results

Molecular Characterization of P2 Receptors in Intact Glomerulus

To identify P2Y receptor subtypes at the molecular level, we performed RT-PCR experiments on RNA extracted from rat glomeruli. Sample gels, shown in figures 1A and B, show the presence of mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, with some variation in intensity between extracts from different rat kidneys.

Immunohistochemistry for P2Y Receptors

$P2Y_2$ and $P2Y_1$ receptors were immunolocalized to the glomerulus (fig. 2A, B and C, D, respectively). In contrast, $P2Y_4$ and $P2Y_6$ receptors were not detected in any glomerular structure (fig. 2E–G). Figure 2A shows fluorescent dual labelling for $P2Y_2$ (green) and anti-WT-1 antibodies (red), co-localizing in yellow in glomerular epithelial cells (podocytes). Although discrete, some staining of the parietal sheet and basolateral membrane of proximal tubule cells could also been detected (fig. 2A). Figure 2B shows no $P2Y_2$ co-localization with an endothelial cell marker (Ox 43). No $P2Y_2$ labelling was clear or strongly visible on mesangial cells. However, the intensity of fluorescence in the glomerulus was high relative to adjacent structures, making it difficult to detect lower levels of specific antibody binding and hence protein expression. Figure 2C shows $P2Y_1$ (red) and anti-Thy1 (green) fluorescent antibody staining co-localizing in yellow in mesangial cells. Figure 2D shows no $P2Y_1$ co-localization with the endothelial cell marker (Ox 43). No staining was visible on podocytes or surrounding tubules (again, at this level of fluorescence intensity). By contrast, $P2Y_1$ labelling as a thin and broken red line was visible in the periglomerular region (thick arrows). This is likely to correspond to interstitial and periglomerular fibroblasts, as suggested by co-localization with ecto-5′-nucleotidase (not shown); intense $P2Y_1$ labelling was also observed on peritubular fibroblasts (not shown) [28]. Figures 2E and F show the absence of glomerular staining for $P2Y_4$, in contrast to the intense labelling of tubules seen in the same sections. Figure 2G shows a lack of glomerular $P2Y_6$ immunostaining, but it does confirm proximal tubule staining, as noted originally by Leipziger [pers. commun.].

Inositol Phosphate Metabolism in Intact Glomeruli

To provide functional confirmation of the molecular findings, we investigated the ability of extracellular nu-

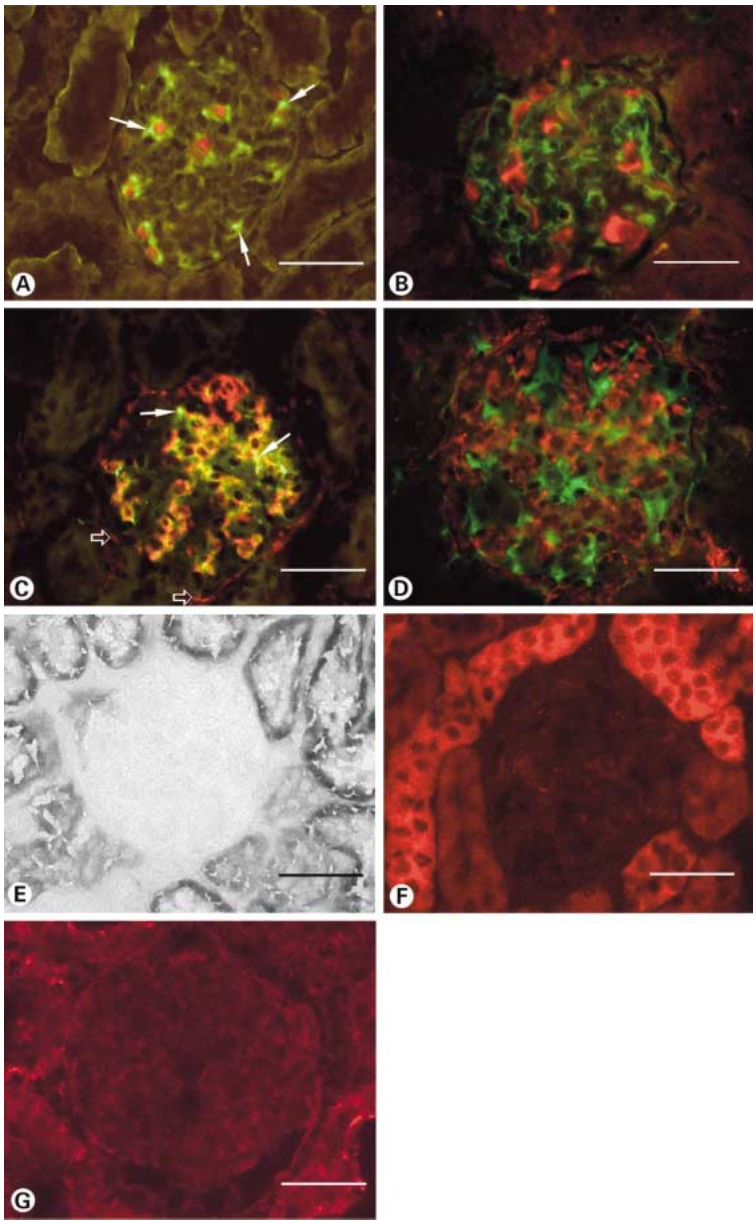

Fig. 2. Photomicrograph showing (**A**) $P2Y_2$ receptor immunopositive podocytes in green (arrows) co-localized with the podocyte nuclear protein Wilms tumour-1 (WT-1) in red – faint staining of the parietal and proximal tubule is also visible; (**B**) no co-localization of $P2Y_2$ receptor immunoreactivity (red) with the endothelial cell marker Ox 43 (green); (**C**) $P2Y_1$ receptor immunoreactivity (red) in a population of mesangial cells co-localized with the mesangial cell marker thymocyte-1 (thy-1) (green) (areas of co-localization appear yellow – white arrows) – peripheral staining (thicker arrows) is not the parietal sheet, but is on periglomerular fibroblasts or interstitial cells (confirmed by co-staining with a ecto-5′-nucleotidase antibody (37) – not shown); (**D**) no co-localization of $P2Y_1$ receptor immunoreactivity (red) with the endothelial cell marker Ox 43 (green); (**E, F**) negative glomerular immunoreactivity for $P2Y_4$, although the renal tubules are strongly immunopositive (scale bars = 50 μm); (**G**) negative glomerular immunoreactivity for $P2Y_6$.

cleotides to stimulate the phosphoinositide pathway in intact glomeruli. In these studies, the distribution of radioactivity between the four inositol-containing pools was similar to that previously reported in rat glomeruli [29]. Approximately 30–40% of the total radioactivity was present in each of the inositol and phosphoinositide fractions, with the remainder distributed between the GPI and IPs pools. In the figures presented, only the data from the IPs fraction are shown.

ATP significantly increased IPs production in the intact glomerulus (fig. 3A; $p < 0.001$) with maximal stimulation of this pathway achieved between 30 and 100 μ*M*, and significant increases in production of IPs being observed at a concentration of 3 μ*M* ($p < 0.01$). Both ATPγS, which binds preferentially to $P2Y_2$, but also to $P2Y_4$, and the $P2Y_1$-selective agonist 2MeSADP, elicited a significant stimulation of this pathway (fig. 3B). In contrast, despite the presence of mRNA, the selective $P2Y_6$ agonist UDP had no effect, consistent with a lack of functional expression of this receptor subtype in the glomerulus. Therefore, these data suggest that at least two PLC-coupled P2Y receptors are expressed in the intact glomerulus:

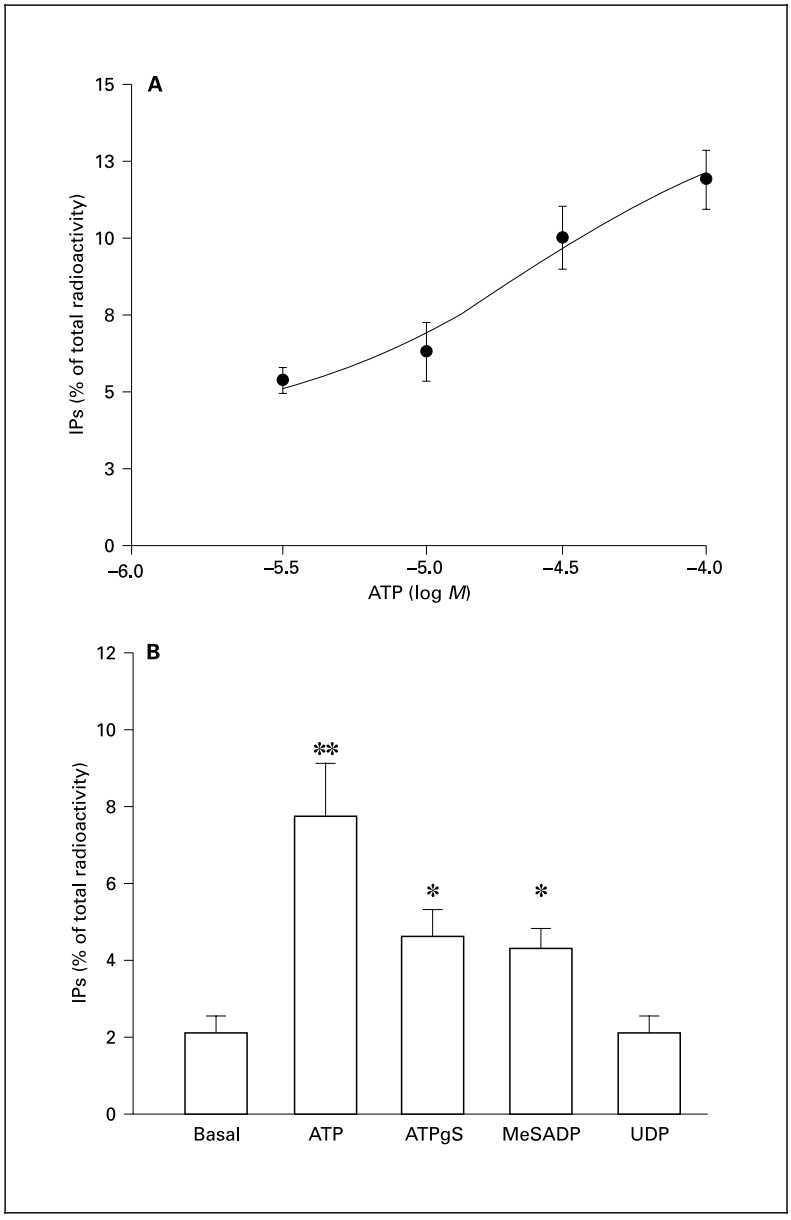

Fig. 3. Inositol phosphates (IPs) production in isolated rat glomeruli expressed as a percentage of total incorporated radioactivity: (**A**) concentration-response curve for ATP (a significant dose-dependent increase above basal – $p < 0.001$); (**B**) response to ATP and receptor-selective agonists (all at 30 μmol/l). Data are means ± SE from 3–6 rats; * $p < 0.05$, ** $p < 0.01$ versus basal level of production.

a $P2Y_1$-like receptor and a receptor with characteristics of $P2Y_2$ (or $P2Y_4$). In support of this, the effects of the receptor-selective agonists were approximately half that of ATP, which can activate all three subtypes.

P2Y-Related Increases in $[Ca^{2+}]_i$

Previous studies by Marchetti et al. [20] have shown that the parietal sheet of Bowman's capsule, which involves a single type of myoepithelial cell, can be separated and studied independently of the glomerular tuft, itself comprised of a mixture of intermingled cells – podocytes, endothelial and mesangial cells. In a separate set of experiments, we compared the functional expression of P2Y receptors in the parietal sheet and the decapsulated glomerulus to try and answer two questions: first, whether the apparent absence of labelling of the parietal sheet by the anti-$P2Y_1$, $P2Y_4$ and $P2Y_6$ antibodies revealed an absence of these receptors, or a limited sensitivity of the immunohistochemical approach; second, whether the lack of effect of UDP on IPs production in intact glomeruli could have been due to limited access of agonists to cell types located deep within the glomerulus. Due to the

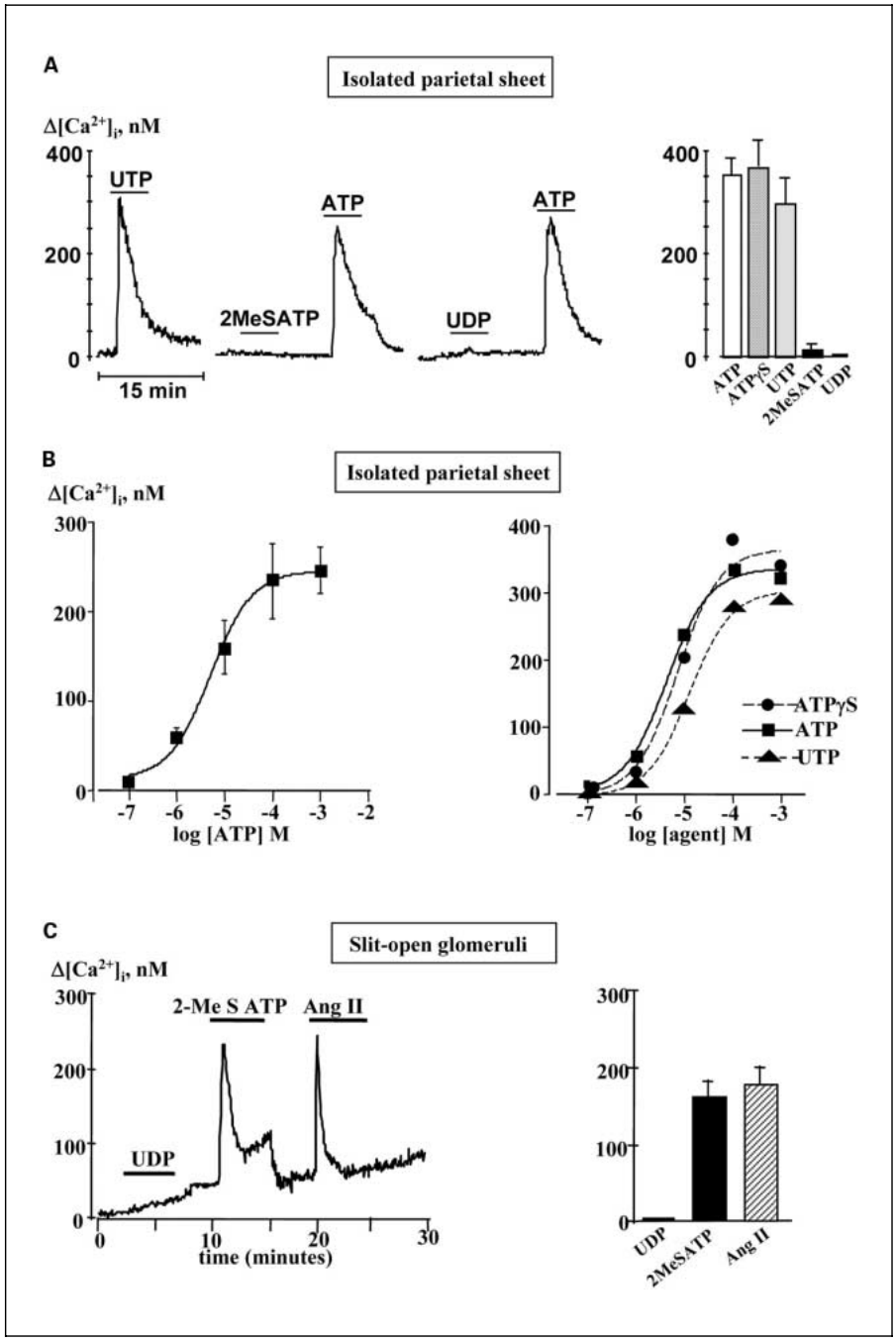

Fig. 4. Experiments on the isolated rat parietal sheet showing (**A**) representative traces of the effects of 2MesATP, ATP, UTP and UDP (all at 100 μmol/l) on $[Ca^{2+}]_i$; (**B**) a mean concentration-response curves for ATP (mean ± SE of at least 3 parietal sheet preparations) and typical agonist concentration-response curves for ATP, ATPγS and UTP (with basal $[Ca^{2+}]_i$ subtracted); (**C**) a trace showing the response of a decapsulated (no parietal sheet) slit-open glomerulus to UDP, 2MeSATP and angiotensin II (Ang II) and mean responses to these agents.

small number of parietal sheets that can be dissected in each experiment, we addressed these points by measuring cytosolic calcium, rather than IPs production, as an index of PLC activation.

The sample traces and mean $[Ca^{2+}]_i$ increases shown in figure 4A illustrate the pattern of response of the parietal sheet to P2Y agonists: 2MeSATP and UDP were without effect on $[Ca^{2+}]_i$, suggesting that neither $P2Y_1$ nor $P2Y_6$ receptors are expressed in this epithelium. As shown by the concentration-response curves for ATP, UTP and ATPγS (fig. 4B), with all agonists, maximal activation of the calcium pathway was achieved at 100 μ*M*, but much lower concentrations still produced significant increases in $[Ca^{2+}]_i$ (EC_{50} of ~ 5–10 μ*M*).

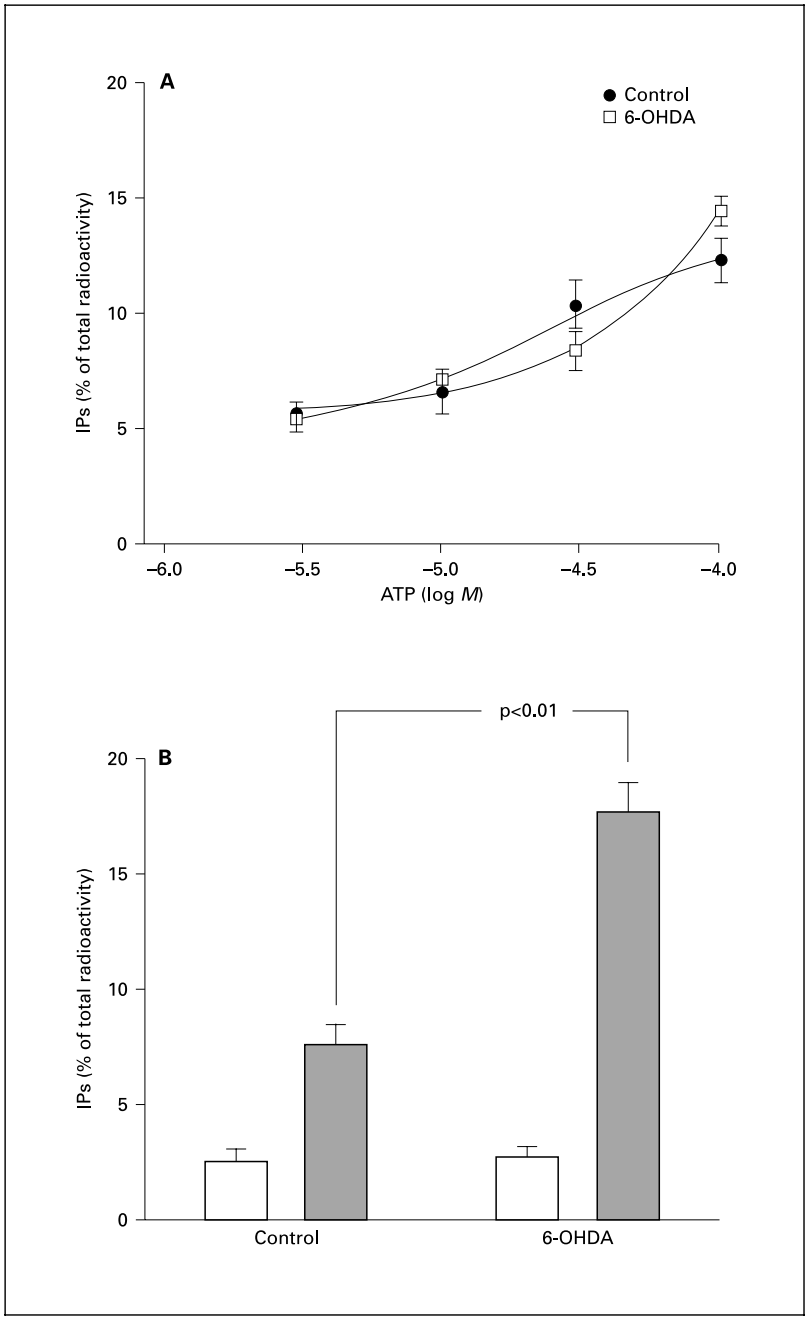

Fig. 5. A ATP-stimulated inositol phosphates (IPs) accumulation in control (closed circles) and 6-hydroxydopamine (6-OHDA)-treated (open squares) rats (a significant dose-dependent increase above basal in each group ($p < 0.001$), but no significant difference between control and 6-OHDA), and **B** basal (open bar) and noradrenaline-stimulated (hatched bar; 10 µmol/l) IPs production in control and 6-hydroxydopamine-treated rats. Data are means ± SE from 5–8 experiments and are expressed as a percentage of total incorporated radioactivity.

ATP, ATPγS and UTP were thus equipotent, suggesting the presence of a receptor with $P2Y_2/P2Y_4$ characteristics.

In decapsulated slit-open glomeruli (fig. 4C), a condition expected to allow free access of agonists to all cell types of the glomerular tuft, UDP was inactive, but the $P2Y_1$ agonist 2MeSATP, and the hormone angiotensin II [30] both increased $[Ca^{2+}]_i$. These data suggest that cell types of the glomerular tuft express $P2Y_1$, but not $P2Y_6$ receptors, again in keeping with our immunohistochemical data.

Effect of Chemical Sympathectomy on ATP-Induced Production of Inositol Phosphates

As a separate and related study, we tested the effect of 6-OHDA-induced sympathectomy on the response to

ATP in the isolated glomerulus. Basal levels of IPs were similar (control 2.52 ± 0.32% vs. 6-OHDA 2.23 ± 0.30%; NS), and the ATP-induced activation of this pathway was not different in the two groups of animals (fig. 5A). In contrast, sympathectomy enhanced the response to noradrenaline (10 μM) in the same experiments (fig. 5B).

Discussion

Receptors for extracellular purines and pyrimidines are grouped into two distinct families: ionotropic P2X receptors are ligand-gated ion channels, whereas P2Y receptors couple to effector pathways linked to G-proteins [3]. In the present study we have focused exclusively on P2Y receptors in the native rat glomerulus, rather than in derived constituent cells in culture and we have tried to combine their detection with evidence of functional expression, which has so far not been done.

Analysis of RNAs extracted from the intact glomerulus identified $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes (fig. 1), a finding in agreement with previous studies in cultured cells [6, 11, 13, 31, 32] and intact glomeruli [5–7]. However, the presence of mRNA does not always mean that the protein is expressed, or that it is functionally active. Up to now, protein detection has been investigated by pharmacological and immunochemical methods, which both have their limitations: immunofluorescence is limited by the availability of antibodies, their specificity and affinity; whereas pharmacological studies are limited by the selectivity of agonists and antagonists, and, for example, cannot distinguish easily between $P2Y_2$ and $P2Y_4$ receptors [33]. Therefore, we combined these techniques to try and provide a more reliable assessment of P2Y receptor subtype presence and activity within the intact glomerulus.

By measurement of nucleotide-stimulated production of IPs, we found evidence for the functional expression of at least two distinct subtypes of P2Y receptor within the intact glomerulus. The effect elicited by 2MeSADP (and to a lesser extent 2MeSATP) was consistent with the presence of $P2Y_1$ receptors [34], while the ability of ATPγS to activate the PLC pathway was more indicative of a receptor with $P2Y_2$/$P2Y_4$-like characteristics [35, 36]. It is noteworthy that ATP-induced production of IPs was approximately twice that of the more receptor-selective agents, consistent with the action of ATP at more than one discrete population of P2 receptor. Of interest, the $P2Y_6$-selective agonist UDP had no effect on IPs production in the intact glomerulus, despite the presence of its mRNA. Since it is believed that the metabotropic P2Y receptor family ubiquitously couples to the hydrolysis of phosphoinositides [3], these data seem to preclude the functional expression of $P2Y_6$ in the intact rat glomerulus.

Immunohistochemically, glomerular staining of $P2Y_1$ appears to be mesangial only (fig. 2C), confirmed by co-localization with the mesangial cell marker, Thy-1 [19]. This receptor also shows some extraglomerular staining in rat kidney (not shown), suggesting a wider expression on periglomerular (see fig. 4B – thick arrows) and peritubular capillary interstitial (rather than endothelial) cells [37], as confirmed by our use of endothelial and interstitial cell marker antibodies. The strong immunohistochemical expression of $P2Y_1$ on mesangial cells is consistent with this receptor mediating the glomerular IPs response to 2MeSADP (fig. 3B) and the calcium response of the decapsulated slit-open glomerulus to 2MeSATP (fig. 4C). It is consistent with the finding of Jankowski et al. [4] on the contraction response of intact glomeruli to $P2Y_1$-selective ATP analogues and also of cultured rat mesangial cells observed by Pavenstadt et al. [38]. However, it is at variance with previous studies on cultured mesangial cells that failed to detect evidence for a $P2Y_1$ pharmacological stimulation of cytosolic calcium [39], or of other signalling pathways [40–42].

In contrast to mesangial cells, we found no evidence for $P2Y_1$ receptors in podocytes, in myoepithelial cells of the parietal sheet (fig. 4A), or in glomerular endothelial cells (fig. 2D). This latter observation is in agreement with available data on cultured cells [5, 12, 13], but it is at odds with a recent study reporting that 2MeSATP relaxes precontracted glomeruli, an effect blocked by endothelial nitric oxide (NO) synthase inhibitors [43]. The authors of this study speculated that intracellular $[Ca^{2+}]_i$ spikes elicited by $P2Y_1$ agonists in endothelial cells could trigger NO release and cause mesangial cells relaxation via a paracrine effect. As already mentioned, our immunohistochemical approach is more likely to detect those receptors that exhibit high levels of protein expression and does not exclude the presence of other P2Y receptors expressed at low levels. Therefore, further studies using more sensitive direct techniques (perhaps as has been done in podocytes using dual photon confocal microscopy [44]) will be necessary to establish definitively whether or not $P2Y_1$ receptors are expressed in native glomerular endothelial cells.

Strong $P2Y_2$ immunostaining of podocytes (as shown by the podocyte WT-1 marker [27]) and faint $P2Y_2$ labelling of the parietal sheet of Bowman's capsule were

detected (fig. 2A). In contrast, we could not detect any $P2Y_4$ immunoreactivity in the glomerulus and it appeared to be expressed exclusively by the renal tubular epithelium (fig. 2E, F). These observations suggest that IPs production induced by ATPγS in glomeruli (fig. 3B) is predominantly $P2Y_2$-mediated and localized to podocytes, in keeping with the reported expression of this receptor in cultured podocytes [5]; calcium transients induced by low concentrations of UTP, ATP and ATPγS in epithelial cells of the isolated parietal sheet (fig. 4A, B) are likely to be due to the presence of $P2Y_2$ receptors. Conversely, the strong $P2Y_4$ antibody staining of cortical tubules suggests that it is this receptor that mediates, for the major part, the calcium responses observed earlier by Bailey et al. [2] in isolated proximal convoluted tubules in vitro. The presence of $P2Y_2$ has been widely reported in cultured mesangial cells on the basis of mRNA [6, 11, 45] and functional data [31, 39, 45–48], as well as probable mesangial cell expression in one immunohistochemical study of intact glomeruli [7]. Several groups have also provided pharmacological evidence for a PLC-coupled receptor in cultured glomerular endothelial cells [12, 13] that could be a $P2Y_2$- or $P2Y_4$-like receptor; however, in our study we could not localize either receptor to mesangial or endothelial cells. This could mean that $P2Y_2$ and/or $P2Y_4$ receptors are normally only weakly expressed in native mesangial and endothelial cells. No published data on native glomerular endothelium are available to confirm this interpretation, but in the study of Rost et al. [7] referred to above, kidney sections from normal rats showed only faint $P2Y_2$ immunolabelling, whereas $P2Y_2$ staining with a stronger and more characteristic mesangial pattern was seen 3–5 days after induction of an inflammatory and proliferative form of glomerulonephritis.

$P2Y_6$ staining was also not found in the glomerulus. Though expression of the $P2Y_6$ receptor has been detected in cultured podocytes [5], we could elicit no $[Ca^{2+}]_i$ or IPs responses to its preferred agonist UDP in the intact glomerulus, the isolated parietal sheet or the decapsulated slit-open glomerulus. This cannot be explained by a failure of agonist access in these models, or to inadequate sensitivity of our functional assays, as all responded to low concentrations of ATP (see fig. 3, 4); moreover, we have previously used changes in $[Ca^{2+}]_i$ to demonstrate renal tubular expression of $P2Y_6$ [22]. Therefore, our data indicate that $P2Y_6$ receptors are not expressed in native glomeruli, at least under normal conditions. Since Rost et al. [7] also found an increase in glomerular $P2Y_6$ mRNA following induction of glomerular inflammation, it is possible that, like $P2Y_2$, $P2Y_6$ protein expression increased as well.

Thus, we have shown that $P2Y_1$ and $P2Y_2$ receptors are expressed in native glomeruli of the normal rat, but we have found no evidence for expression $P2Y_4$ and $P2Y_6$ receptors. Podocytes and epithelial cells of the parietal sheet of Bowman's capsule are the main site of $P2Y_2$ expression and mesangial cells that of $P2Y_1$ expression. Relevant to all P2Y receptors is that their expression level may change in disease states.

Presently, the physiological roles of these P2Y receptors are still unclear. Calcium-mobilizing agents can cause morphological changes in several glomerular cell types, such as mesangial cells [17, 49], podocytes [50, 51] and the parietal sheet of glomerular epithelium [20], and ATP can produce both contraction and relaxation of intact glomeruli [4]. These studies suggest a possible physiological role for extracellular ATP in glomerular ultrafiltration that is independent of any effect it may have on the renal microvasculature [52]. Another reported effect of ATP, at least in cultured mesangial cells, is proliferation and a potential role in the response to glomerular injury [6, 7, 11, 53]. Further studies will be necessary to clarify the part played by each P2Y subtype in the control of glomerular function.

Finally, to investigate one potential source of extracellular ATP in the rat glomerulus, experiments were performed on animals treated with 6-OHDA. Previous studies have shown that this protocol, which destroys sympathetic efferent nerve terminal varicose fibres [54], leads to a reduction in the concentration of catecholamines in the renal cortex and medulla to almost undetectable levels [55]. In particular, 6-OHDA-induced denervation reduces norepinephrine content in the glomerulus [56] and increases α-adrenoceptor expression in the rat kidney as a whole [57]. Increases in the sensitivity to catecholamines in response to this protocol have been reported in other, non-renal tissues [58, 59]: in the rat vas deferens, 6-OHDA promotes supersensitivity to purinergic compounds [59]. In the present study we found that norepinephrine-induced activation of PLC in isolated glomeruli was significantly enhanced in denervated animals, confirming the effectiveness of this protocol. The mechanism of supersensitivity is unclear, but may involve a receptor-based post-synaptic effect [57, 60], as well as a loss of presynaptic norepinephrine uptake and storage [58]. However, in contrast to norepinephrine, ATP-stimulated IPs production was no different in the 6-OHDA-treated rats compared with controls. This apparent lack of effect of 6-OHDA sympathectomy on sensitivity to purines is not

a new finding: in the rabbit saphenous artery, such denervation induces increased sensitivity to norepinephrine, but not to purines [18], which was attributed to continuing endogenous release of ATP from endothelial cells. Thus, while we have not excluded ATP release from renal sympathetic nerves as a source of ATP, we have at least demonstrated that the phenomenon of denervation supersensitivity to ATP, reported in the rat vas deferens [59], does not occur in the glomerulus. Other sources of extracellular ATP are likely: studies of renal tubular cells in culture report that significant amounts of ATP are released across both apical and basolateral membranes [61, 62]. Furthermore, the glomerular endothelium itself may be a source: it has been shown that ATP is released from red blood cells squeezed on passage through the capillary bed [63], and the glomerular filtrate of plasma might also contain ATP [64]. Therefore, although basal cellular release of ATP is likely in the kidney [32], loss of tonic secretion of purines from renal nerve terminals may not lead to any alteration in sensitivity to exogenously applied ATP.

To summarize, we have provided evidence for the presence at both protein and functional levels of two P2Y receptor subtypes, $P2Y_2$ and $P2Y_1$, in the intact rat glomerulus; also, while we could demonstrate the phenomenon of denervation sensitivity to noradrenaline in rat glomeruli, the response to ATP was not enhanced.

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References

1 Cha SH, Sekine T, Endou H: P2 purinoceptor localization along rat nephron and evidence suggesting existence of subtypes $P2Y_1$ and $P2Y_2$. Am J Physiol 1998;274:F1006–F1014.
2 Bailey MA, Imbert-Teboul M, Turner C, Marsy S, Srai K, Burnstock G, Unwin RJ: Axial distribution and characterization of basolateral P2Y receptors along the rat renal tubule. Kidney Int 2000;58:1893–1901.
3 Ralevic V, Burnstock G: Receptors for purines and pyrimidines. Pharmacol Rev 1998;50: 413–492.
4 Jankowski M, Szczepanska-Konkel M, Kalinowski L, Angielski S: Bidirectional action of extracellular ATP on intracapillary volume of isolated rat renal glomeruli. J Physiol Pharmacol 2000;51:491–496.
5 Fischer KG, Saueressig U, Jacobshagen C, Wichelmann A, Pavenstadt H: Extracellular nucleotides regulate cellular functions of podocytes in culture. Am J Physiol 2001;281: F1075–F1081.
6 Harada H, Chan CM, Loesch A, Unwin R, Burnstock G: Induction of proliferation and apoptotic cell death via P2Y and P2X receptors, respectively, in rat glomerular mesangial cells. Kidney Int 2000;57:949–958.
7 Rost S, Daniel C, Schulze-Lohoff E, Baumert HG, Lambrecht G, Hugo C: P2 receptor antagonist PPADS inhibits mesangial cell proliferation in experimental mesangial proliferative glomerulonephritis. Kidney Int 2002;62:1659–1671.
8 Jankowski M, Szczepanska-Konkel M, Kalinowski L, Angielski S: The role of P2Y-receptors in the regulation of glomerular volume. Med Sci Monit 2001;7:635–340.
9 Olivetti G, Anversa P, Melissari M, Loud AV: Morphometry of the renal corpuscle during postnatal growth and compensatory hypertrophy. Kidney Int 1980;17:438–454.
10 Huwiler A, van Rossum G, Wartmann M, Pfeilschifter J: Stimulation by extracellular ATP and UTP of the stress-activated protein kinase cascade in rat renal mesangial cells. Br J Pharmacol 1997;120:807–812.
11 Vonend O, Grote T, Oberhauser V, Von Kugelgen I, Rump LC.: P2Y receptors stimulating the proliferation of human mesangial cells through the MAPK(42/44) pathway. Br J Pharmacol 2003;139:1119–1126.
12 Briner VA, Kern F: ATP stimulates Ca^{2+} mobilization by a nucleotide receptor in glomerular endothelial cells. Am J Physiol 1994;266: F210–F217.
13 Pavenstadt H, Henger A, Briner V, Greger R, Schollmeyer P: Extracellular ATP regulates glomerular endothelial cell function. J Auton Pharmacol 1996;16:389–391.
14 Morel F: Methods in kidney physiology: Past, present and future. Annu Rev Physiol 1992;54: 1–9.
15 Bailey MA, Hillman KA, Unwin RJ: P2 receptors in the kidney. J Auton Nerv Syst 2000;81: 264–270.
16 Schwartz DD, Malik KU: Renal periarterial nerve stimulation-induced vasoconstriction at low frequencies is primarily due to release of a purinergic transmitter in the rat. J Pharmacol Exp Ther 1989;250:764–771.
17 Kon V, Karnovsky MJ: Morphologic demonstration of adrenergic influences on the glomerulus. Am J Pathol 1989;134:1039–1046.
18 Warland JJ, Burnstock G: Effects of reserpine and 6-hydroxydopamine on the adrenergic and purinergic components of sympathetic nerve responses of the rabbit saphenous artery. Br J Pharmacol 1987;92:871–880.
19 Zhang L, Nakazawa K, Shigematsu H: Participation of endothelial cells and transformed mesangial cells in remodeling of glomerular capillary loops in Thy-1 nephritis. Pathol Int 2001;51:227–239.
20 Marchetti J, Meneton P, Lebrun F, Bloch-Faure M, Rajerison RM: The parietal sheet of Bowman's capsule of rat renal glomerulus: A target of endothelin and PAF. Am J Physiol 1995;268:F1053–F1061.
21 Elalouf JM, Buhler JM, Tessiot C, Bellanger AC, Dublineau I, de Rouffignac C: Predominant expression of β_1-adrenergic receptor in the thick ascending limb of rat kidney. Absolute mRNA quantitation by reverse transcription and polymerase chain reaction. J Clin Invest 1993;91:264–272.
22 Bailey MA, Imbert-Teboul M, Turner C, Srai SK, Burnstock G, Unwin RJ: Evidence for basolateral $P2Y_6$ receptors along the rat proximal tubule: Functional and molecular characterization. J Am Soc Nephrol 2001;12:1640–1647.
23 Meneton P, Bloch-Faure M, Guillon G, Chabardes D, Morel F, Rajerison RM: Cholinergic stimulation of phosphoinositide metabolism in isolated rat glomeruli. Am J Physiol 1992;262: F256–F266.
24 Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–3450.

25 Kottgen M, Loffler T, Jacobi C, Nitschke R, Pavenstadt H, Schreiber R, Frische S, Nielsen S, Leipziger J: $P2Y_6$ receptor mediates colonic NaCl secretion via differential activation of cAMP-mediated transport. J Clin Invest 2003; 111:371–379.
26 Llewellyn-Smith IJ, Song ZM, Costa M, Bredt DS, Snyder SH: Ultrastructural localization of nitric oxide synthase immunoreactivity in guinea-pig enteric neurons. Brain Res 1992; 577:337–342.
27 Mundlos S, Pelletier J, Darveau A, Bachmann M, Winterpacht A, Zabel B: Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. Development 1993;119:1329–1341.
28 Dawson TP, Gandhi R, Le Hir M, Kaissling B: Ecto-5′-nucleotidase: Localization in rat kidney by light microscopic histochemical and immunohistochemical methods. J Histochem Cytochem 1989;37:39–47.
29 Bidet M, De Renzis G, Martial S, Rubera I, Tauc M, Poujeol P: Extracellular ATP increases $[Ca^{2+}]_i$ in distal tubule cells. I. Evidence for a $P2Y_2$ purinoceptor. Am J Physiol 2000; 279:F92–F101.
30 Osborne MJ, Droz B, Meyer P, Morel F: Angiotensin II: Renal localization in glomerular mesangial cells by autoradiography. Kidney Int 1975;8:245–254.
31 Huwiler A, Rolz W, Dorsch S, Ren S, Pfeilschifter J: Extracellular ATP and UTP activate the protein kinase B/Akt cascade via the $P2Y_2$ purinoceptor in renal mesangial cells. Br J Pharmacol 2002;136:520–529.
32 Vonend O, Oberhauser V, von K, I, Apel TW, Amann K, Ritz E, Rump LC: ATP release in human kidney cortex and its mitogenic effects in visceral glomerular epithelial cells. Kidney Int 2002;61:1617–1626.
33 Wildman SS, Unwin RJ, King BF: Extended pharmacological profiles of rat $P2Y_2$ and $P2Y_4$ receptors and their sensitivity to extracellular H^+ and Zn^{2+} ions. Br J Pharmacol 2003;140: 1177–1186 (Epub Oct 27, 2003).
34 Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK: Second messenger cascade specificity and pharmacological selectivity of the human $P2Y_1$-purinoceptor. Br J Pharmacol 1996;118:167–173.
35 Nicholas RA, Lazarowski ER, Watt WC, Li Q, Boyer J, Harden TK: Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. J Auton Pharmacol 1996;16: 319–323.
36 Bogdanov YD, Wildman SS, Clements MP, King BF, Burnstock G: Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. Br J Pharmacol 1998;124:428–430.
37 Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ: The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: An immunohistological study. Cells Tissues Organs 2003;175:105–117.
38 Pavenstadt H, Gloy J, Leipziger J, Klar B, Pfeilschifter J, Schollmeyer P, Greger R: Effect of extracellular ATP on contraction, cytosolic calcium activity, membrane voltage and ion currents of rat mesangial cells in primary culture. Br J Pharmacol 1993;109:953–959.
39 Gutierrez AM, Lou X, Erik A, Persson G, Ring A: Ca^{2+} response of rat mesangial cells to ATP analogues. Eur J Pharmacol 1999;369:107–112.
40 Huwiler A, Pfeilschifter J: Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. Br J Pharmacol 1994;113:1455–1463.
41 Pfeilschifter J: Comparison of extracellular ATP and UTP signalling in rat renal mesangial cells. No indications for the involvement of separate purino- and pyrimidino-ceptors. Biochem J 1990;272:469–472.
42 Pfeilschifter J, Merriweather C: Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinase C-ε in rat renal mesangial cells. Br J Pharmacol 1993;110:847–853.
43 Jankowski M, Szczepanska-Konkel M, Kalinowski L, Angielski S: Cyclic GMP-dependent relaxation of isolated rat renal glomeruli induced by extracellular ATP. J Physiol 2001; 530:123–130.
44 Nitschke R, Henger A, Ricken S, Gloy J, Muller V, Greger R, Pavenstadt H: Angiotensin II increases the intracellular calcium activity in podocytes of the intact glomerulus. Kidney Int 2000;57:41–49.
45 Mohaupt MG, Fischer T, Schwobel J, Sterzel RB, Schulze-Lohoff E: Activation of purinergic P2Y2 receptors inhibits inducible NO synthase in cultured rat mesangial cells. Am J Physiol 1998;275:F103–F110.
46 Huwiler A, Wartmann M, van den Bosch H, Pfeilschifter J: Extracellular nucleotides activate the p38-stress-activated protein kinase cascade in glomerular mesangial cells. Br J Pharmacol 2000;129:612–618.
47 Schulze-Lohoff E, Zanner S, Ogilvie A, Sterzel RB: Extracellular ATP stimulates proliferation of cultured mesangial cells via P2-purinergic receptors. Am J Physiol 1992;263:F374–F383.
48 Takeda M, Kawamura T, Kobayashi M, Endou H: ATP-induced calcium mobilization in glomerular mesangial cells is mediated by P2U purinoceptor. Biochem Mol Biol Int 1996;39: 1193–1200.
49 Mene P, Simonson MS, Dunn MJ: Physiology of the mesangial cell. Physiol Rev 1989;69: 1347–1424.
50 Pavenstadt H: Roles of the podocyte in glomerular function. Am J Physiol 2000;278:F173–F179.
51 Huber TB, Gloy J, Henger A, Schollmeyer P, Greger R, Mundel P, Pavenstadt H: Catecholamines modulate podocyte function. J Am Soc Nephrol 1998;9:335–345.
52 Schlatter E, Ankorina I, Haxelmans S, Kleta R: Effects of diadenosine polyphosphates, ATP and angiotensin II on cytosolic Ca^{2+} activity and contraction of rat mesangial cells. Pflügers Arch 1995;430:721–728.
53 Ishikawa S, Kawasumi M, Kusaka I, Komatsu N, Iwao N, Saito T: Extracellular ATP promotes cellular growth of glomerular mesangial cells mediated via phospholipase C. Biochem Biophys Res Commun 1994;202:234–240.
54 Thoenen H, Tranzer JP: Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 1968;261:271–288.
55 Caramona MM, Soares-da-Silva P: The effects of chemical sympathectomy on dopamine, noradrenaline and adrenaline content in some peripheral tissues. Br J Pharmacol 1985;86:351–356.
56 Baines AD, Drangova R, Hatcher C: Dopamine production by isolated glomeruli and tubules from rat kidneys. Can J Physiol Pharmacol 1985;63:155–158.
57 Yamada S, Yamamura HI, Roeske WR: Effects of chemical sympathectomy with 6-hydroxydopamine on α- and β-adrenoceptors and muscarinic cholinoceptors in rat kidney. Eur J Pharmacol 1986;121:345–353.
58 Hirayama T, Ono H, Fukuda H: Functional supersensitivity of α_1-adrenergic system in spinal ventral horn is due to absence of an uptake system and not to postsynaptic change. Brain Res 1991;539:320–323.
59 Rohde GC, Huidobro-Toro JP: Purinergic supersensitivity following sympathectomy adds further support to co-transmission in the rat vas deferens. Arch Int Pharmacodyn Ther 1988;294:99–111:99–111.
60 Krayacich J, Kline RL, Mercer PF: Supersensitivity to norepinephrine in chronically denervated kidneys: Evidence for a postsynaptic effect. Can J Physiol Pharmacol 1987;65:2219–2224.
61 Taylor AL, Kudlow BA, Marrs KL, Gruenert DC, Guggino WB, Schwiebert EM: Bioluminescence detection of ATP release mechanisms in epithelia. Am J Physiol 1998;275:C1391–C1406.
62 Wilson PD, Hovater JS, Casey CC, Fortenberry JA, Schwiebert EM: ATP release mechanisms in primary cultures of epithelia derived from the cysts of polycystic kidneys. J Am Soc Nephrol 1999;10:218–229.
63 Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ: ATP: The red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol 1996;271:H2717–H2722.
64 Jabs CM, Ferrell WJ, Robb HJ: Microdetermination of plasma ATP and creatine phosphate concentrations with a luminescence biometer. Clin Chem 1977;23:2254–2257.