Pharmacological characterization of the P2 receptors profile in the podocytes of the freshly isolated rat glomeruli

Daria V. Ilatovskaya,* Oleg Palygin,* Vladislav Levchenko, and Alexander Staruschenko

Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin

Submitted 15 May 2013; accepted in final form 11 September 2013

Ilatovskaya DV, Palygin O, Levchenko V, Staruschenko A. Pharmacological characterization of the P2 receptors profile in the podocytes of the freshly isolated rat glomeruli. Am J Physiol Cell Physiol 305: C1050–C1059, 2013. First published September 18, 2013; doi:10.1152/ajpcell.00138.2013.—Calcium influx in podocytes is critical for normal and pathophysiological regulation of these types of cells, and excessive calcium signaling results in podocyte damage and improper glomeruli function. Purinergic activation of P2 receptors is a powerful and rapid signaling process; however, the exact physiological identity of P2 receptors subtypes in podocytes remains essentially unknown. The goal of this study was to determine the P2 receptor profile in podocytes of the intact Sprague-Dawley rat glomeruli using available pharmacological tools. Glomeruli were isolated by differential sieving and loaded with Fluo-4/Fura Red cell permeable calcium indicators, and the purinergic response in the podocytes was analyzed with ratiometric confocal fluorescence measurements. Various P2 receptors activators were tested and compared with the effect of ATP, specifically, UDP, MRS 2365, bzATP, αβ-methylene, 2-meSADP, MRS 4062, and MRS 2768, were analyzed. Antagonists (MRS 2500, 5-BDBD, A438079, and NF 449) were tested when 10 μM ATP was applied as the EC50 for ATP activation of the calcium influx in the podocytes was determined to be 10.7 ± 1.5 μM. Several agonists including MRS 2365 and 2-meSADP caused calcium flux. Importantly, only the P2Y1-specific antagonist MRS 2500 (1 nM) precluded the effects of ATP concentrations of the physiological range. Immunohistochemical analysis confirmed that P2Y1 receptors are highly expressed in the podocytes. We conclude that P2Y1 receptor signaling is the predominant P2Y purinergic pathway in the glomeruli podocytes and P2Y7 might be involved in the pathogenesis of glomerular injury and could be a target for treatment of kidney diseases.

kidney; calcium signaling; glomeruli; podocytes; ATP; P2Y1

Nowadays there has been shown a role for ATP in various key physiological processes, such as embryonic development, cell differentiation, apoptosis, sensation, cell signaling, etc. There is growing evidence that changes in ATP signaling contribute to a plethora of physiological and pathophysiological processes in the kidney; therefore, disturbances in purinergic signaling can lead to impaired renal and cardiovascular function (48). In the kidney, purinergic P2 receptors are expressed in both the apical and basolateral membranes of renal tubular cells, the renal vasculature, glomerular mesangial cells, and podocytes (1, 2, 57, 58). For example, in the immunohistochemical study by Turner et al. (57) P2X1, P2X2, and P2Y1 receptors were found in the smooth muscle layer of the intrarenal vessels; P2Y1 was also found on mesangial cells, the brush border membrane of the proximal straight tubule, and peritubular fibroblasts. In the kidney cortex, P2Y4 receptors were found on the tubule epithelium of the proximal convoluted tubule. A number of ex vivo and in vivo evidence revealed a functional role for the P2Y2 receptors in the collecting duct (29, 35, 46, 50, 54, 60, 65). P2X4 and P2X6 receptors were identified throughout the renal tubule epithelium from the proximal tubule to the collecting duct. P2X5 receptors were found to be expressed on medullary collecting duct cells and the apical membrane of the S3 segment of the proximal tubule.

As for the glomeruli, there have been few studies investigating the pattern of the P2 receptors in the different cell types comprising the glomerulus, probably because of the complex structure of this nephron segment, which includes endothelial and mesangial cells encapsulated by visceral (podocytes) and parietal epithelial cells (2, 40). Earlier studies performed in cultured cells report the mRNA expression of the P2Y2, P2Y4, and P2Y6 in mesangial cells (18, 22, 61) and P2Y2 in the endothelium of the glomerulus (3, 42). Other RT-PCR studies have detected the mRNAs of the P2Y1, P2Y2, P2Y6, and P2Y7 receptors within glomeruli, although it was hypothesized that extracellular nucleotides modulate podocyte function mainly by an activation of both P2Y2 and P2Y6 receptors rather than P2Y1 (12). However, a later paper published by Hohenstein et al. (21) revealed that P2Y1 deficiency protects from renal disease progression and capillary rarefaction during the passive crescentic glomerulonephritis. The authors have detected the P2Y1 mRNA in extracts of mouse cortical kidney tissue, laser-microdissected glomeruli, and tubulointerstitium as well as in cultured (undifferentiated) podocytes and mesangial cells (21).

Members of the P2X receptors subfamily were shown to be expressed in rat glomerular mesangial cells (18). P2X1 receptors 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, and renal tubules (5). It has been recently demonstrated that P2X7 receptor-mediated Nlrp3-inflammasome activation is a genetic determinant of macrophage-dependent crescentic glomerulonephritis (7); moreover, P2X7 deficiency has been shown to be renoprotective in mice compared with wild-type controls, as evidenced by a reduction in proteinuria and decreased glomerular injury (56). Importantly, electron microscopy, immunostaining, and mRNA have shown low-level P2X7 receptor expression in the glomeruli under normal conditions, which is significantly upregulated (mainly in podocytes) in diabetic and hypertensive rat models and therefore can be associated with the pathogenesis of glomerular cell injury (62).

Podocytes are highly specialized differentiated cells that are important regulators of the glomerular filtration barrier; these cells contain a plethora of receptors for different hormones and...
auto/paracrine factors, activation of which can affect glomerular filtration (11, 17, 43, 52). Extracellular nucleotides, mainly ATP, are among these factors (4, 24, 31) and play an important role in the regulation of glomerular dynamics via P2 receptors. Under pathophysiological conditions podocytes contribute to the initiation and progression of a variety of glomerular diseases. Membranous nephropathy, minimal change disease, and focal segmental glomerulosclerosis have been especially related to primary or secondary podocyte injury (28, 52).

Although there is growing evidence that purinergic receptors contribute to normal kidney function, until recently not much has been done to exploit the P2 receptors as a potentially useful therapeutic option; the studies have been significantly limited by the lack of the subtype-specific ligands for corresponding receptors. In renal epithelia, particularly, in the glomeruli, there has been shown the presence of multiple receptor subtypes; therefore, the need to differentiate between these receptors has emerged as an essential background for future therapeutic targeting in various disease states associated with impaired purinergic signaling. This study was intended to elucidate which purinoreceptors are particularly responsible for the transduction of the ATP signal in the podocytes of the glomeruli. Using a number of recently developed specific agonists and antagonists of P2 receptors, we revealed that P2Y1 receptor is critical for purinergic signaling in the glomeruli podocytes. The role of the P2X receptors in the mediation of the calcium flux in podocytes requires further investigation.

MATERIALS AND METHODS

Animals. Animal use and welfare adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of the Medical College of Wisconsin. For experiments, male 8- to 10-wk-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used. Rats were provided with food and water ad libitum.

Glomeruli isolation. Glomeruli isolation protocol was described previously (23). Briefly, the rat kidneys were removed, and the cortex was isolated and minced using a single edge razor blade. The minced tissue was sequentially pushed through stainless steel dissociation sieves of 100 and then 140 mesh (04-881-5X; Thermo Fisher) with 5% BSA (Sigma-Aldrich). The suspension was then pipetted onto a 200-mesh sieve (S4145; Sigma-Aldrich) leaving the tissue was sequentially pushed through stainless steel dissociation sieves of 100 and then 140 mesh (04-881-5X; Thermo Fisher) using the culture medium solution RPMI1640 (Invitrogen) with 5% BSA (Sigma-Aldrich). The suspension was then pipetted onto a 200-mesh sieve (S4145; Sigma-Aldrich) leaving the glomeruli on the top surface. The glomeruli were then rinsed into a 15-ml tube and let to settle down on ice for 10–15 min. After sedimentation, the excess of the solution was removed and isolated decapsulated glomeruli were used for microscopy experiments.

Immunohistochemistry. Rat kidneys or lungs were fixed in 10% formalin and processed for paraffin embedding. Tissue sections were cut at 4 μm, dried, and deparaffinized for subsequent labeled streptavidin–biotin immunohistochemistry. After deparaffinization, the slides were treated with a citrate buffer pH 6 for a total of 35 min. The slides were blocked with a peroxidase block (DAKO), avidin and biotin blocks (Vector Laboratories), and serum-free protein block (DAKO). Tissue sections were incubated for 90 min in a 1:50 or 1:100 concentrations of anti-P2Y1 antibodies [H-120 (Santa Cruz Biotechnology) and APR-009, (Alomone Laboratories), respectively]. Secondary detection was performed with goat anti-rabbit biotinylated IgG (Biocare) followed by streptavidin horseradish peroxidase (Biocare) and visualized with DAB (DAKO). All slides were counterstained with Mayers hematoxylin (DAKO), dehydrated, and mounted with permanent mounting media (SAKURA).

Confocal laser-scanning fluorescence microscopy. Calcium imaging was performed with laser scanning confocal microscope system Nikon A1-R. Images were collected in time series (2 μs per frame) with the Nikon imaging software. Changes in intracellular Ca2+ concentration were estimated according to a protocol described previously (44) from ratiometric fluorescence images of Fluo-4–excitation at 488 nm and emission at 520 ± 20 nm) and Fura Red (excitation at 488 nm and emission at >600 nm) loaded glomeruli. Emitted light was collected by the objective lens Plan Apo ×60 oil DIC2. All experiments were performed with the same instrument settings (laser power, offset, gain on both detector channels). The glomeruli suspension was loaded with the dyes by addition of Fluo-4 AM and Fura Red AM (5 μM of each; Invitrogen) dissolved in dimethyl sulfoxide. After loading (~30 min), fluorescent dyes were removed by flushing the glomeruli suspension with the bath solution. Glomeruli were mounted on the poly-L-lysine-covered glass in a registration chamber and washed for ~10 min with bath solution containing the following (in mM): 145 NaCl, 4.5 KCl, 2 MgCl2, and 10 HEPES pH 7.35 (adjusted with NaOH). After the fluorescence signal was stabilized, podocytes were identified on the basis of anatomic considerations, and fluorescence intensity ratios (Fluo-4/Fura Red) were recorded. All cells were carefully checked visually and morphologically selected on the surface of the glomerulus preparation attached to poly-L-lysine to ensure that the cell does not move during the acquisition and the signal is recorded from the podocytes. The fluorescent signal was observed only from the cells on the surface of glomeruli in area attached to glass. Such choice of the sample ensured fluorescence signal stability and eliminated focal plane changes during the application of the drugs. The cells located on the surface of the glomeruli characterized with prominent foot processes were defined as visceral epithelial cells of glomeruli or podocytes. All cells selected for the experiments were checked for the values and time of the response to application of ATP and respective purinergic agents; it was ensured that selected cells had similar values and the response was synchronized in time for all cells of the focal plane. Such precautions allowed concluding that the uniform response recorded corresponded to the same receptors that had equal pharmacological sensitivity. Cells located in the deeper layers of the glomeruli usually have low, close to background fluorescence, or no fluorescence. We observed no significant bleaching of both dyes during the experiments. In each experiment, five to nine podocytes of at least one glomerulus were selected; experiments for every compound tested were repeated at least three times.

Drugs. Adenosine-5′-triphosphate (ATP; cat no A2383) and α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP; cat no M6517) and other salts and chemicals were purchased from Sigma-Aldrich (St. Louis, MO); the rest of the purinergic receptors agonists/antagonists were from the Tocris Bioscience (Minneapolis, MN); UDP (cat. no. 3111), MRS 2768 (cat. no. 3884), bzATP (cat. no. 3312), MRS 2365 (cat. no. 2157), MRS 4062 (cat. no. 4261), 2-methylthioadenosine diphosphate trisodium salt (2-meSADP; cat. no. 1624), 5-BDBD (dissolved in DMSO, cat. no. 3579), NF 449 (cat. no. 1391), A438079 (cat. no. 2972), and MRS 2500 (cat. no. 2159). All pharmacological tools were dissolved in water, unless noted otherwise.

Calculation and statistics. Figure preparation and statistical analyses were carried out using MicroCal Origin software 6.0 (MicroCal Software). Time of decay (τ) and dose responses were calculated using the nonlinear curve fitting (exponential function) and Pharmacology/DoseResp analyses of the Origin 6.0 software, respectively. Fluorescent images were processed with open source software ImageJ 1.42. All summarized data are reported as mean ± SE. Statistical difference was tested with either the Student’s (two-tailed) t-test or a one-way ANOVA. Significance was accepted at P < 0.05.
RESULTS

Ratiometric fluorescence calcium imaging in the podocytes of the freshly isolated rat glomeruli. Initial experiments were designed to establish a method that would allow doing ratiometric calcium imaging on the podocytes of the freshly isolated decapsulated glomeruli. For these experiments, glomeruli of 8- to 10-wk-old Sprague-Dawley rats were loaded with Fluo-4/Fura Red fluorescent dyes and analyzed under the confocal scanning microscope. Addition of ATP into the bath solution resulted in a rapid and fast transient response as shown in corresponding microphotographs on Fig. 1A. Figure 1B demonstrates a representative transient evoked by the addition of 10 μM ATP to the bath solution. In the basic conditions (unless noted otherwise), the glomeruli were kept in the bath solution containing 2 mM CaCl₂. Figure 1B also illustrates the schematics of the calculations done to assess the main parameters of the ATP-evoked transients, the time of decay that represents the dynamic of the signal decrease from the maximum value after addition of ATP, and the area under curve showing the integral intracellular calcium release in response to the drug. The mean amplitude and mean time of decay of calcium concentration in podocytes cells in response to 10 μM ATP were 1.72 ± 0.15-fold and 47.5 ± 14.9 s, respectively. The ATP dose-dependence curve (see Fig. 1C) was measured for the point of the maximal intracellular calcium concentration increase. The EC₅₀ value was calculated according to the Hill’s equation and was determined equal to 10.7 ± 1.5 μM.

To test the implication of the metabotropic P2Y receptors into the purinergic signaling in the podocytes, we probed the ATP response in the calcium-free solution containing a divalent ions chelator (0.2 mM EGTA). Figure 2A shows a typical fast transient evoked by 10 μM ATP in the podocytes in the calcium-free solution (time of decay: 20.02 ± 2.44 s). As may be inferred from the graph, the time of decay is significantly faster (see Fig. 2B) than in calcium-containing solution, whereas the magnitude of the response is even greater in the calcium-free bath solution (Fig. 2C). However, from the comparison of the values of the integral intracellular calcium release represented by the area under curve in Fig. 2D, it is clear that the total calcium release in the calcium-free is smaller than in the calcium-containing solution, which can be explained by deactivation of P2X component of intracellular calcium transient.

Pharmacological effect of the P2 receptors agonists on the intracellular calcium activation in the podocytes. Table 1 provides information about P2X and P2Y receptors agonists and antagonists used in the current study. Among various P2 receptor activators tested here, MRS 2768, MRS 4062, and UDP, which activate P2Y2, P2Y1/P2Y2/P2Y6, and P2Y1/P2Y14, respectively, did not affect intracellular calcium concentration of the podocytes when applied in both low and high concentrations (see Fig. 3); as a control 10–50 μM ATP was added at the end of each experiment to ensure that the podocytes are still responsive (Fig. 3). α,β-Methylene and bzATP,
response to addition of 10
as the most promising candidates for the transduction of the
the next stage of the study was focused on the P2Y receptors
responsible for the calcium flux in the podocytes under normal
conditions. *

Table 1. P2 receptor agonists and antagonists used in the current study and their target receptors as reported in the
literature cited

<table>
<thead>
<tr>
<th>P2 receptor agonists</th>
<th>Target Receptor</th>
<th>IC50/EC50</th>
<th>Applied Concentrations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP</td>
<td>P2Y&lt;sub&gt;6&lt;/sub&gt;, P2Y&lt;sub&gt;14&lt;/sub&gt;</td>
<td>0.2 µM</td>
<td>10 and 50 µM</td>
<td>(13, 47)</td>
</tr>
<tr>
<td>MRS 2768</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.89 µM</td>
<td>5, 10, and 50 µM</td>
<td>(30)</td>
</tr>
<tr>
<td>α,β-methylene</td>
<td>Most P2X (except P2X&lt;sub&gt;3&lt;/sub&gt;), 100 µM affects P2Y</td>
<td>0.5 µM</td>
<td>10 and 50 µM</td>
<td>(25, 27)</td>
</tr>
<tr>
<td>BzATP</td>
<td>P2X&lt;sub&gt;7&lt;/sub&gt;/P2X&lt;sub&gt;1&lt;/sub&gt; and P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.7 µM/8.7 µM</td>
<td>2.5 and 10 µM</td>
<td>(27, 37)</td>
</tr>
<tr>
<td>MRS 2365</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;/P2Y&lt;sub&gt;13&lt;/sub&gt;</td>
<td>0.4 nM/1 µM</td>
<td>10, 50, 200, 500 nM, and 1, 5, 12.5, 20, 50, 100, 200 µM</td>
<td>(6, 49)</td>
</tr>
<tr>
<td>MRS 4062</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;/P2Y&lt;sub&gt;12&lt;/sub&gt;/P2Y&lt;sub&gt;13&lt;/sub&gt;</td>
<td>23/64/70 nM</td>
<td>1, 10, and 50 µM</td>
<td>(36)</td>
</tr>
<tr>
<td>2-meSADP</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;/P2Y&lt;sub&gt;12&lt;/sub&gt;/P2Y&lt;sub&gt;13&lt;/sub&gt;</td>
<td>8.29/9.05/19 nM</td>
<td>10, 20, and 200 µM</td>
<td>(34, 51, 64)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P2 receptor antagonists</th>
<th>Target Receptor</th>
<th>IC50/EC50</th>
<th>Applied Concentrations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-BDBD</td>
<td>P2X&lt;sub&gt;7&lt;/sub&gt;</td>
<td>0.50 µM</td>
<td>10 and 15 µM</td>
<td>(8, 25)</td>
</tr>
<tr>
<td>NF 449</td>
<td>P2X&lt;sub&gt;2&lt;/sub&gt;/P2X&lt;sub&gt;12&lt;/sub&gt;/P2X&lt;sub&gt;13&lt;/sub&gt;/P2X&lt;sub&gt;4&lt;/sub&gt;/P2X&lt;sub&gt;7&lt;/sub&gt;/P2X&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.28 nM/0.69 nM/120 nM/1.820 nM/47 µM/300 µM</td>
<td>10 and 50 µM</td>
<td>(10, 19, 27)</td>
</tr>
<tr>
<td>A438079</td>
<td>P2Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.7 µM</td>
<td>1, 5, 10, and 25 µM</td>
<td>(9, 38)</td>
</tr>
<tr>
<td>MRS 2500</td>
<td>P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.95 nM</td>
<td>0.01, 0.05, 0.1, 1, 5, 30, 50, 200, 400 nM, and 10 µM</td>
<td>(20, 32)</td>
</tr>
</tbody>
</table>

which are established compounds known to activate most of
P2X receptors and P2X<sub>7</sub>/P2X<sub>12</sub>/P2Y<sub>1</sub>, respectively, only slightly elevated the intracellular calcium concentration (Fig. 4). Therefore, it can be hypothesized that the P2X component only represents a minor purinergic receptors component responsible for the calcium flux in the podocytes under normal conditions.

Based on this hypothesis and available specific antagonists, the next stage of the study was focused on the P2Y receptors as the most promising candidates for the transduction of the purinergic signaling. One of the well-known potent purinergic agonists displaying selectivity for P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> receptors is 2-meSADP (34, 51, 64). As seen on Fig. 5A, when applied to the podocytes in the calcium-containing bath solution, 10 µM of 2-meSADP (EC<sub>50</sub> for P2Y<sub>1</sub>, and P2Y<sub>12</sub>) induced a fast and rapid transient with time of decay 8.34 ± 0.77 s and amplitude of 1.88 ± 0.18, compared with the effect of ATP alone (time of decay: 47.5 ± 14.9 s; amplitude: 1.72 ± 0.15). As seen from Fig. 5B, 2-meSADP also activated P2Y receptors in the calcium-free solution, producing a similar transient and kinetics.

From these observations, one can conjecture that P2Y receptors, especially P2Y<sub>1</sub> and probably P2Y<sub>12</sub>, are involved into the regulation of the calcium flux in the podocytes. To further probe this hypothesis with the available pharmacological activators, we have tested the effects of a highly potent, selective P2Y<sub>1</sub> receptor agonist MRS 2365 (6, 49) on the glomeruli preparation that displays no activity at P2Y<sub>12</sub> receptors and only very low agonist activity at P2Y<sub>13</sub>. Five micromoles of MRS 2365 were applied in the calcium-free solution and induced a fast transient with a magnitude of 2.59 ± 0.5-fold; interestingly, following application of 10 µM ATP did not cause any elevations of the calcium concentration (Fig. 6A).

Ten micromoles of ATP and even higher concentration of MRS 2365 (20 µM) were also applied vice versa, and no response to MRS 2365 was recorded in this case (Fig. 6B). Therefore, it can be concluded that amongst P2Y receptors P2Y<sub>1</sub> and (unlikely) P2Y<sub>13</sub> are the main candidates for the purinergic signal transduction in the podocytes.

Effects of the P2 receptors antagonists on the calcium transients evoked by ATP. Taking into consideration that α,β-methylene and bzATP evoked a slight increase in the intracellular calcium concentration, we have tested available P2X receptors antagonists to estimate the contribution of the P2X component into ATP signal transduction. The glomeruli were incubated with either of the antagonists for 10 min before the ratiometric measurements were started.

As seen from Fig. 7A, 5-BDBD, which is known to specifically inhibit P2X<sub>4</sub> receptors subtype, modified the time of decay, however, did not significantly alter the maximum of the increase induced by ATP. This was confirmed with the use of NF 449 (specific for P2X<sub>2</sub>/P2X<sub>3</sub>/P2X<sub>12</sub>/P2X<sub>7</sub>/P2X<sub>13</sub>) and A438079 (specific for P2X<sub>7</sub>) which in a similar way...
affected the parameters of the ATP-evoked transient but did not block the response (Fig. 7, B and C, respectively). Hereof we can conclude that high doses of the P2X blockers known to date can nonspecifically modulate the kinetics of the purinergic response in podocytes, prolong Ca\(^{2+}\) transient, and slightly moderate the integral calcium release. These data indicate that P2X receptors 1 to 4 and P2X7 are most likely not involved into the transduction of the purinergic signaling in the rat podocytes under normal conditions. However, in general, the putative P2X component cannot be excluded, especially in the disease states.

As a result of the described pharmacological screening, P2Y\(_1\) remained as a main target for the ATP signal. To probe this hypothesis, we have utilized a novel selective antagonist of the P2Y\(_1\) receptors, MRS 2500. Incubation with MRS 2500 (in any concentration >1 nM for 10 min) precluded the development of the calcium transient evoked by 10 \(\mu\)M ATP (see Fig. 8A). However, the response to 100 \(\mu\)M ATP in the podocytes of the glomeruli was not completely inhibited by incubation with MRS 2500. The IC\(_{50}\) of MRS 2500 in the podocytes was 1.72 ± 0.59 nM as calculated from the concentration dependency demonstrated in Fig. 8B. The effect of the antagonist was estimated as a magnitude of the ATP-induced calcium transient after incubation with MRS 2500 for 10 min.

P2Y\(_1\) receptor mediates the core purinergic signal transduction in the podocytes of the rat glomeruli. Figure 9 summarizes the action of various P2 receptors agonists (Fig. 9A) and antagonists (Fig. 9B) on the intracellular calcium levels in the podocytes of the rat glomeruli. As seen from Fig. 9A, several receptors could be excluded from the list of potential candidates for mediation of the purinergic signaling in the podocytes. The absence of the effect of UDP, MRS 2768, and MRS 4062 allowed eliminating P2Y\(_2\), P2Y\(_4\), P2Y\(_6\), and P2Y\(_{14}\), especially taking into consideration the overlapping targeting effects of these drugs. Low responses to \(\alpha,\beta\)-methylene adenosine triphosphate (\(\alpha,\beta\)-methylene ATP) in the podocytes, these being P2X1/P2X1/5/P2X2/3/P2X3/P2X2/P2X3 (proved with the application of NF449). The absence of the P2X4 and P2X7 receptor effect in the studied pathway was also confirmed with 5-BDBD and A438079, respectively. Interestingly, among the activators and inhibitors applied in this study the most potent proved to be ones targeting P2Y\(_1\). This leaves the G protein-coupled P2Y\(_1\) receptor as the most likely pharmacologically selected P2 family member to be responsible for the purinergic signaling and consequent calcium flux in the podocytes; the contribution of P2Y\(_{12}\) and P2Y\(_{13}\) receptors can be excluded due to the results of the use of MRS 2500. Figure 10 shows the immunohistochemical staining for P2Y\(_1\) receptors (APR-009 antibody; Alomone Laboratories) in the Sprague-Dawley rat kidney and lung. As can be seen from the representative images (Fig. 10B), P2Y\(_1\) receptor is highly expressed in the podocytes of glomeruli. We also performed staining in isolated glomeruli to avoid background signals from the tubules surrounding glomeruli (Fig. 10C). Negative (Fig.

---

**Fig. 3.** Representative curves showing the absence of the effects of the specific P2 receptors agonists MRS 2768 (P2Y\(_2\); A), MRS 4062 (P2Y\(_4\), P2Y\(_2\), and P2Y\(_{14}\); B), and UDP (P2Y\(_6\), and P2Y\(_{14}\); C) on [Ca\(^{2+}\)]\(_i\) in the podocytes of the freshly isolated glomeruli in the solution containing 2 mM CaCl\(_2\). Control addition of ATP (10–50 \(\mu\)M) is shown at the end of each trace.

**Fig. 4.** Representative curves illustrating the summarized effects of the specific P2 receptors agonists \(\alpha,\beta\)-methylene (specific for most of P2X receptors; A) and bzATP (P2X\(_5\), P2X\(_7\), and P2Y\(_1\); B) on [Ca\(^{2+}\)]\(_i\) in the podocytes of the freshly isolated glomeruli in the solution containing 2 mM CaCl\(_2\); 10 \(\mu\)M ATP was added at the end of each experiment as control.
and positive controls (Fig. 10D, lung section) demonstrated specificity of used antibodies. Additional experiments with P2Y1 receptors obtained in Santa Cruz Biotechnology revealed similar staining in podocytes.

**DISCUSSION**

We provide here a comprehensive pharmacological analysis of P2 receptors profile in the podocytes, which are critical for glomeruli functions. On the basis of the reported results it can be concluded that the main receptor responsible for the purinergic signaling transduction in the podocytes of the normal rat glomeruli is P2Y1, which belongs to the metabotropic P2Y subfamily of the P2 receptors. However, it should be noted that the data reported here unveil the P2 receptor expression under basal conditions, which could be altered by certain pathophysiological states. It was reported that P2Y1 receptor deficiency leads to enhanced survival and preservation of renal kidney histology and function during the crescentic nephrotic glomerulonephritis (21). As reported by Hohenstein et al. (21) this histology and function during the crescentic nephrotic glomerulonephritis (21) leads to enhanced survival and preservation of renal kidney histology and function during the later course of the disease. Determination of the P2Y1 receptor as the major purinergic signal transducer in the podocytes opens new perspectives for the future studies of the P2 involvement into the calcium signal regulation and disease progression.

In the recent years, several new classes of purinergic modulators, which are highly specific for certain P2Y or P2X subfamily members, have been discovered. However, most of the purinergic modulators are concentration-dependent specific and high concentrations of the drugs can target several homologous receptors. One of the best examples of such effect is a popular P2X7 activator BzATP, which can also activate both P2X1 and P2Y1 receptors in concentrations >10 μM (see Table 1). Here we have implicated various cutting-edge pharmacological tools to identify the receptor that would mediate ATP-induced calcium increase in the podocytes of the normotensive rat glomeruli. As has been noted in several reviews relevant to the regulation of the renal function by purinergic signaling, specific antagonists are very much needed to better characterize the functional role of the P2 receptors (48, 53, 59). In the current study we have applied the most specific recently developed pharmacological tools, as well as the classic compounds that have been long used in the field of the purinergic signaling, to differentiate between the P2 signals in the glomerulus. As summarized in Fig. 9, pharmacological screening allowed us to discriminate between the P2X- and P2Y-mediated calcium release and to show the major role of the P2Y1.

Our observations based on the effects of various agonists with the partially overlapping target receptors allow concluding that P2Y2, P2Y4, P2Y6, and P2Y14 are most likely not involved into the transduction of the ATP-induced signaling in the podocytes. In contrast to some of the previously published data (2), we found no evidence of P2Y2 receptor involvement into the ATP signal transduction in the podocytes. However, Bailey et al. (2) recognize that further studies using a more sensitive direct technique will be necessary to establish definitely whether P2Y1 receptors are expressed in the glomeruli.

Our results reveal that apart from P2Y1, P2Y12 and P2Y13 could be involved into the transduction of the ATP signal, however, as a minor P2Y component. Inhibition of the P2Y12 receptor with clopidogrel in a rat model of the angiotensin II-induced hypertension reduced cellular proliferation and...
macrophage infiltration and lessened tissue injury (14). These data suggest that P2Y<sub>12</sub> could be implicated into the renal processes that result in glomerular injury in the angiotensin-induced form of hypertension.

The P2X component of the purinergic signaling in the podocytes has been speculated upon in the previous decade. Most studies showed low expression profiles of the P2X receptors in the glomeruli, which are, however, increased under pathological conditions. For example, P2X<sub>7</sub> receptor expression in the glomeruli is significantly enhanced in the diabetic and hypertensive rats (62). Generally, P2X<sub>7</sub> receptors mediate apoptotic, necrotic, and “aponecrotic” cell death (16, 55), so this could be important in the disease states associated with proteinuria and podocytes death. In accordance with these observations, our data show that there is a P2X<sub>7</sub>-mediated component in the calcium release stimulated by ATP, which is, however, quite small compared with the P2Y-mediated release. We have not detected any significant P2X receptors activity in the podocytes of the rat glomeruli under normal conditions; application of the P2X receptors antagonists revealed that neither of the P2X receptors 1 to 4 nor 7 is involved into the pathological conditions. Our experiments revealed that upon inhibition of the P2Y<sub>1</sub> component with MRS 2500 the response to high concentration of ATP (100 µM) is sustained, this allowing us to suggest a putative role of the P2X receptors in the disease states accompanied with abnormally high ATP production.

Interestingly, in our experiments, total intracellular Ca<sup>2+</sup> concentration release is always higher in the outer solution containing 2 mM Ca<sup>2+</sup>. In the 2-mM Ca<sup>2+</sup> solution, the response is longer and total calcium release is increased. However, the peak value of the ATP-induced response is higher in zero extracellular calcium, which could be potentially mediated by the gap junction channel coupling. However, we believe that this mechanism is not involved in these effects, as the response in 0 mM Ca<sup>2+</sup> would not involve calcium entry through the channels from the extracellular side. The higher peak in zero Ca<sup>2+</sup> that we consistently observed can be potentially associated with more efficient depot storage capacity in the presence of low calcium.

It should be noticed that in general there is a significantly less calcium release in the absence of the extracellular calcium, whereas in the 2 mM Ca<sup>2+</sup> solution we observed a longer (“slow”) component of the transient, which could have been assigned to the P2X signaling. As we have tested the P2X component responsible for the calcium release and concluded that the P2X component cannot be excluded, the longer calcium release could be explained by the activity of the P2X receptors. However, taking into consideration that tested P2X<sub>7</sub> inhibitors did not alter the magnitude of the response to 10 µM ATP (Fig. 9B) and, moreover, under MRS 2500 treatment we could only detect the response to 100 µM ATP, longer calcium

---

**Fig. 7.** Representative graphs demonstrating the development of the increase in the [Ca<sup>2+</sup>], in the podocytes of the freshly isolated glomeruli in response to 10 µM ATP following the incubation of the sample with the specific P2X antagonists 5-BDBD (P2X<sub>4</sub>; A), NF449 (P2X<sub>1</sub>/P2X<sub>1/5</sub>/P2X<sub>2/3</sub>/P2X<sub>3</sub>/P2X<sub>4</sub>; B), and A438079 (P2X<sub>7</sub>; C) in the solution containing 2 mM CaCl<sub>2</sub>. Each point represents an averaged effect from at least 5 cells from 4 to 5 different rats.
release transient in the calcium-containing solution can be explained by the stimulation of various intracellular signaling pathways (like activation of the transient receptor potential channels) by the P2Y-triggered cascade. This assumption is consistent with the results obtained in the experiments presented on Fig. 6, when the P2Y1 signaling was blocked with MRS 2500 in 2 mM Ca2⁺-containing solution; inhibition of the P2Y1-triggered cascade causes further silencing of the transient receptor potential channel signaling.

It is well-known that multiple G protein-coupled receptors have been implicated in the pathogenesis of glomerular disease processes. P2Y1 receptors identified here are coupled to Gq-activated cascades (41, 63). Activation of P2Y1 receptors could be a strong regulator of podocyte function and signaling and through the Gq-mediated intracellular cascades would trigger the second intracellular messengers and could further lead to activation of calcineurin or calmodulin-dependent mechanisms (39, 63). Gi-coupled receptors of the P2Y family P2Y12 and P2Y13 are known to inhibit adenylyl cyclase and to reduce the intracellular levels of cAMP. Moreover, it has been recently shown that Gi proteins can activate members of the Src family of tyrosine kinases (33). However, our experiments could not show any clear presence of these Gi-coupled P2Y12 and P2Y13 in the podocytes, and thus Gi signaling could be excluded from the signal transduction pathway, although this purinergic mechanism is quite important for other physiological processes, like platelet aggregation.

It has been reported that the release of reactive oxygen species leads to proteinuria by affecting glomerular endothelial and epithelial cells and disturbing normal glomerular permse-

---

**Fig. 9.** Summary graphs illustrating the effects of the specific P2-receptors agonists (A) and antagonists (B) on the calcium concentration in the podocytes of the freshly isolated rat glomeruli. For the antagonists the effect was estimated as an increase in calcium concentration in response to 10 μM ATP in presence of the inhibitor. Each column represents an averaged effect of at least 15 podocytes from at least 4 animals.

**Fig. 10.** Representative immunohistochemical staining of the rat tissues with the antibodies against P2Y1 receptors. A: control staining with secondary and without primary antibodies. B: representative staining of the rat cortical renal tissue; podocytes of the glomerulus are marked with arrows. C: representative staining of the isolated decapsulated rat glomerulus; D: control staining of the rat lung tissue shows clear localization of the P2Y1 receptor in the epithelium of the airway capillaries. Scales shown in A = 50 μm (common for A–C); scale in D = 100 μm.
pectivity (4, 24). Importantly, it was demonstrated that NADH/NADPH oxidase system that represents the main source for reactive oxygen species generation in the podocytes is activated by the P2 receptor-mediated pathway (15, 45). Therefore, activation of P2 receptors may finally restore cell energy homeostasis in different pathophysiological conditions preceding the depression of intracellular ATP (e.g., oxidative stress, ischemia, inflammation, and diabetes). Moreover, recent evidence indicates that diabetic milieu, represented mainly by high glucose concentration, may lead to diabetic podocytopathy, abnormalities in podocytes numbers, and structure or function; glucose transport activity in podocytes may be modulated by P1 and P2 receptors under this pathological condition (26). Nevertheless, targeting the specific P2 receptors may be a means of treatment of various renal diseases associated with podocyte injury. Over-induced purinergic signaling may prove detrimental in this regard and specific inhibition of the P2 receptors could be of useful means to modulate the disease state. Of much interest is the involvement of the P2 receptors into the pathophysiology and developments of renal diseases such as glomerulonephritides. However, there is an emerging need for the development of the ligands that would not degrade in vivo to explore this in the prospective of the disease treatment. Pharmacological targeting of the P2 receptors, especially P2Y1, in the podocytes of the renal glomeruli might be considered as a promising strategy to further delineate the role of the P2 receptors in the regulation of the glomerular transport mechanisms under physiological and pathophysiological conditions and assessing their therapeutic potential.

ACKNOWLEDGMENTS

For technical assistance, we thank Glenn Slocum (microscopy analysis), Christine Duris (immunohistochemistry) from the Medical College of Wisconsin, and Colleen A. Lavin from Nikon Instruments for invaluable help with the microscope setup.

GRANTS

This research was supported by the American Diabetes Association Grant 1–10-BS-168, National Heart, Lung, and Blood Institute Grant HL-108880 (to D. Ilatovskaya), and Ben J. Lipps Research Fellowship from the American Society of Nephrology (to D. Ilatovskaya).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.V.I., O.P., and A.S. conception and design of research; D.V.I., O.P., and V.L. performed experiments; D.V.I. and O.P. analyzed data; D.V.I., O.P., and A.S. interpreted results of experiments; D.V.I. and O.P. contributed to reagent development. All authors reviewed drafts of the manuscript.

REFERENCES


