Extracellular nucleotides regulate cellular functions of podocytes in culture

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Fischer, Karl-Georg, Ulrich Saueressig, Claudius Jacobshagen, Arndt Wichelmann, and Hermann Pavenstädt. Extracellular nucleotides regulate cellular functions of podocytes in culture. Am J Physiol Renal Physiol 281: F1075–F1081, 2001.—Extracellular nucleotides are assumed to be important regulators of glomerular functions. This study characterizes purinergic receptors in podocytes. The effects of purinergic agonists on electrophysiological properties and the intracellular free Ca²⁺ concentration of differentiated podocytes were examined with the patch-clamp and fura 2 fluorescence techniques. mRNA expression of purinergic receptors was investigated by RT-PCR. Purinergic agonists depolarized podocytes. Purinergic agonists similarly increased intracellular free Ca²⁺ concentration of podocytes. The rank order of potency of various nucleotides on membrane voltage and free cytosolic calcium concentration; ion currents; nucleotides; P2X7 receptor

EXTRACELLULAR ATP IS AN IMPORTANT signaling molecule that regulates distinct cellular functions via P2 receptors. These receptors have been divided into two families of ligand-gated ion channels and G protein-coupled receptors, termed P2X and P2Y receptors, respectively (23). Up until now, seven subtypes of P2X receptors and eight subtypes of P2Y receptors have been identified (11). Within the glomerulus, ATP is involved in the regulation of glomerular hemodynamics and renal autoregulation. ATP is released by sympathetic nerve stimulation. Other sources of ATP are erythrocytes, platelets, mast cells, and endothelial cells (2). During glomerular inflammation, ATP is released from damaged resident glomerular and infiltrating cells, thereby modulating cellular responses during glomerular injury (2). A P2Y receptor has been pharmacologically characterized in cultured glomerular endothelial cells (1), and P2Y receptors and a P2X7 receptor have been identified in cultured mesangial cells (25, 26). Knowledge about the expression of P2 receptors in podocytes is limited. The glomerular basement membrane possesses ATP- and ADPase activity, which is assumed to play an antiproteinuric role in the early phase of anti-Thy1 nephritis. ATP possibly plays a role in podocyte function and influences its barrier function (22). The podocyte forms a crucial part of the glomerular filtration barrier. Its foot processes possess contractile structures, which may respond to vasoactive hormones and thereby regulate the ultrafiltration coefficient (Kf) (4, 12). Podocytes thus contribute to size and charge selectivity of the glomerular filtration barrier; their injury leads to proteinuria (10, 17). Here we investigate expression and functional properties of P2 receptors in differentiated podocytes.

METHODS

Cell culture. Immortalized mouse podocytes were derived from mice that have a thermosensitive variant of the SV40-T antigen inserted into the mouse genome. These mouse podocytes proliferate at 33°C in the presence of γ-interferon (SV40-T antigen active). At 37°C and after removal of γ-interferon, cells transform into the quiescent, differentiated phenotype (14). Within the present study, cells then stained positive for the podocyte markers Wilm’s tumor protein (WT-1) (15), synaptopodin (13), nephrin (28), and p57 (16). In addition, mRNA expression of the podocyte marker CD2AP (27) was detected by RT-PCR. For experiments, cells between passages 15 and 25 were seeded at 37°C into sixwell plates and cultured in standard RPMI media containing 1% FCS (Boehringer, Mannheim, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (both Gibco, Egggenstein, Germany) for at least 7 days until cells were differentiated, showing an arborized morphology.

Patch-clamp experiments. Most of the patch-clamp experiments were performed in the slow whole cell configuration (SWC), which has been described in detail elsewhere (5, 6). In

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brief, podocytes were placed in a bath chamber on the stage of an inverted microscope, kept at 37°C, and superfused with a phosphate-buffered Ringer-like solution containing (in mM) 145 NaCl, 1.6 K2HPO4, 0.4 KH2PO4, 1.3 CaCl2, 1.03 MgCl2, and 5 D-glucose, pH 7.4. The patch pipettes were filled with a solution containing (in mM) 95 K-gluconate, 30 KCl, 4.8 Na2HPO4, 1.2 NaH2PO4, 0.73 CaCl2, 1.03 MgCl2, 1 EGTA, and 5 D-glucose, as well as 50–100 mg/l nystatin, pH 7.2 (10−7 M Ca2+ activity). The patch pipettes had an input resistance of 2–3 MΩ. A flowing (10 µl/h) KCl (2 M) electrode was used as a reference. The data were recorded using a patch-clamp amplifier (Froeb and Busche, Physiologisches Institut, Freiburg, Germany) and were continuously displayed on a pen recorder. The access conductance (Gao) was measured whole cell current (Gm), the voltage of the respective cell was clamped in the voltage direction. S, sense; AS, antisense.

To vary the free Ca2+ concentration ([Ca2+]i) peak refer to the highest value of the fluorescence ratio.

RT-PCR for evaluation of expression of purinergic receptor mRNA in mouse glomeruli and podocytes. The RNA preparation, reverse transcription, and PCR amplification were performed according to the method recently described (7). In brief, total RNA from mouse glomeruli, which were obtained with the sieve technique and from cultured mouse podocytes, was isolated with guanidinium-acid phenol-chloroform extraction, and the amount of RNA was measured by spectrophotometry. For first-strand synthesis, total RNA from podocytes and mouse glomeruli was mixed in 5× reverse transcription buffer and completed with 0.5 mM dNTP, 10 µM random hexanucleotide primer, 10 mM dithiothreitol, 0.02 U RNase inhibiting RNA, and 100 U Moloney murine leukemia virus RT/µg RNA (RT was omitted in some experiments to check for amplification of contaminating DNA). The reverse transcription was performed at 42°C for 1 h, followed by a denaturation at 95°C for 5 min. PCR was performed in duplicate with a total volume of 20 µl, each containing 40 ng RNA/4 µl of reverse transcription template, 16 µl of PCR master mixture, and 10 pmol each of sense and antisense primer. The mixture was overlaid with mineral oil and heated for 2 min at 94°C. The samples were kept at 80°C until 1 U Taq DNA polymerase and the primer were added. The cycle profile consisted of 1 min of denaturation at 94°C, annealing for 1 min for the P2Y1 at 62°C, P2Y2 at 63°C, P2Y6 at 60°C, and for the P2X7 receptor at 57°C, respectively, and extension for 1 min at 72°C. To amplify P2Y1 receptor mRNA, 37 cycles were performed, 35 cycles for P2Y2, 40 cycles for P2Y6, 35 cycles for P2X7 in glomeruli, and 40 cycles for P2X7 in podocytes, respectively. The amplification products of 10 µl of each PCR reaction were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet irradiation. The primer oligonucleotides were selected from published cDNA sequences and are depicted in Table 1. All primers are noted in the 5′-3′ direction.

Measurements of intracellular calcium concentration. Measurements of intracellular Ca2+ concentration ([Ca2+]i) were performed in single podocytes with an inverted fluorescence microscope as recently described (bath temperature: 37°C, bath solution changes within 1 s) (24). In short, podocytes were incubated with the Ca2+-sensitive dye fura 2-acetoxymethyl ester (AM) (5 µM, Sigma, Deisenhofen, Germany) for 30 min at 37°C. Thereafter, they were mounted in a bath chamber on the stage of an inverted microscope and perfused with a Ringer-like solution. The light from a 75-W xenon lamp was directed through an infrared light filter (Tempax, Schott, Mainz, Germany) to avoid thermal damage of the three excitation filters mounted in a motor-driven filter wheel (10 cycles/s). The excitation filters were band-pass filters with excitation filters described by Grynkiewicz et al. (8). A dissociation constant (Kd) of the fura 2-Ca2+ complex of 224 nM (37°C) was assumed. The given concentrations for the [Ca2+]i peak refer to the highest value of the fluorescence ratio.

Measurements of lactate dehydrogenase (LDH) release from podocytes. To assess cytotoxicity, LDH release was measured with a routine autoanalyzer (Modular I, Hitachi). Total LDH content of the cells was measured after incubation of cells in 1% Triton X-100. LDH release from control and stimulated cells was expressed as a percentage of total LDH release.

Chemicals. Unless otherwise indicated, chemicals were purchased from Sigma.

Statistical analyses. Data are given as means ± SE, where n refers to the number of experiments. Student’s t-test was used to compare mean values within one experimental series. A P value ≤ 0.05 was considered statistically significant.

Table 1. Primers used to identify P2 receptor subtypes

<table>
<thead>
<tr>
<th>P2 Receptor Subtype</th>
<th>Accession No.</th>
<th>Sequence of Primer Pairs</th>
<th>Sense/Antisense</th>
<th>Length, bp</th>
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<tr>
<td>P2Y1</td>
<td>U22829</td>
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<td></td>
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<td>AS</td>
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<td>L14751</td>
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<td>D63665</td>
<td>TGG TAT GTG GAG TCG TGG</td>
<td>S</td>
<td>346</td>
</tr>
<tr>
<td>P2X7</td>
<td></td>
<td>GCT GTG TGG GAC GTA GA</td>
<td>AS</td>
<td></td>
</tr>
</tbody>
</table>

The sequence of primer pairs for P2X7 receptor mRNA was chosen according to Kaiho H et al. (9a). All primers are noted in the 5′-3′ direction. S, sense; AS, antisense.
Results
Extracellular purinergic agonists depolarize differentiated podocytes in culture. The resting $V_m$ of podocytes was $-65 \pm 1$ mV ($n = 39$). ATP (100 μM) induced a depolarization of podocytes from $-64 \pm 2$ to $-40 \pm 2$ mV ($n = 16$, $P < 0.05$). In the presence of ATP, an increase of $G_m$ from 2.7 ± 0.4 to 4.3 ± 0.7 nS in the inward direction and from 2.3 ± 0.3 to 3.3 ± 0.6 nS in the outward direction was detected ($n = 16$, $P < 0.05$ both for the inward and outward direction). After removal of ATP, the $V_m$ response was completely reversible. Figure 1A shows an original recording of the effect of 100 μM ATP on $V_m$ of a single differentiated podocyte. UTP and, interestingly, 2'- and 3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP; each 100 μM) similarly depolarized $V_m$ from $-58 \pm 2$ to $-34 \pm 2$ mV ($n = 12$, $P < 0.05$) and from $-63 \pm 2$ to $-42 \pm 2$ mV ($n = 18$, $P < 0.05$), respectively (Fig. 1, B and C). ATP (100 μM) led to a $G_m$ increase from 3.99 ± 1.16 to 4.36 ± 0.85 nS (inward direction) and from 4.25 ± 1.19 to 5.04 ± 1.05 nS (outward direction), respectively ($n = 12$, $P < 0.05$ both for the inward and outward direction). BzATP increased $G_m$ from $2.21 \pm 0.32$ to $3.62 \pm 0.64$ nS (inward direction) and from $2.73 \pm 0.46$ to $4.41 \pm 0.77$ nS (outward direction), respectively ($n = 18$, $P < 0.05$ both for the inward and outward direction).

To further pharmacologically characterize the P2 receptors involved, the influence of additional P2 receptor agonists on the $V_m$ response of podocytes was investigated. Figure 2 shows the concentration-response curves of the $V_m$ response of podocytes to the different purinergic agonists tested. On the basis of nonlinear regression analysis, the rank order of potency for the depolarizing effect of the purinergic agonists was UTP (EC50 $1.8 \times 10^{-6}$ M) ≈ UDP (EC50 $2 \times 10^{-6}$ M) > ATP-γ-S (EC50 $3.8 \times 10^{-6}$ M) > ATP (EC50 $7.4 \times 10^{-6}$ M) > 2-methylthioadenosine 5’-triphosphate (2-MeS-ATP) (EC50 $4.9 \times 10^{-5}$ M) > BzATP (EC50 $8.1 \times 10^{-5}$ M) > ADP-β-S (EC50 $1.1 \times 10^{-4}$ M) ($n = 3–18$). α,β-Me-ATP did not depolarize $V_m$ of podocytes (100 μM, $n = 5$).

ATP activates a Cl− conductance in podocytes. The effect of ATP on $V_m$ and $G_m$ was examined in the nominal absence of intracellular Cl−. Pipettes were filled with 145 mM Cs2SO4. After fast whole cell configuration was achieved, cells were dialyzed with Cs2SO4 for ~5 min, and then extracellular Cl− was replaced by 145 mM Na+ gluconate. The addition of ATP (100 μM) led to a depolarization from $-32 \pm 5$ to $-21 \pm 4$ mV in this setting, but no significant increase of $G_m$ was observed ($n = 6$). Figure 3 summarizes the data.

Effect of the purinoceptor antagonists suramin and pyridoxal phosphate 6-azophenyl-2’, 4’-disulfonic acid on the $V_m$ response to ATP. Pretreatment of podocytes with suramin (100 μM, 5 min) depolarized resting $V_m$ of cells from $-62 \pm 3$ to $-58 \pm 3$ mV. Suramin (100 μM) led to a depolarization from $-60 \pm 2$ to $-48 \pm 2$ mV ($n = 18$, $P < 0.05$), respectively (Fig. 3, A and B). ATP (100 μM) led to a $G_m$ increase from 3.47 ± 1.09 to 4.46 ± 1.39 nS (inward direction) and from 4.30 ± 1.08 to 5.13 ± 1.30 nS (outward direction), respectively ($n = 18$, $P < 0.05$ both for the inward and outward direction).
μM) inhibited the depolarization induced by 100 μM ATP by 50 ± 8% (n = 5). Pyridoxal phosphate 6-azophenyl-2', 4'-disulfonic acid (PPADS; 100 μM, 5 min) did not change resting Vm of podocytes. PPADS (100 μM) inhibited ATP-mediated depolarization by 88 ± 4% (n = 5). Figure 4 summarizes the data.

**Nucleotides increase [Ca2+]i in podocytes.** To further characterize cellular responses of podocytes to extracellular nucleotides, microfluorescence experiments using the Ca2+-sensitive fluorescent dye fura 2 were performed. Resting [Ca2+]i of podocytes was 72 ± 4 nM (n = 61). Apart from α,β-Me-ATP, all nucleotides increased [Ca2+]i of podocytes (each 100 μM, Table 2). This [Ca2+]i response varied between the different agonists tested. Figure 5 shows an original recording of the [Ca2+]i response of a single podocyte to the extracellular nucleotides ATP (n = 14), UTP (n = 12), and BzATP (n = 11), respectively (each 100 μM). An additional set of experiments addressed the question of whether this [Ca2+]i increase was due either to the Ca2+ release from intracellular stores or to a Ca2+ influx from the extracellular space. Figure 6 shows original recordings of the effect of ATP (6A), UTP (6B), and BzATP (6C; each 100 μM) on [Ca2+]i, with normal or low (from 1 mM to 1 μM) extracellular Ca2+ concentration. All three agonists elicited a biphasic [Ca2+]i response consisting of an initial Ca2+ peak followed by a sustained Ca2+ plateau, the latter being dependent on extracellular Ca2+. Ca2+ peaks were not affected by lowering extracellular Ca2+. Repetitive stimulation with 10 μM ATP in the presence of normal and reduced extracellular Ca2+ resulted in Ca2+ peaks of 258 ± 42 (1 mM extracellular Ca2+), 267 ± 57 (1 μM extracellular Ca2+), and 284 ± 79 nM (1 mM extracellular Ca2+), respectively (n = 6). Similar results were obtained with UTP and BzATP (each 10 μM, n = 6, data not shown). These results indicate initial Ca2+ peaks to result from Ca2+ release from intracellular stores.

**Podocytes and glomeruli express mRNA for P2Y1, P2Y2, P2Y6, and P2X7 receptors.** Figure 7 shows ethidium bromide-stained agarose gel electrophoresis of PCR products for the P2Y1, P2Y2, P2Y6, and P2X7 receptors in mouse glomeruli (top). Similar results were also found in mouse podocytes, where PCR products for the P2Y1, P2Y2, P2Y6, and P2X7 receptors were also detected (bottom).

**Is there a functional role for the P2X7 receptor in podocytes?** Among the P2 receptor family, the P2X7 receptor is unique in that it constitutes a ligand-gated...
ion channel, which on sustained activation opens large-conductive nonselective pores, ultimately resulting in cell lysis (11, 23). P2X7 receptor mRNA expression was detected in podocytes (cf. Fig. 7). BzATP is the most potent P2X7 receptor agonist presently available. BzATP induced a $V_m$ response in podocytes that was comparable to the cellular responses initiated by ATP and UTP, respectively (cf. Fig. 1). In addition, BzATP also induced a biphasic $Ca^{2+}$ transient in podocytes, its morphology being similar to that elicited by ATP and UTP, respectively (cf. Fig. 6). To further elucidate whether the P2X7 receptor is functionally present in podocytes, resulting in the aforementioned pore formation, supplementary experiments were performed. In the presence of UTP, the addition of BzATP did not result in an additional depolarization of podocytes, indicating the lack of functional activity of a P2X7 receptor (Fig. 8, $n = 5$). On top of that, treatment of podocytes with ATP or BzATP (both 0.3 mM) for 1, 4, and 8 h, respectively, did not result in an increase in LDH release from podocytes (Fig. 8; 1, 4, 8 h: $n = 9$ each). Only after a prolonged exposure over 24 h, a slight, albeit significant, increase in LDH release was detected in podocytes (ATP: 14 ± 3%, BzATP: 25 ± 4% vs. control: 17 ± 2%; Fig. 9, 24 h: $n = 12$).

**DISCUSSION**

Extracellular ATP is considered to be an important regulator of biological functions in glomerular cells. ATP might play a role in mesangial cell injury during glomerulonephritis because it increases $[Ca^{2+}]_i$ and inositol phosphates in mesangial cells, resulting in their depolarization and contraction (18, 21). It also stimulates proliferation of mesangial cells via a P2 receptor (26). In addition, ATP concentrations >300 μM mediate apoptosis and necrosis of cultured mesangial cells via the P2X7 receptor (25). P2 receptor-induced $Ca^{2+}$ signaling has also been reported in bovine glomerular endothelial cells and human glomerular epithelial cells with a cobblestone appearance (1, 20).

In the past, it was not possible to identify cultured glomerular epithelial cells as podocytes, and it was suggested that glomerular epithelial cells in culture were not podocytes, but in fact were glomerular parietal epithelial cells (14). Recently, successful cul-

Fig. 6. Original fluorescence recordings of the effect of ATP (A), UTP (B), and BzATP (C) on $[Ca^{2+}]_i$, in podocytes both in normal or low (from 1 mM to 1 μM = $Ca^{2+}$ 10^{-6}) extracellular $Ca^{2+}$ concentration, respectively. The sustained $[Ca^{2+}]_i$ plateau induced by ATP, UTP, and BzATP depends on extracellular $Ca^{2+}$.

![Image](image1.png)

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![Image](image4.png)

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tivation of differentiated podocytes was demonstrated; undifferentiated podocytes with a cobblestone appearance developed into differentiated podocytes with cell processes expressing podocyte-specific markers (14). In the present study, podocytes showing a differentiated phenotype were used. Apart from exhibiting an arborized morphology, cells stained positive for the podocyte markers WT-1 (15), synaptopodin (13), nephrin (28), and p57 (16). In addition, mRNA expression of the podocyte marker CD2AP (27) was detected by RT-PCR. However, although cultured differentiated podocytes possess many in vivo properties of podocytes, biological functions of the cells may change during culture, and therefore results obtained from these cells have to be interpreted with care.

We show that ATP regulates ion currents and \([Ca^{2+}]_i\) in differentiated podocytes. ATP depolarized \(V_m\) and increased \(G_m\) of podocytes. In addition, ATP led to an increase in \([Ca^{2+}]_i\), which was due to the release of \(Ca^{2+}\) from intracellular stores and to an influx of \(Ca^{2+}\) from the extracellular space. An increase of \([Ca^{2+}]_i\) is known to activate a Cl\(^{-}\) current in podocytes (17). The Cl\(^{-}\) replacement experiments in this study showed that in the nominal absence of Cl\(^{-}\), ATP failed to activate ion currents. An ATP-induced activation of Cl\(^{-}\) current has been reported in mesangial cells, whereas in glomerular endothelial cells ATP has been assumed to open nonselective ion currents (18, 19).

In this study, both the purines ATP and ATP-g-S, as well as the pyrimidines UTP and UDP, were potent agonists in mediating \(V_m\) response and increase of \([Ca^{2+}]_i\) in podocytes. Cellular responses to 2-MeS-ATP were small; \(\alpha,\beta\)-Me-ATP almost had no effect. The pharmacological profile fits well to that of a P2Y\(_2\) receptor (23), whose mRNA expression was detected both in glomeruli and podocytes by RT-PCR. In addition, the marked cellular response of both \(V_m\) and \([Ca^{2+}]_i\) to extracellular uridine nucleotides hints at a functional relevance of the uridine nucleotide-specific receptor P2Y\(_6\) (see below). P2Y\(_1\) receptors seem to play a minor functional role, because the P2Y\(_1\) receptor has been reported to be strongly activated by 2-MeS-ATP, but not by UTP or UDP (23). Moreover, adenine nucleotide diphosphates are potent agonists to P2Y\(_1\) receptors (23). Here ADP-\(\beta\)-S elicited only weak responses of both \(V_m\) and \([Ca^{2+}]_i\) in podocytes. Despite mRNA expression of the P2Y\(_1\) receptor found both in glomeruli and podocytes, our data clearly indicate that it is functionally less active than its P2Y\(_2\) counterpart.

Apart from P2Y\(_1\) and P2Y\(_2\) receptor mRNA expression, P2Y\(_6\) receptor mRNA expression similarly was demonstrated in both glomeruli and podocytes. Coexpression of P2Y\(_6\) mRNA, along with P2Y\(_1\) and P2Y\(_2\) mRNA, has also been demonstrated in adult rat cardiac myocytes (29). The P2Y\(_6\) receptor has also been detected in other cell types, and it seems to be more widely distributed than the P2Y\(_4\) receptor, for instance (23). In this regard, the P2Y\(_4\) receptor was not detected in podocytes. The P2Y\(_6\) receptor has been reported to be activated most potently by UDP (3), and it was assumed that it accounts for endogenous uridine nucleotide-specific responses (23). Among the nucleotides tested in the present study, the marked cellular responses of podocytes elicited by UDP and UTP clearly hint at a functional role of P2Y\(_6\) receptors in podocytes.

Among the P2 receptor family, the P2X\(_7\) receptor is unique in that it constitutes a ligand-gated ion channel, which, on sustained activation, opens large-conductive nonselective pores, ultimately resulting in cell lysis (11, 23). In our sets of experiments, BzATP, the most potent agonist of the P2X\(_7\) receptor known so far (23), was shown to be less potent than ATP and UTP in stimulating cellular responses of podocytes. In this regard, in mouse tissue BzATP has been described to be 10–100 times more potent than ATP in activating P2X\(_7\) receptors (23). To further clarify the role of the P2X\(_7\) receptor in podocytes, several sets of experiments were performed with the following results. First, mRNA of the P2X\(_7\) receptor could be detected in mouse glomeruli and podocytes. Second, in the presence of UTP, BzATP did not induce an additional depolarization of podocytes. An additional depolarization induced by BzATP would have been expected in the case of a separate P2X\(_7\) receptor being functionally active in podocytes. Third, BzATP released \(Ca^{2+}\) from intracellular stores, suggesting that it might act via a P2Y receptor. Apart from differences in magnitude, both the time course and morphology of \(Ca^{2+}\) transients induced by BzATP were almost similar to those elicited by ATP or UTP. Fourth, P2X\(_7\) receptor-induced pore formation with subsequent cell lysis has been reported to occur only after prolonged receptor activation. One might thus argue that the cellular responses reported here were only reversible due to short exposure to BzATP. To further address this question, LDH release was tested in podocytes. Prolonged exposure to high concentrations of ATP or BzATP did not cause LDH release in podocytes within 8 h, indicating that in contrast to mesangial cells (25), the P2X\(_7\) receptor did not mediate cytotoxicity in podocytes. Therefore, although some mRNA expression of the P2X\(_7\) receptor could be detected in podocytes, the present experi-
ments do not support a functional role of the P2X7 receptor in this cell type. This is in agreement with a recent study by Harada et al. (9), in which weak mRNA expression and very-low-intensity immunoreactivity of the P2X7 receptor were detected in the glomerulus, including mesangial cells and podocytes.

In summary, the data indicate that the nucleotide P2Y1, P2Y2, P2Y6, and P2X7 receptors are expressed in podocytes. The effects of extracellular nucleotides on \( V_m \) and \([Ca^{2+}]_i\) in podocytes, however, mainly are mediated by P2Y2 and P2Y6 receptors.

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