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Research Article

Endocytosis mechanism of $P2Y_2$ nucleotide receptor tagged with green fluorescent protein: clathrin and actin cytoskeleton dependence

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Abstract. Extracellular nucleotides exert a large number of physiological effects through activation of P2Y receptors. We expressed rat $P2Y_2$ ($rP2Y_2$) receptor, tagged with green fluorescent protein (GFP) in HEK-293 cells and visualized receptor translocation in live cells by confocal microscopy. Functional receptor expression was confirmed by determining $[Ca^{2+}]_i$ responses. Agonist stimulation caused a time-dependent translocation of the receptor from the plasma membrane to the cytoplasm. Rearrangement of the actin cytoskeleton was observed during agonist-mediated $rP2Y_2$-GFP receptor internalization. Colocalization of the internalized receptor with early endosomes, clathrin and lysosomes was detected by confocal microscopy. The inhibition of receptor endocytosis by either high-density medium or chlorpromazine in the presence of UTP indicates that the receptor was internalized by the clathrin-mediated pathway. The caveolin-mediated pathway was not involved. Targeting of the receptor from endosomes to lysosomes seems to involve the proteasome pathway, because proteasomal inhibition increased receptor recycling back to the plasma membrane.

Key words. Human embryonic kidney (HEK-293) cells; receptor regulation; translocation; internalization; proteasomal system.

Metabotropic P2Y receptors belong to the family of G protein-coupled receptors (GPCRs). Eight subtypes in this family, namely $P2Y_{(1,2,4,6,11,12,13,14)}$, have been characterized [1, 2]. In the P2Y receptor family, $P2Y_1$ and $P2Y_{11}$ are selectively activated by purines [3], $P2Y_4$ [4] and $P2Y_6$ [5] are stimulated by pyrimidines, and $P2Y_2$ responds equipotently to purine and pyrimidine triphosphates [6]. All of these receptors, upon stimulation with specific agonists, cause an increase in intracellular inositol 1,4,5-trisphosphate ($InsP_3$) via the phospholipase C (PLC) pathway, whereas $P2Y_{11}$ is also positively coupled to adenylyl cyclase [3]. The $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors are negatively coupled to adenylyl cyclase via the G_i protein [1]. The $P2Y_{14}$ receptor is activated by UDP-glucose, while ATP, UTP, ADP or UDP are inactive [1]. Stimulation of P2Y receptors in different cell types results in a multitude of effects including proliferation and apoptosis. In addition to purine or pyrimidine nucleoside triphosphates, P2Y receptors can also be activated by diadenosine polyphosphates [7].

The $P2Y_2$ receptor is expressed in a wide variety of cells and tissues. Through this receptor subtype, extracellular uridine nucleotides regulate airway epithelial mucocil-

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iary clearance. Mucosal administration of UTP, and to a lesser extent of UDP [8], increases ciliary beat frequency and mucin secretion [9]. The $P2Y_2$ receptor has also been shown to modulate proliferation of different cell types [10–12]. Under conditions of stress or injury, the $P2Y_2$ receptor has been found to be upregulated in activated thymocytes, salivary gland epithelial cells and models of vascular tissue injury [13].

The mechanism of internalization and the intracellular trafficking of P2Y receptors has not been studied in detail. Internalization pathways have been reported for the β_2-adrenergic receptor, including ligand-stimulated endocytosis [14] and downregulation that is independent of endocytosis [15]. Receptor endocytosis is a complex process that involves the recruitment of a number of proteins to the plasma membrane, such as arrestins, clathrin and AP2 [14], and translocation to the early endosomal compartment and lysosomes [16]. This process is also known to be regulated by the cholesterol content and fluidity of the plasma membrane [17]. Extraction of cholesterol from the plasma membrane with methyl-β-cyclodextrin (MβCD) has been reported to inhibit clathrin-mediated receptor endocytosis [18]. Besides clathrin-mediated receptor endocytosis it can occur through caveolin-mediated pathways [16]. Receptor endocytosis from the plasma membrane and intracellular trafficking require massive rearrangement of the cytoskeleton mediated by cytoskeleton-associated proteins [19]. Agonist stimulation of P2Y receptors has been reported to result in the reorganization of the actin cytoskeleton in myocytes [20]. However, a role for the actin cytoskeleton in P2Y receptor endocytosis was not clearly established.

Prolonged stimulation of P2Y receptors results in agonist-induced receptor desensitization, but not known is whether this is the direct result of receptor internalization, or if it is due to changes in interactions with intracellular signaling modulators. Site-directed mutations of probable phosphorylation sites of the murine $P2Y_2$ receptor revealed different structural determinants for receptor desensitization and sequestration [21]. The targeting of the mannose-6-phosphate receptor from early endosomes to other compartments, such as late endosomes, lysosomes, or recycling endosomes is directed via the multivesicular body [22]. An interesting regulatory step that could play a role in directing receptors from endosomes to lysosomes is the proteasome complex. Ubiquitin and the proteasomal pathway have been implicated in lysosomal targeting and degradation of the growth hormone receptor [23]. However, little is known about the pathways involved in $P2Y_2$ receptor trafficking.

Thus, we decided to investigate the trafficking of the $P2Y_2$ receptor and its interaction with intracellular compartments and the cytoskeleton to elucidate the subcellular localization of the $P2Y_2$ receptor upon agonist stimulation. We were able to demonstrate that endocytosis of the $P2Y_2$ receptor involves a reversible rearrangement of the actin cytoskeleton and the involvement of myosin light-chain kinase in live cells. Endocytosis of the $P2Y_2$ receptor proceeds via the clathrin-mediated pathway. Stimulation of the receptor with agonist for different time periods resulted in targeting of the receptor to early endosomes and subsequently to lysosomes. Trafficking of the receptor from endosomes to lysosomes was inhibited by the proteasomal inhibitor MG-132, suggesting a proteasomal pathway for the degradation of the $P2Y_2$ receptor.

Materials and methods

Materials

Geneticin (G418 sulfate), ML-9, FTI-277, MG-132 (Calbiochem); poly-L-lysine, (PLL), ATP, UTP, BSA, chlorpromazine, cytochalasin-D (Cyto-D), filipin III, methyl-beta-cyclodextrin (Sigma); Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (10,000/10,000 IU/ml), Trypsin/EDTA (0.05%/0.02%), fetal calf serum (FCS) (Seromed); N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammoniummethyl sulfate (DOTAP) (Roche); cell culture dishes (Nunc); coverslips (22 mm, OmniLab); fura 2-AM, LysoTracker Red (Biomol/Molecular Probes); mouse monoclonal antibody against the clathrin heavy chain, early endosome antigen-1 (EEA-1), lysosome-associated marker protein-1 (LAMP-1) (Pharmingen); anti-MycHis-IgG (Invitrogen); cholera toxin subunit B conjugated to Alexa Fluor 555 (CTxB), goat anti-mouse IgG conjugated to Alexa Fluor488/555/568 (Molecular Probes); Aquatex (Merck).

Cell culture and transfection

$rP2Y_2$ receptor cDNA was kindly provided by Dr. Rice [24] and subcloned into pcDNA 3.1A(+) myc-his (MH) (Invitrogen) and pEGFPN2 (Clontech). Cell culture and transfection of human embryonic kidney (HEK-293) cells were carried out as described previously [25]. Briefly, HEK-293 wild-type cells were transfected with 5 µg/ml DNA in serum-free medium using DOTAP for 8 h. After this time, the medium was replaced with complete medium and the cells were selected with 1 mg/ml G418. Transfected cells were grown in medium consisting of DMEM/Ham's F12 (1:1), supplemented with 10% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin (referred to in subsequent text as culture medium) in a 5% CO_2/95% air, humidified atmosphere at 37 °C.

Cytosolic Ca^{2+} measurements

The cells were plated on PLL-coated plates and single-cell measurements were made after 3 days, when the cells were 40–60% confluent. The changes in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured by prein-

cubation of the cells with 2 µM fura-2AM for 30 min in NaHBS (HEPES-buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose and 20 mM HEPES/Tris pH 7.4). Cells were then stimulated under continuous superfusion of prewarmed NaHBS at 37 °C with different concentrations of UTP or other agonists at 37 °C. Fluorescence intensity was recorded alternatively at 340 nm and 380 nm excitation and 520 nm emission. Changes were monitored in single cells bathed in a perfusion chamber which was placed on the microscope stage (Zeiss) of a fluorescence imaging system from TILL Photonics with a ×40 oil immersion objective and a flow rate of 1 ml/min [25].

Agonist-induced internalization

The cells were plated at a density of 5×10^5 to 1×10^6 cells/dish (diameter, 50 mm) on PLL (0.01%)-precoated coverslips (diameter, 22 mm). The cells were stimulated with 100 µM ATP or UTP in medium at 37 °C. Lysosomes were labeled by preincubating the cells for 30 min with 100 nM LysoTracker Red prior to agonist stimulation. Labeling of other subcellular compartment markers such as LAMP-1 (lysosomes) and EEA-1 (early endosomes) was performed in fixed cells by immunofluorescence, as described below.

To study inhibition of receptor internalization, the cells were incubated with medium containing 0.45 M sucrose or at 4 °C in the presence of 100 µM UTP [15]. When using inhibitors such as Cyto-D, ML-9, FTI-277, methyl beta cyclodextrin, chlorpromazine and filipin III, the preincubation time was 30 min, since that pretreatment time did not affect morphology of the cells.

Cell staining for immunofluorescence

Immunohistochemistry was performed as suggested by the manufacturer (Pharmingen). Briefly, cells grown on coverslips were fixed using methanol:acetone (1:1) for 10 min at –20° C after agonist stimulation. The cells were then placed in blocking buffer (1% BSA in PBS) for 1 h. Cells were then incubated with primary antibody (e.g. anti-clathrin heavy chain, anti-EEA-1, anti-LAMP-1 or anti-myc antibody) in blocking buffer for 1 h at room temperature, washed three times in PBS and incubated with Alexa Fluor 568 secondary antibody in blocking buffer for 1 h at room temperature. The cells were then washed three times in PBS and the coverslip was inverted onto a slide covered with mounting medium. For F-actin staining, the cells were stimulated with agonist, fixed as above, and incubated for 10 min with phalloidin-Alexa Fluor 546. The cells were then washed three times with PBS and the coverslip was placed onto a slide and covered with mounting medium.

Confocal imaging

Images were taken on a Zeiss inverted LSM 510 laser scanning confocal microscope equipped with a Plan-Apochromat ×63 objective. The green fluorescence protein (GFP) was excited using a 488-nm argon/krypton laser, and the emitted fluorescence was detected with a 505–530 band pass filter. LysoTracker Red and Alexa Fluor 555/568 were detected using a 543-nm helium/neon laser for excitation and a 560-nm long-pass filter. In live-cell imaging, the cells were incubated on stage in a chamber of 5% CO_2 at 37 °C in complete culture medium. Thirteen to fifteen sections 1 µm apart were taken serially from the bottom to the top of each cell. The sections were projected onto one plane, and XZ- or YZ-plane images were constructed from all optical sections for three-dimensional images. Images were processed with Zeiss confocal microscopy software, release 3.2. The loss of fluorescence from the plasma membrane was determined by quantitating the fluorescence intensity in user-defined regions of interest (ROI) that encompass either the plasma membrane or cytoplasmic regions. The calculations took into consideration the relative intensities of the pixels in the respective ROI and the value obtained was the mean of the fluorescence intensities of the pixels in the ROIs.

Data analysis

Unless stated otherwise, results are presented as the mean ± SE, and statistical analysis was achieved by Student's unpaired t test using SigmaPlot (Jandel Scientific).

Results

Functional expression of GFP- and MH-tagged $P2Y_2$ receptors in HEK-293 cells

The $P2Y_2$-GFP receptor was directly visualized in live cells using a confocal laser scanning microscope (fig. 1A). The receptor was localized mainly at the plasma membrane of the cells and not concentrated in any other cellular compartment. We also expressed the $P2Y_2$ receptor with an MH tag which was detected by immunostaining of fixed cells with anti-myc antibody as primary antibody and Alexa Fluor 488-conjugated secondary antibody. The MH-tagged $P2Y_2$ receptor was similarly detected at the plasma membrane of the cells (data not shown). Thus, incorporation of either the GFP or MH tag at the C terminus of the $P2Y_2$ receptor did not prevent the targeting of the receptor to the plasma membrane.

Functional coupling of the transfected $P2Y_2$-GFP receptor was determined by measuring changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in fura 2-loaded cells upon stimulation with $P2Y_2$ receptor agonists. Transfected and untransfected cells were challenged with the nucleotide agonists UTP or ATP, at different concentrations ranging from 10^{-9} to 10^{-4} M. The cells were visualized at 340 nm to monitor changes in $[Ca^{2+}]_i$ by fura-2 fluorescence and at 460 nm to detect the $P2Y_2$-GFP receptor. The simultaneous visualization of fura-2 and GFP

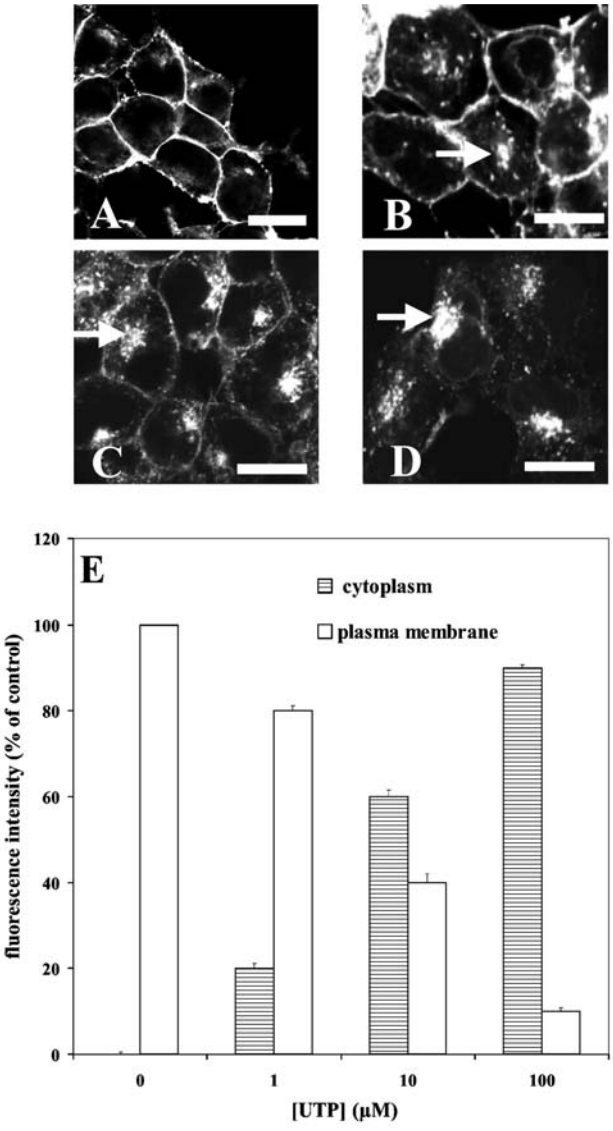

Figure 1. Stable expression of the $rP2Y_2$ receptor in HEK-293 cells and agonist concentration-dependent internalization of the $rP2Y_2$-GFP receptor. (*A–D*) HEK-293 cells were stably transfected with cDNA encoding the $rP2Y_2$-GFP gene, as described in Materials and methods. Expression and localization of the receptor were visualized in live cells using confocal laser scanning microscopy (CLSM). The $rP2Y_2$-GFP receptor was visualized in stably transfected HEK-293 cells that were unstimulated (*A*, control cells), or stimulated for 30 min at 37°C with 1 μM (*B*), 10 μM (*C*) or 100 μM UTP (*D*). The increase in the amount of internalized receptor is indicated by bold arrows. Scale bar, 20 μm. (*E*) The fluorescence intensity at the plasma membrane and in the cytoplasm was calculated by selecting the appropriate region of interest, using Zeiss software. The distributions of cellular fluorescence intensity between the cytoplasm (hatched bars) and the plasma membrane (open bars) were determined in unstimulated and stimulated cells. The data are normalized to initial fluorescence intensity at the plasma membrane (100%). The data presented are the means ± SD from at least three independent experiments with 20 cells each.

fluorescence allowed the selection of double-positive, transfected cells with a high receptor expression level for measurement of the Ca^{2+} response. In untransfected cells, the calcium response to nucleotide is due to stimulation of endogeneous $P2Y_2$ and $P2Y_1$ receptors, since mRNAs for these receptors were previously detected in HEK-293 cells by RT-PCR [12, 25]. In untransfected cells, the agonist-induced calcium response was submaximal at agonist concentrations below 10^{-5} M, and at 10^{-7} M there was only a negligible response. The EC_{50} value for either UTP or ATP in untransfected cells was 1.8 μM. In $P2Y_2$-GFP receptor-transfected cells, the sensitivity of the cells to agonist was increased, as observed by the leftward shift of the curves in comparison to untransfected cells. The EC_{50} values for nucleotides in cells expressing the $rP2Y_2$-GFP receptor or the $rP2Y_2$-MH receptor, respectively, were 200 and 350 nM for UTP, and 200 and 150 nM for ATP (data not shown). These results confirm that the tagged receptors were functional when expressed in HEK-293 cells.

Effect of agonist concentration on receptor localization

The $rP2Y_2$-GFP receptor expressed in HEK-293 cells was stimulated with UTP, an agonist of the $P2Y_2$, but not the $P2Y_1$ receptor. In unstimulated cells, the receptor was localized mainly to the plasma membrane (fig. 1A). Stimulation of $rP2Y_2$-GFP receptor-transfected cells with 1–100 μM UTP resulted in a concentration-dependent increase in the extent of receptor internalization. This was observed as a loss of receptor-associated fluorescence from the plasma membrane and the simultaneous accumulation of fluorescence in intracellular organelles (fig. 1B–D). The increase in the amount of internalized receptor is indicated by bold arrows (fig. 1B–D). The loss of fluorescence from the plasma membrane was determined by quantitating the fluorescence intensity in user-defined ROI that encompassed either the plasma membrane or cytoplasmic regions. As shown in figure 1E, at 100 μM UTP, there was almost no receptor left at the plasma membrane but it appeared entirely in the cytoplasmic compartment. Qualitatively similar results were obtained for internalization of the $rP2Y_2$-MH receptor (data not shown), indicating that the presence of either a GFP or MH tag did not affect receptor internalization. In our further studies of receptor endocytosis we used the agonist UTP at 100 μM, because this concentration induced complete receptor endocytosis and the amount of receptor present at the plasma membrane and in the cytoplasm could be clearly distinguished.

Compartmentalization of the receptor after stimulation with agonist

The agonist-promoted loss of surface immunoreactivity in cells expressing the $P2Y_2$ receptor has been investigated previously [6], but the time-dependent compart-

mentalization of the $P2Y_2$ receptor and the mechanism responsible for internalisation had not yet been investigated. HEK-293 cells stably expressing $rP2Y_2$-GFP receptors were stimulated with 100 µM UTP for 30 or 60 min. In these experiments, after stimulation, the cells were fixed and incubated with antibodies raised against either EEA-1 [26] or LAMP-1. After 30 min with UTP, the internalized receptors (green) colocalized with early endosomes (red) (colocalization shown by yellow, and indicated by arrows in fig. 2A3), but not with lysosomes (data not shown). After 60 min with UTP, $P2Y_2$-GFP receptors colocalized with lysosomes (red), as indicated by the yellow signal and indicated by arrows in figure 2A6, but were no longer detected in early endosomes (data not shown).

Lysosomal localization of $P2Y_2$-GFP receptors after stimulation with UTP for 60 min was seen in fixed cells (fig. 2A6) and confirmed by live imaging using LysoTracker (see large yellow structure in fig. 8B, as indicated by the bold arrow). Cells were preincubated for 30 min with 100 nM LysoTracker Red to label lysosomes and 10 µM of the farnesyltransferase inhibitor FTI-277, which blocks farnesylation of endosomal proteins and prevents trafficking of internalized membrane proteins between endosomes and lysosomes [27]. Preincubation of the cells with FTI-277 did not affect the localization of the $rP2Y_2$-GFP receptor (fig. 2B1) and did not prevent the UTP-induced endocytosis of the receptor (fig. 2B2). However, FTI-277 prevented the colocalization of internalized receptors with lysosomes stained with LysoTracker Red, even after 60 min of UTP stimulation (fig. 2B3), a time normally sufficient to target the $rP2Y_2$-GFP receptor to lysosomes (fig. 2A6).

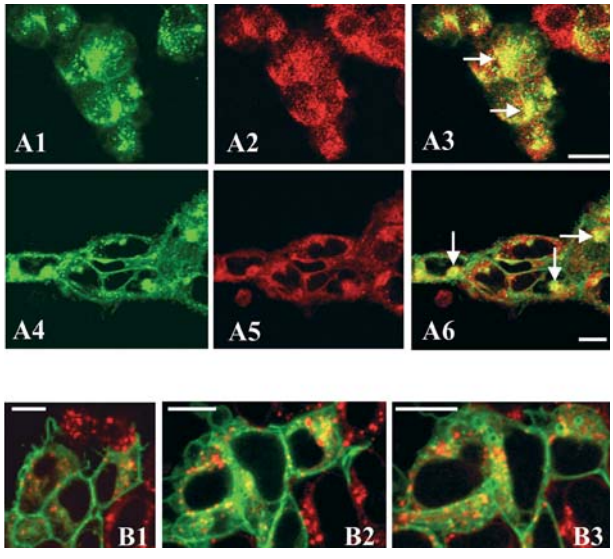

Figure 2. Compartmentalization of the $P2Y_2$ receptor after stimulation with agonist. (*A*) Colocalization of the $rP2Y_2$-GFP receptor with early endosomes and lysosomes after UTP stimulation. HEK-293 cells stably expressing the $rP2Y_2$-GFP receptor were stimulated with 100 µM UTP at 37 °C in culture medium for 30 min (*A1*) or 60 min (*A4*). Colocalization of the GFP-tagged receptor (green) with early endosomes or lysosomes was determined in fixed cells with mouse anti-EEA-1 antibody (*A2*; red) or anti-LAMP-1 antibody (*A5*; red), respectively, visualized with goat anti-mouse Alexa Fluor 568 as secondary antibody. *A3* is an overlay picture from *A1* and *A2*, *A6* is merged from *A4*, and *A5*. Colocalization is indicated in yellow (*A3*, *A6*), marked with arrows. Scale bar shown for each figure is 20 µm. Results shown are representative of at least 3 individual experiments. (*B*) Effect of the farnesyltransferase inhibitor FTI-277 on trafficking of the $rP2Y_2$-GFP receptor by live-cell imaging. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 100 nM LysoTracker (red fluorescence) and 10 µM FTI-277 for 30 min at 37 °C. Cells were unstimulated (*B1*), or stimulated with 100 µM UTP for 30 min (*B2*) or 60 min (*B3*) and receptors and lysosomes were visualized. FTI-277 prevents colocalization of the internalized receptor with lysosomes. The results shown are representative of three different experiments. Scale bar, 20 µm in *B1, 2, 3.*

Nucleotides induce actin cytoskeleton rearrangements

Rearrangements of the cytoskeleton play an important role in cellular processes such as endocytosis and exocytosis [19]. Stimulation of HEK-293 cells expressing the $rP2Y_2$-GFP receptor with 100 µM UTP caused massive reorganization of the actin cytoskeleton counterstained in fixed cells with Alexa Fluor 546-labeled phalloidin (red). In unstimulated cells, the $rP2Y_2$-GFP receptor and actin were colocalized to the plasma membrane with few actin fibers in the cytoplasm (fig. 3A2), indicating that the cells were not under stress [28]. For the first 5 min of UTP stimulation, the $rP2Y_2$-GFP receptor remained localized to the plasma membrane, whereas actin was redistributed to the cytoplasm (fig. 3B1,2). After 10 min of UTP stimulation, initial receptor internalization was observed and actin was diffusely distributed in the cytoplasm (fig. 3C1,2). Figure 3C3 shows that there is no colocalization of actin with the receptor in comparison to figure 3A3, and a diffuse staining of actin is seen. This demonstrates that actin rearrangement preceded endocytosis of the receptor. After 20 min of UTP stimulation, only 20% of the receptors remained at the plasma membrane, as compared to unstimulated cells, whereas actin had redistributed to the plasma membrane (fig. 3D1,2). The $rP2Y_2$-GFP receptor was completely internalized at 30 min (data not shown) and 60 min after UTP stimulation, whereas actin reappeared concentrated at the plasma membrane (fig. 3E1,2).

To confirm the role of the actin cytoskeleton in receptor endocytosis, HEK-293 cells expressing the $rP2Y_2$-GFP receptor were pretreated with an actin-disrupting agent, Cyto-D [29] prior to UTP stimulation. Figure 4 indicates that a 30-min treatment with UTP (fig. 4A2) was insufficient to induce receptor internalization in Cyto-D-treated cells, whereas the receptor was completely internalized by a 20-min treatment with UTP in the absence of Cyto-D, as seen in figure 3D1. Compared to unstimulated cells (fig. 4A1), a 30-min stimulation with UTP induced a

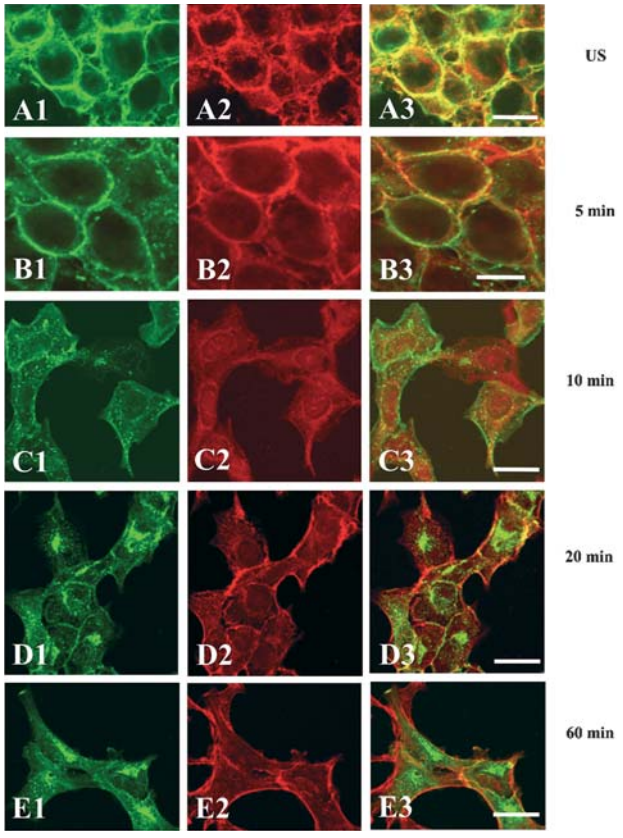

Figure 3. Actin cytoskeleton rearrangement in UTP-stimulated HEK-293 cells expressing the rP2Y_2-GFP receptor. HEK-293 cells expressing the rP2Y_2-GFP receptor were incubated in culture medium at 37 °C in the absence (*A*) or presence (*B–E*) of 100 µM UTP for 5 (*B*) 10 (*C*), 20 (*D*) or 60 (*E*) min. The cells were then fixed and counterstained with actin-binding phalloidin coupled to Alexa Fluor 546 (as described in Materials and methods) and actin (red) and rP2Y_2-GFP receptor (green) were visualized. In *A* to *E*, the respective picture with number 1 represents green fluorescence of P2Y_2-GFP receptor, the picture with number 2 represents red fluorescence of actin staining, and the picture with number 3 gives the overlay. Colocalization of actin and rP2Y_2-GFP receptor is indicated in yellow. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

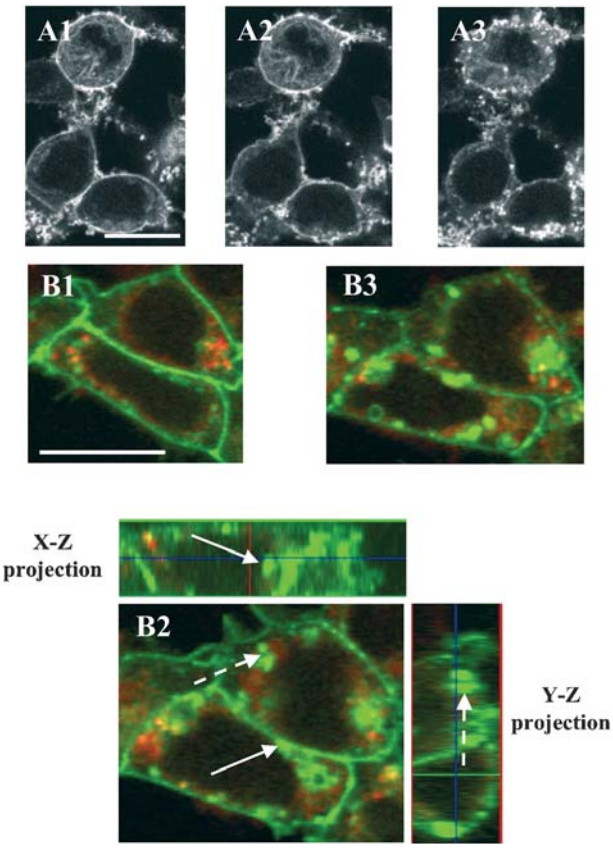

Figure 4. Effect of Cyto-D (*A*), and the myosin light-chain kinase inhibitor ML-9 (*B*) on endocytosis of the rP2Y_2-GFP receptor. Scale bar, 20 µm. Results shown are representative of at least three individual experiments. (*A*) HEK-293 cells stably expressing the rP2Y_2-GFP receptor were preincubated with 100 nM cyto-D at 37 °C for 30 min and live-cell confocal imaging was performed. Images were collected from cells before stimulation (*A1*), and stimulated with 100 µM UTP for 30 min (*A2*) or 60 min (*A3*). (*B*) HEK-293 cells expressing the rP2Y_2-GFP receptor were preincubated with 100 µM ML-9 and 100 nM LysoTracker Red for 30 min at 37 °C and then live-cell imaging was performed. Images were collected from cells before stimulation (*B1*), and stimulated with 100 µM UTP for 30 min (*B2*) or 60 min (*B3*). In ML-9-treated cells, the receptor was clustered upon UTP treatment in small vesicles below the plasma membrane (arrows in *B2*). This is clearly visible in the X-Z and Y-Z projections. There was no colocalization of the endocytosed receptor with lysosomes (as seen in *B3*).

punctate receptor distribution in the membrane (fig. 4A2). In Cyto-D-treated cells stimulated with UTP for 60 min, the receptor was internalized in small vesicles (fig. 4A3), in contrast to the large receptor aggregates internalized in cells treated with UTP in the absence of Cyto-D (fig. 1D). We next examined the effect of the myosin light-chain kinase inhibitor, ML-9 [30], on P2Y_2 receptor internalization. HEK-293 cells expressing the rP2Y_2-GFP receptor were preincubated with 100 µM ML-9 and 100 nM LysoTracker Red for 30 min and then stimulated with 100 µM UTP. In comparison to the membrane localization of rP2Y_2-GFP receptors in ML-9-treated controls (fig. 4B1), a 30-min UTP stimulation in the presence of ML-9 induced receptor localization (green) in small vesicular clusters below the plasma membrane (marked by arrows in fig. 4B2), which is different from the effect of Cyto-D in UTP-treated cells (fig. 4A2). For three-dimensional analysis, YZ- and XZ images were constructed from XY-plane images, which were taken from the bottom to the top of each cell. The presence of the vesicles, as indicated by arrows in figure 4B2, just below the plasma membrane, is visible in these XZ and the YZ projections. After a 60-min UTP stimulation in the presence of ML9, receptor endocytosis had occurred although there was little localization of receptor in lyososmes (red) in figure 4B3, in contrast to results in the absence of ML-9 (cf data for fixed cells shown in fig. 2A6). Taken to-

gether, these findings indicate that the actin cytoskeleton and cytoskeletal-associated proteins (e.g. myosin) mediate the endocytosis of the $rP2Y_2$-GFP receptor.

Inhibition of receptor internalization

UTP-mediated $rP2Y_2$-GFP receptor internalization in HEK-293 cells was completely inhibited at 4 °C (data not shown). In addition, there was very little receptor internalization at 14 or 28 °C (data not shown), indicating that $P2Y_2$-GFP receptor endocytosis is temperature dependent. The temperature effect indicates that there is no constitutive endocytosis of the receptor.

$P2Y_2$ receptor endocytosis was also sensitive to the density of the medium, since addition of 0.45 M sucrose prevented UTP-induced receptor internalization (data not shown), and after a 30-min UTP stimulation, most of the receptor remained localized to the plasma membrane in small punctate structures (data not shown). The inhibition of endocytosis of the receptor by 0.45 M sucrose suggests the role of a clathrin-dependent mechanism [16].

Colocalization of the $P2Y_2$ receptor and clathrin

Unstimulated cells displayed a diffuse distribution of clathrin (red) in the cytoplasm, and the receptor (green) was localized to the plasma membrane (fig. 5A1,2). After a 5-min UTP stimulation when some receptor internalization was detected, there was a reorganization of clathrin in the cytoplasm (data not shown). After a 10-min UTP stimulation, colocalization of the internalized receptor and clathrin was first detected (fig. 5B3). After a 20-min UTP stimulation, most of the receptor signal colocalized with clathrin (fig. 5C3). Interestingly, $rP2Y_2$-GFP receptor and clathrin colocalization was no longer detectable after 30 min UTP stimulation (data not shown). Thus, these results strongly suggest that UTP-dependent endocytosis of the $P2Y_2$-GFP receptor occurs via clathrin-coated pits, prior to receptor transport to early endosomes or lysosomes depending on the duration of agonist exposure.

To confirm the involvement of a clathrin-mediated pathway in agonist-induced $P2Y_2$-GFP receptor endocytosis, cells were preincubated with 100 µM chlorpromazine, an inhibitor of clathrin-mediated endocytosis [31], and then stimulated with 100 µM UTP for 30 min. Chlorpromazine by itself did not affect cell shape or $P2Y_2$-GFP receptor distribution in the absence of UTP (fig. 6A1), but it prevented UTP-induced receptor internalization (fig. 6A2). When cells were preincubated with 100 µM chlorpromazine for 30 min and 5 nM cholera toxin subunit B coupled to Alexa Fluor 555, a marker for the caveolin-rich lipid rafts in the plasma membrane [32], the $rP2Y_2$-GFP receptor colocalized with cholera toxin subunit B in un-

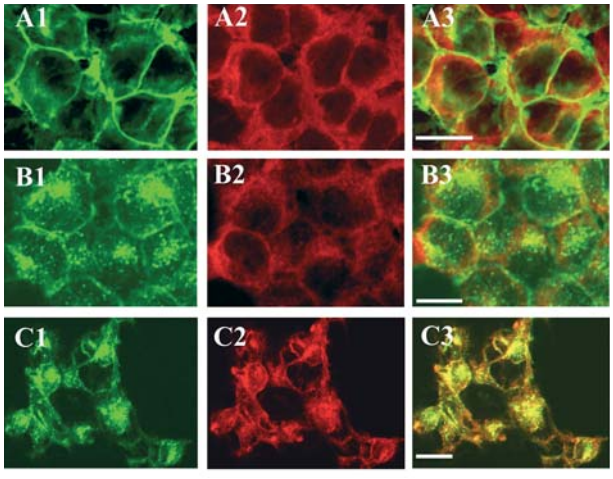

Figure 5. UTP induces colocalization of the $rP2Y_2$-GFP receptor with clathrin. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were incubated in the absence (*A*) or presence (*B*, *C*) of 100 µM UTP at 37 °C for 10 min (*B*), or 20 min (*C*). The cells were fixed, immunolabeled with mouse monoclonal anti-clathrin antibody and with goat anti-mouse Alexa Fluor 568 as secondary antibody, and images were collected. The receptor is visualized by green fluorescence, the clathrin by red fluorescence. The pictures numbered 1, 2 and 3 in *A* to *C* show $P2Y_2$-GFP receptor in 1, clathrin staining in 2, and the overlay in 3. Colocalization is indicated by yellow. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

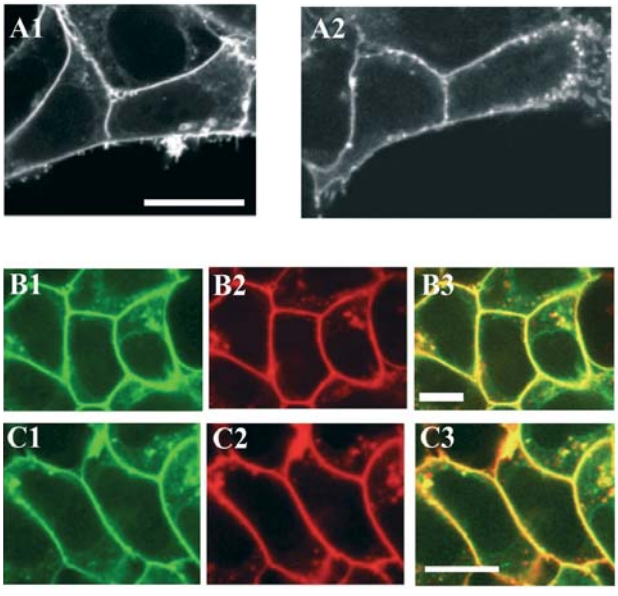

Figure 6. Effect of chlorpromazine and cholera toxin on internalization of the $rP2Y_2$-GFP receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 100 µM chlorpromazine alone (*A1*) or together with 5 nM cholera toxin subunit B coupled to Alexa Fluor 555 (*B*) for 30 min at 37 °C, then incubated in the presence (*A2*, *C*) of 100 µM UTP (*A2* and *C*) for 30 min at 37 °C, and images were collected. The numbering of pictues in *B* and *C* corresponds to that in Fig. 3, with $P2Y_2$-GFP receptor (green fluorescence) in 1, cholera toxin subunit B staining (red fluorescence) in 2, and the overlay in 3. In *B* and *C* the same cells are shown before (*B*) and after (*C*) UTP incubation. Scale bar is 20 µm. Results shown are representative of at least three individual experiments.

stimulated cells (fig. 6B3). After a 30-min stimulation with 100 µM UTP, neither receptor endocytosis nor redistribution of cholera toxin was detected (fig. 6C3), indicating that inhibition of clathrin-mediated endocytosis by chlorpromazine does not lead to $P2Y_2$-GFP receptor internalization by the caveolin-mediated pathway. Cholera toxin internalization is known to occur by a caveolin-mediated pathway. However, cholera toxin was not internalized upon activation of the $P2Y_2$-GFP receptor in HEK-293 cells. Therefore, $P2Y_2$ receptors apparently do not couple to this pathway.

The cholesterol content of the plasma membrane is important for receptor endocytosis. MβCD is an agent known to extract cholesterol from the plasma membrane [18], thereby inhibiting invagination of clathrin-coated pits [18]. When HEK-293 cells expressing the $rP2Y_2$-GFP receptor were pretreated with 5 mM MβCD to deplete plasma membrane cholesterol, the $rP2Y_2$-GFP receptor did not internalize either in unstimulated cells (fig. 7A1) or in cells stimulated with 100 µM UTP for 30 min (fig. 7A2). After a 60-min UTP stimulation in the presence of MβCD (fig. 7A3), however, internalized receptors were observed close to the plasma membrane (bold arrows) and in some cells there was a punctate appearance (broken arrows) of receptor-containing vesicles, indicating that depletion of plasma membrane cholesterol significantly altered the rate and extent of $P2Y_2$-GFP receptor endocytosis. The endocytosed receptor exhibits a punctate distribution indicating that the receptor is not targated to lysososmes. This punctate distribution of the receptor indicates that cholesterol depletion does affect the targeting of the receptor.

To further confirm that endocytosis of the $P2Y_2$-GFP receptor was independent of caveolin, cells were preincubated with 10 µM filipin III, an inhibitor of caveolin-mediated pathways [32], prior to stimulation with 100 µM UTP. Filipin III did not affect the shape or induce receptor internalization in unstimulated cells (fig. 7B1), but receptors were completely internalized after a 30-min (fig. 7B2) or 60-min UTP stimulation (fig. 7B3), consistent with the conclusion that internalization of the $P2Y_2$-GFP receptor is mediated by a clathrin-dependent pathway.

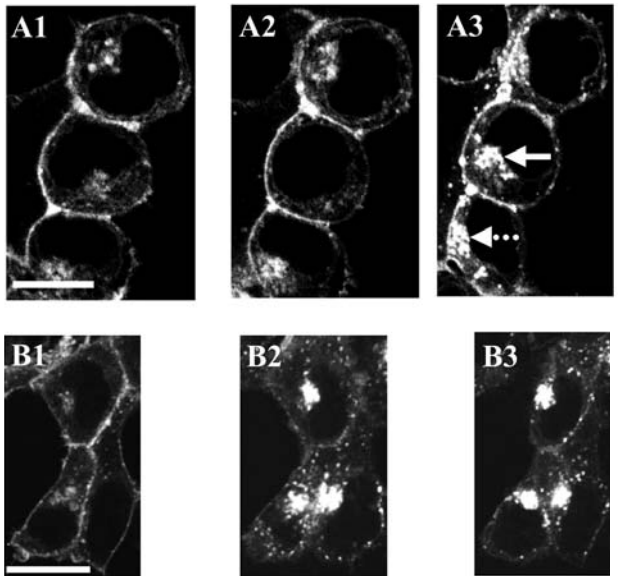

Figure 7. Effect of (*A*) the cholesterol-extracting agent methyl-β-cyclodextrin (MβCD), and (*B*) filipin III on $rP2Y_2$-GFP receptor endocytosis. Scale bar is 20 µm. Results shown are representative of at least three individual experiments. (*A*) HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 5 mM MβCD for 30 min at 37 °C (*A1*), then incubated in the presence of 100 µM UTP for 30 min (*A2*) and 60 min (*A3*), and images were collected. In MβCD-treated cells, receptor endocytosis is inhibited for the first 30 min (as seen in *A2*) and at the end of 60 min there is a punctate distribution of the endocytosed receptor (arrows in *A3*). (*B*) HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 10 µM filipin III for 30 min at 37 °C (*B1*), then incubated in the presence of 100 µM UTP for 30 min (*B2*) and 60 min (*B3*), and images were collected. Filipin III is an inhibitor of caveolin-mediated endocytosis. It has no effect on the endocytosis of the receptor, as complete endocytosis of the receptor is observed after 30 min in filipin III-treated cells.

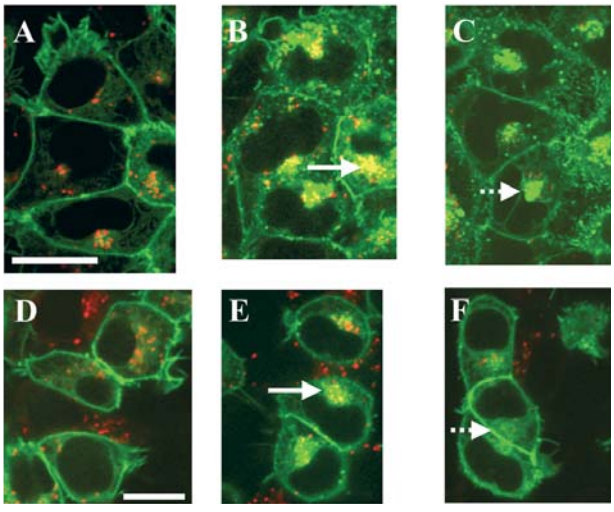

Figure 8. Recyling of the internalized $rP2Y_2$-GFP receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 100 nM LysoTracker (red) for 30 min at 37 °C (*A*), then cells were incubated for 60 min in the presence (*B*) of 100 µM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 4 h 30 min in agonist-free medium, and images were collected (*C*). Other cells were pretreated with 100 nM LysoTracker and 10 µM MG-132 (*D*), then incubated in the presence of 100 µM UTP for 60 min at 37 °C (*E*), allowed to recover for 4 h 30 min in the continued presence of MG-132 (*F*), and images were collected. MG-132 is a proteasome inhibitor. In cells treated with MG-132 there is an inhibition of the targeting of the endocytosed receptor to lysosomes (as seen in *E*, indicated by bold arrow), different from *B* with colocalization of endocytosed receptor with lysosomes (bold arrow). A complete reappearance of the receptor back to the plasma membrane (as seen in F, broken arrow) was observed in MG-132-treated cells, whereas in untreated cells there are aggregates of endocytosed receptor (*C*, broken arrow). Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

Reappearance of the $P2Y_2$ receptor after receptor internalization

HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated for 30 min with 100 nM LysoTracker (fig. 8A) and incubated for 60 min in the presence of 100 µM UTP to induce receptor internalization (fig. 8B). The internalized receptor was colocalized with lysosomes (fig. 8B, bold arrows). The cells were then washed and allowed to recover in agonist-free medium for 4 h 30 min prior to cell imaging, whereupon receptors remained internalized as aggregates outside of lysosomes (fig. 8C, broken arrows).

Trafficking of receptors from endosomes to lysosomes is known to occur via an intermediate multivesicular body complex [22]. To investigate the proteasome-dependent trafficking of $rP2Y_2$-GFP receptors from endosomes to lysosomes, we preincubated the cells with 10 µM MG-132, a proteasome inhibitor [33]. Pretreatment of the cells with MG-132 did not affect cell morphology or receptor distribution in unstimulated cells (fig. 8D), whereas in cells stimulated with 100 µM UTP for 60 min, there was complete endocytosis of the receptor in large aggregates outside lysosomes (fig. 8E, bold arrows). This is different from the situation seen in the absence of inhibitor where the endocytosed receptor was colocalized with lysosomes (fig. 8B, bold arrows). After a 4 h 30-min recovery period, $P2Y_2$-GFP receptors had reappeared at the plasma membrane (fig. 8F, broken arrows). The pattern observed in figure 8F is different from that exhibited in figure 8C, as there are no vesicular structures present in the cytoplasm, indicating that MG-132 prevented trafficking of the endocytosed receptors to lysosomes and promoted receptor distribution in cytoplasmic vesicles that facilitated recycling of internalized receptors back to the plasma membrane. Thus, targeting of the $P2Y_2$-GFP receptor to lysosomes probably involves a proteasome-dependent pathway.

Discussion

Prolonged stimulation of cells with agonist can lead to agonist-induced desensitization of the receptor and termination of downstream signals. Endocytosis is one of the pathways by which desensitization of a receptor can occur. Receptor internalization is a dynamic process that involves the modification and translocation of a number of proteins to the plasma membrane [14].

In the present study, the $rP2Y_2$ receptor, subcloned into a GFP vector, was functionally expressed in HEK-293 cells. The $P2Y_2$ receptor is the only subtype of the P2Y family that responds equipotently to both UTP and ATP. The $rP2Y_2$-GFP receptor was detected mainly at the plasma membrane. The mechanism of $P2Y_2$ receptor internalization has been investigated here. We found a strong correlation between the concentration of the agonist and endocytosis of the $P2Y_2$-GFP receptor. We stimulated the cells with UTP that is an agonist only for the $P2Y_2$ receptor subtype expressed in HEK-293 cells. The time course of internalization of the human $P2Y_2$ receptor [6] is similar to what we observe with the rat $P2Y_2$ receptor. However, the trafficking of the receptor, the compartmentalization of the receptor after endocytosis and the mechanism of internalization have not yet been elucidated.

One of the molecular players in receptor endocytosis is actin, which is part of the cytoskeleton that undergoes a massive reorganization to enhance endocytosis [34]. We did not see actin stress fibers in unstimulated HEK-293 cells expressing the $rP2Y_2$-GFP receptor, which is consistent with a normal distribution of the actin cytoskeleton in unstimulated HEK-293 cells [28]. In these cells we studied $rP2Y_2$ receptor internalization. Our data indicate that in unstimulated HEK-293 cells, actin and the $P2Y_2$-GFP receptor are colocalized in the plasma membrane, most likely in a complex involving extracellular matrix-binding proteins and integrins. Colocalization of actin and the $P2Y_2$-GFP receptor decreased after stimulation of cells with UTP, indicating that actin depolymerization precedes the endocytosis of the receptor and facilitates its endocytosis. Changes in the distribution of the actin cytoskeleton are necessary for endocytosis, since endocytotic vesicles have been shown to have an actin tail that helps in their propulsion [19]. In $rP2Y_2$-GFP receptor-expressing cells, there was complete reappearance of actin at the plasma membrane after 60 min of UTP stimulation. These observations strongly suggest that actin plays a role in the internalization of the $P2Y_2$ receptor.

To confirm the role of actin in $P2Y_2$-GFP receptor endocytosis, we demonstrated the inhibitory effects of Cyto-D, an actin-disrupting agent [29], and ML-9, a myosin light-chain kinase inhibitor [30]. In astrocyte cultures, incubation of the cells with 1 µM Cyto-D for 3 h also decreased ATP-induced Ca^{2+} mobilization [29]. Preincubation of the $rP2Y_2$-GFP receptor-transfected HEK-293 cells with a low inhibitor concentration (100 nM) for 30 min did not affect cell morphology. A clear inhibition of receptor endocytosis was observed 30 min after stimulation of the cells with UTP. In the presence of Cyto-D, the activated receptors were not internalized, but exhibited a vesicular appearance in the plasma membrane, probably due to the absence of nucleotide-induced actin depolymerization. However, after 60 min of UTP stimulation there was internalization of the $P2Y_2$-GFP receptor in small vesicles despite of the presence of Cyto-D, suggesting that there are proteins other than actin which play a role in $P2Y_2$ receptor endocytosis.

Actin remodeling is rapid and is involved in processes like cell shape change, cell movement and transport of vesicles in the cytoplasm. Myosin, an enzyme that hydrolyzes ATP, aids in actin remodeling. Myosin light-chain kinase (MLCK) phosphorylates myosin and regulates its

activities. ML-9 inhibited the internalization of the $P2Y_2$-GFP receptor after 30 min of UTP stimulation, indicating that MLCK plays an important role in remodeling the cytoskeleton. On further stimulation of the cells for 60 min, an inhibition in targeting of the endocytosed receptor to the lysososmes was also observed. These facts indicate that a normal remodeling of the cytoskeleton plays an important role in the formation of the endocytotic complex at the plasma membrane which further influences the targeting of the endocytosed receptor.

Endocytosis of receptors can take place via different pathways. The two most commonly known routes of endocytosis are via clathrin or caveolin [35]. The clathrin-dependent pathway involves the recruitment of arrestin and adapter protein-2 to form an internalization vesicle. In addition to these two proteins, the GTPase dynamin is required for pinching off the vesicle from the plasma membrane. In our study, $P2Y_2$ receptor internalization was inhibited in the presence of 0.45 M sucrose, indicating that internalization of the receptor occurs via the clathrin-mediated pathway that is sensitive to the density of the medium [16]. Furthermore, $P2Y_2$-GFP receptor endocytosis in HEK-293 cells was prevented by chlorpromazine, an inhibitor of the clathrin-dependent pathway [31]. This confirms the role for clathrin in the receptor internalization. Another possible pathway for endocytosis in HEK-293 cells is caveolin mediated. Cholera toxin subunit B is known to be endocytosed exclusively by the caveolin-mediated pathway [32]. Stimulation of $P2Y_2$-GFP receptor-expressing cells with 100 μM UTP in the presence of cholera toxin subunit B and chlorpromazine revealed an absence of internalization of both the $P2Y_2$-GFP receptor and cholera toxin, suggesting that the caveolin-mediated pathway cannot be activated by $P2Y_2$ receptors in HEK-293 cells. The lack of a contribution of the caveolin pathway was confirmed by the lack of effectiveness of filipin III, a known inhibitor of the caveolin-dependent process [32]. Thus, endocytosis of the $P2Y_2$ receptor clearly proceeds via a clathrin-mediated pathway.

The role of plasma membrane lipids in the endocytosis of the $P2Y_2$ receptor was also investigated. These lipids are normally associated with the caveolin-mediated endocytosis pathway [18]. However, pretreatment of the cells with MβCD resulted in delayed endocytosis of the receptor and a punctate distribution of the endocytosed receptor. These facts indicate that although the endocytosis of the receptor proceeds via the clathrin-mediated pathway, the plasma membrane cholesterol content does have an influence on this pathway and that there could be lipids involved in the clathrin-mediated pathway. The content of plasma membrane cholesterol does have an influence on actin reorganization in the cell, indicating that treatment of the cells with MβCD not only affects the cholesterol content but also alters the reorganization of actin cytoskeleton. This effect is revealed by the punctate distribution of the endocytosed receptor, a pattern which resembles the distribution of the receptor observed after treatment of the cells with Cyto-D.

The trafficking of the $P2Y_2$ receptor to different intracellular compartments determines the signals that are transmitted and the subsequent response of the cell to external stimuli. After chronic stimulation with agonist, the time required for receptor resensitization is greater than after an acute stimulus. This effect might be due to differential targeting of the receptor. We observed that after 30 min of UTP stimulation there was targeting of the internalized $P2Y_2$-GFP receptor to early endosomes and upon prolonged stimulation there was colocalization of the receptor with lysosomes. Thus, different receptor trafficking pathways are activated, depending on the duration of stimulation with agonist. The localization of the $P2Y_2$ receptor in early endosomes after a 30-min UTP stimulation indicated the possibility that the receptor could be recycled back to the plasma membrane. In contrast, stimulation of the cells for 60 min with the same concentration of UTP resulted in receptors targeting to lysosomes, where they would be degraded, requiring new receptor synthesis for resensitization. These observations could explain the differences in time necessary for resensitization of the cells and reappearance of the receptor at the plasma membrane after different lengths of receptor stimulation. The slower resensitization of the receptors stimulated for 60 min could also be attributable to the time required for transport of presynthesized receptors from the Golgi to the plasma membrane. Importantly, these variations in resensitization rate with duration of agonist stimulation are not due to the modification of $P2Y_2$-GFP receptors, since a similar dependency for receptor resensitization has been observed for endogenously expressed $P2Y_2$ receptors in human U937 cells [36].

Receptor endocytosis is an important process by which extracellular signals regulate cellular functions. Regulation by receptor endocytosis has been observed for the epidermal growth factor receptor that signals from endosomes to promote cell survival [37]. Trafficking of receptors from endosomes to lysosomes requires the presence of associated proteins, like Rabs, whereas the farnesylation of RhoB is necessary for targeting to early endosomes [38]. Incubation of $P2Y_2$-GFP receptor-expressing cells with the farnesyltransferase inhibitor FTI-277 did not affect UTP-induced internalization of the receptor, but due to the absence of farnesylation of the early endosomal coat proteins, the endocytosed receptor was not further trafficked to lysosomes. Thus, the absence of the appropriate vesicle coat proteins led to mistrafficking of the $P2Y_2$-GFP receptor. FTI-277 can affect the reorganization of actin via the inactivation of Ras, thus affecting the intial step of endocytosis, namely the reorganization of the cellular cytoskeleton [39]. Trafficking of receptors from the early endosomes to other compartments, such as

late endosomes, lysosomes, or recycling endosomes, is directed via the multivesicular body [22, 40]. The proteasome-mediated degradation of GPCRs is one of the methods for termination of receptor signaling [23]. For the first time, we give here indication for a proteasomal-dependent degradation pathway for $P2Y_2$ receptor trafficking, as indicated by the blockage of receptor targeting to lysosomes by the proteasomal inhibitor MG-132. Taken together, our results support a model for endocytosis of the $P2Y_2$ receptor that involves active participation of the cytoskeleton via a clathrin-dependent pathway, and trafficking of the receptor from endosomes to lysosomes that most likely involves receptor ubiquitination. The clear association of defined molecular steps in $P2Y_2$ receptor trafficking found here will enable us to design strategies to allow long-term stimulation and signaling of this receptor subtype. The $P2Y_2$ receptor is an important target for several pathological situations [8] for which an understanding of the consequences of continuous stimulation of this nucleotide receptor is important.

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