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Agonist-induced Phosphorylation and Desensitization of the P2Y₂ Nucleotide Receptor.

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Summary

Purification of HA-tagged P2Y₂ receptors from transfected human 1321N1 astrocytoma cells yielded a protein with a molecular size determined by SDS-PAGE to be in the range of 57–76 kDa, which is typical of membrane glycoproteins with heterogeneous complex glycosylation. The protein phosphatase inhibitor, okadaic acid, attenuated the recovery of receptor activity from the agonist-induced desensitized state, suggesting a role for P2Y₂ receptor phosphorylation in desensitization. Isolation of HA-tagged P2Y₂ nucleotide receptors from metabolically [³²P]-labeled cells indicated a 3.8 ± 0.2-fold increase in the [³²P]-content of the receptor after 15 min of treatment with 100 μM UTP, as compared to immunoprecipitated receptors from untreated control cells. Receptor sequestration studies indicated that ~40% of the surface receptors were internalized after a 15 min stimulation with 100 μM UTP. Point mutation of three potential GRK and PKC phosphorylation sites in the third intracellular loop and C-terminal tail of the P2Y₂ receptor (namely, S243A, T344A, and S356A) extinguished agonist-induced receptor phosphorylation, caused a marked reduction in the efficacy of UTP to desensitize P2Y₂ receptor signaling to intracellular calcium mobilization, and impaired agonist-induced receptor internalization. Activation of PKC isoforms with phorbol 12-myristate 13-acetate that caused heterologous receptor desensitization did not increase the level of P2Y₂ receptor phosphorylation. Our results indicate a role for receptor phosphorylation by phorbol-insensitive protein kinases in agonist-induced desensitization of the P2Y₂ nucleotide receptor.

Abbreviations

AAA-P2Y₂, S243A, T344A, and S356A triple mutant of P2Y₂; BSA, bovine serum albumin; [Ca²⁺]_i, intracellular free calcium ion concentration; DMEM, Dulbecco's modified Eagles Medium; EC₅₀, agonist concentration that produces 50% of a response; FITC, fluorescein isothiocyanate; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; Hepes, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; IC₅₀, agonist concentration that inhibits 50% of the response; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PLC-β, phospholipase C-β; P2Y₂-1321N1, HA-tagged P2Y₂ transfected 1321N1 cells; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SEM, standard error of the mean; WT-P2Y₂, wild type P2Y₂

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Introduction

The heptahelical P2Y₂ nucleotide receptor couples to the activation of mitogen-activated protein kinases (MAPK), the mobilization of intracellular stores of calcium, the activation of phospholipase C- β (PLC- β), protein kinase C (PKC) isoforms, focal adhesion kinase, and c-Src kinase, and has been implicated in a number of physiological and pathological processes including inflammation (1,2), reactive astrogliosis (3), and neuroprotection (4). Nucleotides acting through the P2Y₂ receptor have been shown to induce Cl⁻ secretion in airway epithelial cells that is not dependent on the cystic fibrosis transmembrane-conductance regulator (5), and P2Y₂ receptor ligands have been proposed as novel class of medication for treatment of retinal detachment (6) and improved mucociliary clearance in clinical conditions such as cystic fibrosis (7).

There is limited information regarding the regulation of signaling by the P2Y₂ nucleotide receptor. Like in other G protein-coupled receptors (GPCRs), the P2Y₂ nucleotide receptor signaling is regulated by agonist-induced desensitization (8,9). The mechanisms of GPCR desensitization have been studied most extensively with the β_2 -adrenergic receptor (10,11). G protein-coupled receptor kinase (GRK)-mediated phosphorylation increases the binding of cytosolic proteins termed arrestins to the β_2 -adrenergic receptor, leading to its internalization (12–16). It has been shown that GRK-mediated phosphorylation targets the arrestin-bound β_2 -adrenergic receptor towards sequestration *via* clathrin-coated pits (17). Truncation of GPCR C-terminal regions containing consensus sequences for potential phosphorylation by GRKs has been shown to impair β_2 -adrenergic receptor sequestration (14).

The P2Y₂ nucleotide receptor is a Gq protein-coupled receptor that is stimulated equipotently by UTP and ATP, mediating activation of phospholipase C-beta (PLC- β) and mitogen-activated protein kinase (MAPK) (18,19). We have previously reported that the P2Y₂ receptor undergoes rapid agonist-induced desensitization (8,9,20). Significantly, treatment of desensitized cells with the protein phosphatase inhibitor, okadaic acid, inhibited resensitization of the receptor, suggesting a role for protein phosphorylation in the regulation of P2Y₂ receptor signaling (9,20). Moreover, truncation mutants indicated an important role for the C-terminal tail of the P2Y₂ receptor in desensitization and sequestration of the receptor (8).

In this paper, we have investigated the UTP-induced desensitization, phosphorylation and sequestration of a hemagglutinin (HA) epitope-tagged P2Y₂ receptor transfected in 1321N1 human astrocytoma cells. Antibodies directed towards the HA-tag facilitated detection of the P2Y₂ receptor by flow cytometry, confocal microscopy, immunoprecipitation, and Western blot in sequestration and phosphorylation assays. Agonist-mediated desensitization of the P2Y₂ receptor mediated Ca²⁺ mobilization correlated with an increase in receptor phosphorylation and sequestration. We confirmed that inhibition of protein phosphatases with okadaic acid decreased receptor resensitization. Furthermore, point mutation of potential sites for GRK and PKC phosphorylation diminished agonist-induced phosphorylation and internalization of the P2Y₂ receptor, and the efficacy of UTP to induce desensitization. Interestingly, heterologous desensitization induced by phorbol 12-myristate 13-acetate occurred in the absence of increased receptor phosphorylation. This study yields a better understanding of the molecular mechanisms for desensitization of the P2Y₂ nucleotide receptor.

Experimental Procedures

Materials

[³²P]-orthophosphate (HCl-free, carrier-free) and Protein A-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech. Anti-HA 12CA5 and Anti-HA-peroxidase 3F10

monoclonal antibodies were provided by Roche Molecular Biochemicals. Horse radish peroxidase-protein markers for Western blots were purchased from New England Biolabs. Phosphate-free Dulbecco's modified Eagles Medium (DMEM) and Geneticin were supplied by Invitrogen Life Technologies. Tissue culture reagents were from Hyclone. All other reagents were obtained from Sigma.

P2Y₂ receptor gene cDNA construct

The P2Y₂ receptor cDNA was subcloned into the retroviral vector pLXSN at the *EcoRI* / *BamHI* sites of the multiple cloning site. The open reading frame of the P2Y₂ receptor cDNA was modified using polymerase chain reaction to include the hemagglutinin (HA) epitope (YPYDVPDYA) from influenza virus at the amino terminus of the expressed protein, as described previously (8). The forward and reverse HA primers designed for this study were (HA-tag encoded by underlined sequence): 5'–
 gatcgtgaattctgatgtatccatgatgttccagattatgctgcagcagacctggaaccctgg–3' and 5'–
 gatcgtggatcccctgactgaggtgctatagccg–3', respectively. A mutant P2Y₂ receptor was constructed by changing serine-243, threonine-344, and serine-356 to alanine (AAA-P2Y₂). Recombinant cDNA constructs were sequenced on both strands to ensure that mutagenesis had occurred as expected using an ABI Prism automated sequencing apparatus (Perkin-Elmer) and fluorescence dideoxynucleotide technology.

Cell culture and transfection

1321N1 astrocytoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Fetalclone III bovine serum, 100 units/mL penicillin, 100 pg/mL streptomycin, 5 mM Hepes and 500 pg/mL Geneticin (G418) (21). Cells were seeded at 2.5×10^6 per 75cm² tissue culture flasks and grown to ~90% confluency in a humidified atmosphere of 5% CO₂ and 95% air. The 1321N1 cells were transfected with HA-tagged P2Y₂ receptor cDNA using the pLXSN retroviral vector as described previously (22).

Intracellular free calcium ion mobilization

Activation of the P2Y₂ receptor was monitored by detecting changes in intracellular free calcium ion concentration (i.e., [Ca²⁺]_i) in fura2 labeled 1321N1 cells using a dual-excitation spectrofluorimeter, as described previously (9,23). Cells were assayed in 15 mM Hepes-buffered saline (pH 7.4) containing 1 mM CaCl₂, 1 mM MgCl₂, 0.1% (w/v) bovine serum albumin (BSA), and 0.18% (w/v) glucose. Desensitization experiments were performed by incubating the transfected 1321N1 cell suspension with varying concentrations of UTP or PMA for 15 min at 37°C. The cells were pelleted in a microcentrifuge and resuspended in 2 mL Hepes buffer. A concentration of UTP equal to the EC50 value for each cell line was used to rechallenge the cells in receptor desensitization experiments. Calcium mobilization is reported as a ratio relative to the maximal response. Concentration-response data was analyzed with the Prism curve-fitting program (GraphPAD Software for Science, San Diego, CA).

Receptor immunoprecipitation

1321N1 cells were seeded at 2.5×10^6 cells / 25 mL in 25mm x 150mm tissue culture dishes and allowed to grow to ~90% confluency. On the day of the experiment, each cell monolayer was washed two times with 5 mL of serum-free DMEM, leaving 5 mL of the medium over the cells. Then, the cells were either stimulated for 15 min with 100 μM UTP or control-treated with serum-free medium. The culture dishes were placed on ice and the medium was aspirated followed by two washes with ice-cold PBS. The final wash was aspirated and 700 pL of RIPA Buffer (2x) [300 mM NaCl; 100 mM Tris-HCL; 10 mM EDTA; 2% (v/v) NP-40; 1% (w/v) sodium deoxycholate; 0.2% (w/v) SDS; 20 mM NaF; 20 mM disodium pyrophosphate; 20 pg/mL of benzamidine, leupeptin, and soybean trypsin inhibitor; 10 pg/mL aprotinin; 2 pg/mL

pepstatin A; and 0.2 mM PMSF; pH 8] was added. The cells were scraped in this volume and solubilized for 1 h at 4°C with gentle rocking. After solubilization, the extract was centrifuged at 200,000 x g for 45 min at 4°C. The supernatant was mixed with 100 mL 10% (w/v) Protein A-Sepharose prepared in 3% BSA/RIPA (1x) buffer and incubated for 1 h at 4°C with gentle rocking. The suspension was centrifuged at 12,000 x g for 2 min at 4°C and the precleared supernatant was mixed with 50 pg anti-HA antibody and 100 pL 10% Protein A-Sepharose. The samples were incubated overnight at 4°C with gentle rocking. On the next day, the gel matrix was washed three times using RIPA (1x) buffer at 4°C and the final pellet was resuspended in 50 pL SDS Loading Buffer (1x). The samples were incubated at 65°C for 10 min to elute the antigen-antibody complexes and the gel matrix was pelleted as before. The supernatant was removed and saved at -20°C until SDS-PAGE (10%) analysis was performed.

SDS-PAGE and Western blot

The immunoprecipitated samples were analyzed in a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred electrophoretically to nitrocellulose membranes using trisglycine transfer buffer and 15V constant voltage in a Semi-dry Transfer System (Bio-Rad) for 1.5 h. The nitrocellulose membrane was blocked for 1 h with 5% nonfat dry milk in PBS-0.1% Tween 20 pH 7.2 (PBS-T), followed by three quick rinses with PBS-T. The membrane was then incubated with 1 pg of anti-HA-peroxidase 3F10 antibody (Roche Molecular Biochemicals) in 10 mL of 5% nonfat dry milk in PBS-T pH 7.2 for 1 h at room temperature or overnight at 4°C with gentle agitation. Anti-biotin-peroxidase (10 mL, New England BioLabs) was also added in order to detect the biotinylated protein standards. The membrane was washed three times for 10 min with PBS-T before adding the luminol detection solution (SuperSignal West Dura, Pierce). The drained nitrocellulose membrane covered with plastic wrap was exposed for 15 min to the Molecular Imager Screen-CH (GS-525 Bio-Rad, Hercules, CA) for chemiluminescence analysis of total protein.

Receptor phosphorylation

HA-tagged P2Y₂ transfected 1321N1 cells (P2Y₂-1321N1) were seeded and grown as mentioned in the receptor immunoprecipitation assay. On the day of the experiment, each cell monolayer was washed two times with 5 mL of phosphate-free, serum-free DMEM, leaving 5 mL of the medium over the cells. Then, 2.5 mCi of [³²P]-orthophosphate was added to each dish and incubated at 37°C for 2 h. Okadaic acid (1 μM final concentration) was added 10 min prior to the UTP or PMA which was incubated for the final 15 min of the 2 h labeling period according to Freedman *et al* (24). The P2Y₂ receptor was immunopurified and analyzed by SDS-PAGE and Western blot as described earlier. Phosphorylation data was acquired prior to chemiluminescence analysis by exposing the nitrocellulose membrane to the Molecular Imager Phosphor Screen-BI™ (GS-525 Bio-Rad, Hercules, CA) for 18 h. Total protein was determined *via* chemiluminescence by exposing the same membrane to the Molecular Imager Screen-CH for 15 min after visualization of bands with anti-HA peroxidase-coupled antibodies as described earlier. Phosphorylation data was normalized to total protein data by dividing [³²P] counts by chemiluminescence counts.

Flow cytometry sequestration assay

The procedure was performed essentially as described previously (8). Briefly, P2Y₂-1321N1 cells were grown to ~90% confluency in 35-mm² culture dishes and incubated with 100 μM UTP for various time periods. Control cells were incubated without UTP to allow an estimation of the total detectable cell-surface P2Y₂ receptors. UTP-treated cells were then washed with ice-cold Hepes buffer and incubated at 4°C for 1 h with the same buffer containing 10 pg of anti-HA 12CA5 antibody. The cells were washed and incubated with PBS containing 30 pg/mL of Fc-specific fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Sigma)

in the dark at 4°C for 1 h. Control cells were incubated with primary or secondary antibody alone to detect any nonspecific fluorescence. The PBS-washed P2Y₂-1321N1 cells were detached from the dishes using Hepes buffer containing 2 mM EDTA. They were centrifuged and resuspended in 1 mL of 1% (v/v) formaldehyde in PBS and incubated in the dark at 4°C for 10 more minutes. Finally, the cells were centrifuged and resuspended in 0.5 mL PBS, and analyzed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL) with a 5-watt argon laser tuned at 488 nm using 150-milliwatt output.

Confocal microscopy

1321N1 cell transfected with either wild type or AAA mutant P2Y₂R were cultured in Laboratory-Tek chamber slides (Nalge Nunc Int., Rochester, NY, USA) for 3 days in culture medium. On the day of the assay, cells were equilibrated in serum-free medium for 1 h at 37°C. Nonspecific protein sites were blocked with 1% (w/v) bovine serum albumin in PBS (blocking buffer) for 15 min at 4°C. Cells were then incubated with 10 pg/mL anti-HA mouse monoclonal antibody (Roche Molecular Biochemicals) in blocking buffer for 1 h at 4°C to form receptor/antibody complexes and washed three times with ice-cold PBS. Cells were incubated with or without 10 µM UTP at 37°C for 5, 15, or 30 min. The cells washed three times with ice-cold PBS and fixed with 2% formaldehyde in PBS for 10 min at room temperature. Plasma membrane staining with Wheat Germ Agglutinin-Texas Red (Molecular Probes) was performed as described by Holloway *et al.* (25). Images were acquired using a Zeiss (Thornwood, NY, USA) LSM-5 Pascal scanning confocal microscope equipped with an Alpha-Fluar 100×1.45 DIC oil immersion objective. Argon and helium-neon lasers were used for excitation of Alexa Fluor 488 and Texas Red, respectively. Emission from Alexa Fluor 488 and Texas Red was detected using BP 505–530 and LP 560 filters, respectively using multitracking scan mode. P2Y₂ receptor sequestration was analyzed by determining colocalization of receptor with the plasma membrane marker Wheat Germ Agglutinin-Texas Red (WGA). The weighted colocalization coefficient of immunostained P2Y₂ receptor with Wheat Germ Agglutinin-Texas Red (WCC:P2Y₂/WGA) was determined using Zeiss LSM 5 Pascal Software Release 3.2 which calculates the coefficient according to the following equation:

$$\text{WCC : P2Y}_2 / \text{WGA} = S(\text{intensities for colocalizing pixels}) / S(\text{total pixel intensities})$$

Statistical analysis

One-way multiple Tukey comparison post tests analysis of variance test (ANOVA) and unpaired Student's t-test were used for comparison of multiple groups and two groups, respectively. A probability <0.05 between control and experimental groups was considered to be statistically significant. All analyses were performed with InStat software Version 3.06 (GraphPad Software Inc. San Diego, CA).

Results

N-terminally hemagglutinin (HA)-tagged recombinant P2Y₂ receptors were constructed by polymerase chain reaction and expressed in 1321N1 astrocytoma cells to investigate UTP-mediated receptor desensitization, phosphorylation and sequestration. The HA tag (YPYDVDPYA) is specifically recognized by the commercially-available 12CA5 and 3F10 monoclonal antibodies.

Immunoprecipitation of the P2Y₂ receptor reveals non-uniform receptor protein distribution

Solubilized protein extracts of HA-tagged P2Y₂ transfected 1321N1 cells (P2Y₂-1321N1) were immunoprecipitated with 12CA5 anti-HA monoclonal antibody. Western blots of the immunoprecipitated products revealed heterogeneity of receptor protein ranging in size from

57 to 76 kDa, and a single protein band of 45 kDa (Figure 1, lane 2). Vector-transfected 1321N1 cells yielded no immunoprecipitated products nor did the eluent from washed Sepharose matrix (Figure 1, lanes 3 and 4, respectively). No bands were detected in a sample consisting of 12CA5 anti-HA antibody indicating that the detected bands originated from the P2Y₂-1321N1 cells and were not contaminants introduced by the antibody used in the immunoprecipitation protocol (Figure 1, lane 6).

Agonist-mediated P2Y₂ receptor desensitization involves phosphorylation

Extracellular UTP caused a concentration-dependent activation of P2Y₂ receptors that mediated rapid and transient increases in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) as previously reported (9). The agonist concentration that produces 50% of a response (EC₅₀) value was 0.32 μ M (log EC₅₀ = -6.5 ± 0.1) (Table 1). Desensitization of the UTP-mediated calcium response was determined after a 15 min pretreatment of the cells with various UTP concentrations. The desensitization was rapid exhibiting an agonist concentration that inhibits 50% of the response (IC₅₀) value of 6.3 μ M (log IC₅₀ = -5.2 ± 0.2) (Table 1). Flow cytometry sequestration analysis of HA-tagged P2Y₂ receptors was performed using mouse anti-HA 12CA5 antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody. About 40% of the receptors were sequestered within 15 min of incubation with 100 μ M UTP (Figure 2).

Previous reports have suggested that receptor phosphorylation plays a role in the modulation of P2Y₂ receptor desensitization and recovery (9,20). Consistent with this idea, we observed that recovery of P2Y₂ receptor signaling after agonist-induced desensitization was inhibited in 50% upon treatment with 1 μ M of the protein phosphatase inhibitor okadaic acid (Figure 3). Okadaic acid treatment had no effect on receptor desensitization. However, to directly assess phosphorylation of the P2Y₂ receptor, we developed a receptor phosphorylation assay using an immunopurification protocol to examine metabolically [³²P]-orthophosphate labeled P2Y₂-1321N1 cells. Treatment of [³²P]-orthophosphate labeled cells with 100 μ M UTP agonist caused a significant increase in the [³²P]-radioactivity associated with immunoprecipitated proteins in the 57–76 kDa region as compared to control unstimulated cells (Figure 4A).

The [³²P]-radioactivity data was normalized to the total protein detected by chemiluminescence analysis of the same nitrocellulose membrane using anti-HA-peroxidase antibody and a luminol substrate. Thus, the ratio of [³²P]-radioactivity counts to chemiluminescence counts was determined for immunoprecipitated samples from UTP-treated cells and control unstimulated cells (Figure 4B). A 3.8 ± 0.2 -fold increase in [³²P]-content (phosphorylation) was obtained for the P2Y₂ receptor after 15 min of stimulus with UTP.

Elimination of potential GRK and PKC phosphorylation sites attenuates agonist-induced but not phorbol ester-induced P2Y₂ receptor desensitization and phosphorylation

Examination of the primary structure of the P2Y₂ receptor revealed the presence of serine and threonine residues within GRK/PKC consensus substrate sequences (26,27) in the intracellular domains (28). A mutant HA-tagged P2Y₂ receptor was constructed by changing serine-243, threonine-344 and serine-356 to alanine (AAA-P2Y₂) to test the hypothesis that phosphorylation of the P2Y₂ receptor may be implicated in agonist-induced desensitization. We transfected the AAA-P2Y₂ receptor cDNA into 1321N1 cells and tested the receptor's capacity to signal through calcium mobilization and its desensitization. The AAA-P2Y₂ receptor induced the mobilization of intracellular stores of calcium with an EC₅₀ value for UTP of 0.20 μ M (log EC₅₀ = -6.7 ± 0.1) (Table 1) which is comparable to that for the wild type P2Y₂ receptor (WT-P2Y₂). Desensitization of the Ca²⁺ response was determined in cells expressing either wild type or AAA-P2Y₂ receptors. Cells were pretreated for 15 min with various concentrations of UTP, as indicated in Figure 5A, and then rechallenged with a

concentration of UTP equal to the corresponding EC₅₀ value for each cell line, i.e., 0.32 μ M and 0.20 μ M for wild type and AAA-P2Y₂ receptors, respectively. Interestingly, the IC₅₀ value for UTP was ~10-fold higher for the AAA-P2Y₂ receptor (IC₅₀ = 79 μ M, log IC₅₀ = -4.1 $\pm$ 0.2) than for the wild type receptor (IC₅₀ = 6.3 μ M, log IC₅₀ = -5.2 $\pm$ 0.2) (Figure 5A and Table 1). Moreover, when cells were pretreated for 15 min with phorbol 12-myristate 13-acetate (PMA), known to activate PKC isoforms and to cause heterologous desensitization of the P2Y₂ receptor (8,9), we found no significant difference between the IC₅₀ values for PMA determined in cells expressing AAA-P2Y₂ receptors (IC₅₀ = 2.0 μ M, log IC₅₀ = -5.7 $\pm$ 0.3) and those expressing wild type P2Y₂ receptors (IC₅₀ = 3.2 μ M, log IC₅₀ = -5.5 $\pm$ 0.2) (Figure 5B and Table 1).

We found that UTP-induced phosphorylation of the AAA-P2Y₂ receptor was markedly reduced as compared to the wild type P2Y₂ receptor (Figure 6). No increase in receptor phosphorylation was detected upon treatment with 10 μ M PMA for 15 min in either wild type or AAA-P2Y₂ receptors (Figure 6). Importantly, agonist-induced internalization was significantly impaired in AAA-P2Y₂ receptors compared to wild type P2Y₂ receptors (Figure 7). Taken together, these results implicate the phosphorylation of potential GRK/PKC phosphorylation sites in agonist-induced desensitization of the P2Y₂ receptor. Our data also indicate that PMA induces heterologous desensitization of the P2Y₂ receptor independent of receptor phosphorylation.

Discussion

The therapeutic potential of nucleotides and P2Y₂ receptor ligands may depend on our understanding of the desensitization process that modulates signaling by this receptor. Desensitization of G protein-coupled receptor (GPCR) may require the activities of several protein kinases such as protein kinase A (PKA), protein kinase C (PKC) and G protein-coupled receptor kinases (GRKs) (29,30). Although the molecular basis for desensitization of the adenylyl cyclase-linked b₂-adrenergic receptor has been well characterized, less is known about the regulatory mechanisms involved in the desensitization of signaling by the P2Y₂ nucleotide receptor. Immunoprecipitation of the HA-tagged P2Y₂ receptor expressed in 1321N1 astrocytoma cells revealed a non-uniform distribution of the receptor protein that is typical of membrane glycoproteins with heterogeneous complex glycosylation (Figure 1). This most likely results from asparagine-linked high mannose carbohydrates as well as hybrid and complex oligosaccharides since immunoprecipitated P2Y₂ receptors were found to be susceptible to hydrolysis by peptide *N*-glycosidase F (data not shown). A similar heterogeneous pattern was reported for the b₂-adrenergic receptor which has a molecular mass of 46,556 and was detected in the 47–90 kDa size range (31).

UTP-mediated activation of the P2Y₂ receptor triggers the phospholipase C (PLC) signaling pathway leading to the production of inositol-1,4,5-triphosphate which increases [Ca²⁺]_i and diacylglycerol which activates PKC. EC₅₀ values for Ca²⁺ mobilization determined with the HA-tagged P2Y₂ receptor transfected cells (0.32 μ M; Table 1) were similar to those obtained by us (20) for endogenous P2Y₂ receptors in human monocytic U-937 cells (0.44 μ M). A 15 min treatment with 100 μ M UTP produced rapid desensitization showing an IC₅₀ value of 6.3 μ M (Table 1). We confirmed that treatment of desensitized cells with the protein phosphatase inhibitor okadaic acid (1 μ M) inhibited the recovery of receptor activity from the desensitized state by ~50% (9), suggesting a role for P2Y₂ receptor phosphorylation in the desensitization process (Figure 3). These results prompted us to investigate whether UTP-mediated desensitization involved an increase in receptor phosphorylation. Indeed, the P2Y₂ receptor was phosphorylated after an agonist treatment that caused desensitization and receptor internalization by 40% (Figures 2 and 4). These results suggest a link between receptor phosphorylation and agonist-induced desensitization. Interestingly, immunoprecipitation of

detergent-solubilized total cell extracts from UTP-stimulated cells yielded an unphosphorylated ~45 kDa protein band. Receptors from non-plasmatic membranes (e.g., non-glycosylated receptors in endoplasmic reticulum membranes) would not be accessible to extracellular ligands and thus, remain un-phosphorylated upon agonist challenge

Studies of other GPCRs have indicated the importance of the C-terminus in agonist-mediated receptor regulation, since this portion of the receptor contains potential phosphorylation sites for protein kinases. For example, truncation of the C-terminus of the α_{1b} -adrenergic receptor exhibited decreased agonist-mediated desensitization, phosphorylation and sequestration when compared to wild type receptors (32). C-terminus truncations of other GPCRs such as angiotensin II, neurotensin and the parathyroid hormone receptor, have been observed to inhibit receptor sequestration as well (33–35). Studies from our laboratory have shown that the C-terminus of the P2Y₂ receptor is important for both agonist-mediated desensitization and sequestration (8). Therefore, we constructed a mutated AAA-P2Y₂ receptor by changing three potential GRK and PKC phosphorylation sites to alanine (S243, T344, S356) located in the C-terminus tail and the third intracellular loop of the P2Y₂ receptor. While the triple mutation did not affect the receptor's ability to activate calcium mobilization, it dramatically reduced the extent of phosphorylation of the receptor upon activation with agonist as well as the efficacy of agonist towards inducing receptor desensitization. It is very likely that agonist-mediated homologous desensitization of P2Y₂ receptors involves protein kinases other than PMA-activated PKC isoforms, such as the GRKs; although PMA-insensitive PKC isoforms have not been ruled out. Receptor phosphorylation may modulate its affinity to agonist, its ability to couple with signal transducers, and mediates its internalization by promoting receptor trafficking towards endocytic pathways. It is possible that PKC-mediated heterologous desensitization of the P2Y₂ receptor involves phosphorylation of signaling molecules other than the heptahelical receptor, such as phospholipase C (36), regulator of G protein signaling (RGS) (37,38), and GRK2 (39,40).

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Figure 1. Immunoprecipitation of the HA-tagged P2Y₂ receptor

1321N1 cells stably transfected with HA-tagged P2Y₂ receptor or with pLXSN vector without receptor were detergent solubilized and immunoprecipitated with 12CA5 anti-HA monoclonal antibody. Immunoprecipitated products were resolved on 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted using 3F10 anti-HA-peroxidase monoclonal antibody. Samples in the blot are: protein standards (lanes 1 and 5), immunoprecipitation from HA-tagged P2Y₂ transfected cells (lane 2), immunoprecipitation from pLXSN vector transfected cells (lane 3), products eluted from the anti-HA crosslinked gel matrix (lane 4), and 12CA5 anti-HA antibody only (used for immunoprecipitation reactions) (lane 6). Results shown are representative of three separate experiments.

Figure 2. UTP-induced sequestration of P2Y₂ receptors expressed in 1321N1 cells

1321N1 cells expressing HA-tagged P2Y₂ receptors were incubated at 37°C with 100 μ M UTP for the indicated times. Control cells were incubated without UTP to determine the total detectable cell-surface P2Y₂ receptors. The cells were washed in assay buffer and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody at 4°C in the dark for 1 h. Cells were washed again and analyzed by flow cytometry as described in Experimental Procedures. Each value shown is expressed as a percentage of the total P2Y₂ receptors detected in control cells that were treated with UTP-free carrier buffer. The data presented are the average $\pm$ SEM of results from three independent experiments.

Figure 3. Effect of the phosphatase inhibitor okadaic acid on recovery of P2Y₂ receptor from UTP-induced desensitization

Fura2 labeled P2Y₂-1321N1 cells were incubated with 100 μ M UTP for 15 min at 37°C in the presence of 1 μ M okadaic acid. The cells were washed, resuspended in buffer and either assayed immediately for Ca²⁺ mobilization (open bars), or incubated for 30 min in UTP-free medium (shaded bars), in the presence or absence of 1 μ M okadaic acid prior to measuring changes in [Ca²⁺]_i in response to 1 μ M UTP as described in Experimental Procedures. Data are expressed as a percentage of the maximal response to 100 μ M UTP in control untreated cells. Data are the average $\pm$ SEM of results from three independent experiments. Cells with okadaic acid treatment were compared to parallel cultures without okadaic acid treatment (*** $p < 0.001$; ns, $p > 0.05$ one-way ANOVA).

Figure 4A

Figure 4B

Figure 4. UTP-induced phosphorylation of P2Y₂ receptors

1321N1 cells expressing the HA-tagged P2Y₂ receptor gene were metabolically labeled with [³²P]-orthophosphate for 2 h in serum-free and phosphate-free medium and then challenged with 100 μM UTP for 15 min as described in Experimental Procedures. Control-unstimulated cells were challenged with carrier medium. Cells were lysed and detergent-solubilized cell extracts were analyzed by anti-HA immunoprecipitation, SDS-PAGE (10%), and Western blot. The [³²P]-radioactivity data were acquired prior to chemiluminescence analysis by exposing the nitrocellulose membrane to the Molecular Imager Screen-BI for 18 h. Total protein in the membrane was then analyzed after chemiluminescence detection by incubating the same nitrocellulose membrane with peroxidase-conjugated anti-HA antibody followed by luminol substrate incubation and exposure to the Molecular Imager Screen-CHEMI for 15 min. (A) Chemiluminescence (left panel) and [³²P]-radioactivity (right panel) signals were detected in

the same nitrocellulose membrane. Molecular weight standards are indicated on the left. **(B)** Chemiluminescence and [^{32}P]-radioactivity counts (arbitrary units) were detected in the nitrocellulose membrane (thick line, UTP-treated cells; thin line, control unstimulated cells) and are expressed as a function of protein migration distance (in mm) from top to bottom of the scanned membrane. [^{32}P]-radioactivity counts from phosphorylated proteins were normalized by calculating the ratio to chemiluminescence counts from total immunoprecipitated protein. UTP treatment caused a 3.8 ± 0.2 -fold increase in phosphorylation as compared to control unstimulated cells. Results shown are representative of three separate experiments.

Figure 5. Desensitization of wild type and AAA-P2Y₂ receptors mediated by UTP and PMA

Potential serine/threonine phosphorylation sites for GRK and PKC in the receptor (S243, T344, S356) were mutated to alanine to create the AAA-P2Y₂ receptor. 1321N1 cells expressing either wild type (WT,●) or AAA-P2Y₂ (○) receptor were labeled with fura2 and incubated for 15 min with various concentrations of either UTP (**A**) or phorbol 12-myristate 13-acetate (PMA) (**B**). Ca²⁺ mobilization was determined after rechallenging the cells with the EC₅₀ concentration for UTP of each transfected cell (wild type EC₅₀ = 0.32 μM and AAA-P2Y₂ mutant EC₅₀ = 0.20 μM). The data are expressed as a percentage of the maximal response to 100 μM UTP in cells that were not preincubated with UTP or PMA. Values shown are the mean ± SEM of three separate experiments.

Figure 6. Phosphorylation of wild type and AAA-P2Y₂ receptors mediated by UTP and PMA
1321N1 cells expressing either wild type or AAA-P2Y₂ receptor were labeled with [^{32}P]-orthophosphate for two hours in serum-free and phosphate-free medium. Cells were challenged for 15 min with 100 μM UTP or 10 μM phorbol 12-myristate 13-acetate (PMA). Control unstimulated cells were treated with carrier medium. Detergent solubilized cell extracts were immunoprecipitated with anti-HA antibody followed by SDS-PAGE (10%) and Western blot as described in Methods. [^{32}P]-radioactivity counts from phosphorylated proteins were normalized by calculating the ratio to chemiluminescence counts from total immunoprecipitated protein as described in Figure 6. Results are the mean $\pm$ SEM of three separate experiments (** $p < 0.01$, significantly different from unstimulated control).

Figure 7A

Figure 7B

Figure 7. Internalization of wild type P2Y₂ and AAA-P2Y₂ receptors transfected in 1321N1 cells
(A) 1321N1 cells expressing either HA-tagged wild type or AAA-P2Y₂ receptors were treated with or without 10 μ M UTP for 30 min as described in Experimental Procedures. Laser scanning confocal microscopy images were acquired as described in Experimental Procedures. The HA-tagged receptors were detected by immunofluorescence with Alexa Fluor 488 staining (shown in green) and cell surface *N*-acetylglucosamine and *N*-acetylneuraminic acid residues in the same cells were detected with Texas Red-conjugated wheat germ agglutinin (WGA) (shown in red). Merge images are shown. Scale bar in lower left corner = 20 μ m. **(B)** The weighted colocalization coefficient of the HA-tagged P2Y₂ receptors with WGA were determined as described in Experimental Procedures. Receptor internalization (fraction surface HA-tagged) was determined at the indicated times after treatment with 10 μ M UTP. The surface HA-tagged receptors were calculated from the ratio of WCC values to WCC in unstimulated control cells. Data presented are the average $\pm$ SEM of *n* individual cells (as indicated inside each bar) determined from at least three independent experiments. Cells expressing wild type P2Y₂ receptors were compared to cells expressing AAA-P2Y₂ receptors (*** $p < 0.001$; ns, $p > 0.05$ one-way ANOVA).

Table 1

EC₅₀ and IC₅₀ values for different P2Y₂ receptor constructs expressed in 1321N1 cells.

P2Y ₂ Receptor	EC ₅₀ , μ M (log EC ₅₀)	IC ₅₀ , μ M log (IC ₅₀)	
	UTP	UTP	PMA
wild type	0.32 (−6.5±0.1)	6.3 (−5.2±0.2)	3.2 (−5.5±0.2)
AAA-P2Y ₂	0.20 (−6.7±0.1)	79 (−4.1±0.2)	2.0 (−5.7±0.3)

EC₅₀ (agonist concentration that produces 50% of the maximal response), IC₅₀ (agent concentration that inhibits 50% of a response), log EC₅₀, log IC₅₀ and standard error of the mean (SEM) were determined as described in Experimental Procedures using the Prism 4.0 software, GraphPad, San Diego, CA, USA.