

Agonist-Induced Internalization of the P2Y₂ Receptor

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ABSTRACT

The G_q/phospholipase C-linked human P2Y₂ receptor was tagged at its amino terminus with the hemagglutinin A (HA) epitope sequence (P2Y₂-HA) and stably expressed in 1321N1 human astrocytoma cells. Neither the pharmacological selectivity nor the signaling properties of the receptor were altered by the presence of the epitope. An enzyme-linked immunosorbent assay was developed to quantify cell surface levels of P2Y₂-HA receptors using an anti-HA antibody. Incubation of cells with P2Y₂ receptor agonists resulted in a concentration of agonist- and time-dependent decrease in cell surface immunoreactivity. Methodology for indirect immunofluorescence confocal microscopy was developed and applied to demonstrate that the agonist-promoted decreases in cell surface immunoreactivity paralleled increases in intracellular immunoreactivity. Agonist-induced internalization of P2Y₂ receptors was demonstrated directly by prelabeling P2Y₂-HA receptors with antibody before

agonist challenge and then quantifying the movement of receptors from a cell surface to intracellular localization in the presence of agonist. Removal of agonist from the medium resulted in recovery of cell surface immunoreactivity to control levels within ~1 hr. Incubation of P2Y₂-HA receptor-expressing cells with P2Y₂ receptor agonists also resulted in receptor-specific desensitization of nucleotide-promoted inositol phosphate accumulation. This loss of responsiveness occurred more rapidly and to a greater extent than did the agonist-promoted loss of surface receptors. Inhibition of receptor internalization by reduction of temperature to 16° had no effect on the capacity of nucleotides to induce P2Y₂ receptor-specific desensitization. These results illustrate that the P2Y₂ receptor undergoes agonist-promoted movement to an intracellular compartment. This receptor internalization is not required for agonist-induced desensitization.

Extracellular nucleotides produce a broad range of physiological responses as a consequence of activation of P2 receptors (Dubyak and El-Moatassim, 1993; Harden *et al.*, 1995). Ionotropic responses to nucleotides are mediated by ligand-gated ion channels (P2X receptors), whereas metabotropic responses are mediated by heptahelical receptors (P2Y receptors) coupled to heterotrimeric G proteins. The five mammalian P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors) cloned to date all activate phospholipase C (Communi *et al.*, 1997; Fredholm *et al.*, 1997). The P2Y₁, P2Y₄, P2Y₆, and P2Y₁₁ receptors are specifically activated by either adenine or uridine nucleotides (Communi *et al.*, 1997; Fredholm *et al.*, 1997; Harden *et al.*, 1998), but the P2Y₂ receptor is equipotently activated by both ATP and UTP (Lustig *et al.*, 1993; Lazarowski *et al.*, 1995). The P2Y₂ receptor exhibits a broad tissue distribution (Parr *et al.*, 1994; Lazarowski *et al.*, 1995). For example, activation of P2Y₂ receptors on human airway epithelial cells promotes chloride secretion (Mason *et al.*, 1991).

Activation of most G protein-coupled receptors, including the P2Y₂ receptor, results in a progressive decrease in the capacity of the receptor to respond to agonists (i.e., the receptor desensitizes) (Brown *et al.*, 1991; Wilkinson *et al.*, 1994; Fisher, 1995; Lazarowski *et al.*, 1995). Studies on adenylyl cyclase-coupled receptors, such as the β_2 -adrenergic receptor, indicate that agonist-promoted receptor phosphorylation is at least partially responsible for decreased responsiveness (Lefkowitz, 1993; Lohse, 1993). However, the cellular and molecular mechanisms underlying agonist-induced desensitization of G_q/phospholipase C-coupled receptors are less well understood (Wojcikiewicz *et al.*, 1993; Fisher, 1995), and essentially nothing is known about P2Y receptor desensitization in general and desensitization of the P2Y₂ receptor in particular.

To begin to address the steps underlying P2Y receptor desensitization, we examined the possibility that the P2Y₂ receptor internalizes as a consequence of agonist occupancy. This study required the construction and expression of an epitope-tagged P2Y₂ receptor because no specific and selective radioligands or antibodies are available with which to

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ABBREVIATIONS: ATP γ S, adenosine-5'-O-(3-thio)triphosphate; HA, hemagglutinin A; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' buffered saline solution; P2Y₂-HA receptor, human P2Y₂ receptor engineered with the hemagglutinin A sequence in the amino terminus; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction.

follow this receptor in intact cell assays. Using this epitope-tagged P2Y₂ receptor stably expressed in mammalian cells, we determined the relationship between agonist-promoted P2Y₂ receptor redistribution and changes in agonist-promoted receptor function under identical experimental conditions in intact cells. The results directly illustrate nucleotide-promoted internalization of the P2Y₂ receptor and establish that movement of receptors away from the cell surface *per se* is not responsible for agonist-induced alteration in P2Y₂ receptor responsiveness.

Materials and Methods

Construction of P2Y₂-HA. Insertion of the sequence TAC CCA TAT GAC GTT CCA GAC TAC GCA, which encodes the HA amino acid sequence YPYDVPDYA, between the fourth and fifth codons of the P2Y₂ receptor sequence was accomplished using four-primer PCR. pBlueScript containing the wild-type human P2Y₂ receptor cDNA was used as template (Parr *et al.*, 1994). Vent DNA polymerase (New England Biochemical) was used according to manufacturer's protocol, except that the final MgSO₄ concentration was increased to 5 mM. A 538-bp nucleotide fragment spanning the *EcoRI* site in the vector to the *SacII* site in the P2Y₂ sequence was generated and used to replace the corresponding fragment in the original vector. Ligations and transformations were performed using standard methods (Sambrook *et al.*, 1989). The sequence of the PCR-amplified portion of the P2Y₂-HA receptor cDNA was verified by dideoxy-sequencing according to manufacturer's instructions (Sequenase Version 2.0).

Retroviral expression of receptors. P2Y₂ and P2Y₂-HA constructs were subcloned into the *EcoRI* and *XhoI* sites of the retroviral expression vector pLXSN (Miller and Rosman, 1989). Purified plasmids were used to transfect the murine fibroblast packaging cell line PA317 by calcium phosphate precipitation for production of retrovirus (Comstock *et al.*, 1997). Retroviral supernatant was collected 3 days after transfection and used to infect subconfluent 1321N1 human astrocytoma cells for receptor expression. Infected cells were selected by neomycin resistance using G418 sulfate (0.6 mg/ml, GIBCO BRL, Grand Island, NY). Single cells were cloned from receptor-expressing cell populations, and a single clonal cell line was used for all desensitization and receptor redistribution studies.

Inositol phosphate accumulation assays. Cells were grown in DMEM-high glucose supplemented with 5% FBS and 0.6 mg/ml G418 to maintain selection. After 3 to 4 days in culture, the cells were incubated overnight in 0.2 ml of serum-free, inositol-free, phenol red-free RPMI containing 0.2 μ Ci of *myo*-[³H]inositol (14 Ci/mmol). Assays were carried out on the following day after equilibration with 50 mM HEPES, pH 7.4, for 60 min. LiCl (15 mM) was added to each well 10 min before the addition of agonist. Reactions were terminated by adding an equal volume of 10% trichloroacetic acid to lyse the cells and release [³H]inositol phosphates. Samples were extracted with ethyl ether to remove the trichloroacetic acid. [³H]inositol phosphates were separated by anion exchange chromatography on Dowex AG1-X8 columns and quantified by liquid scintillation counting (Brown *et al.*, 1991).

For desensitization assays, cells were seeded, cultured, and labeled with [³H]inositol as described above. Cells were buffered with 50 mM HEPES, pH 7.4, and equilibrated for 60 min. Three sets of cells were incubated with 30 μ M ATP γ S for 25 min. One set of these cells was challenged for an additional 5 min with 20 mM LiCl to determine the amount of inositol phosphate accumulation that was due to challenge with LiCl in the absence of additional agonist (this amount of accumulation was subtracted from values obtained with the following two sets of cells). The second set of ATP γ S-pretreated cells was challenged for 5 min with fresh ATP γ S (30 μ M, final concentration) in the presence of 20 mM LiCl to establish desensitization of P2Y₂-HA receptors. The third set of ATP γ S-pretreated cells

was challenged for 5 min with a combination of 1 mM carbachol and 2 units/ml human thrombin (Sigma Chemical, St. Louis, MO) in the presence of 20 mM LiCl to measure the activity of the phospholipase C-linked muscarinic cholinergic and thrombin receptors. The activity of muscarinic cholinergic and thrombin receptors in the absence of ATP γ S pretreatment was measured in another set of cells incubated for 5 min with 20 mM LiCl without or with carbachol/thrombin. All reactions were terminated by the addition of TCA, and [³H]inositol phosphates were separated and quantified as described above.

Indirect immunofluorescence microscopy with antibody-postlabeled cells. Cells were seeded onto poly-D-lysine-coated (10 μ g/ml) glass coverslips and cultured for 4–5 days in DMEM. On the day of the assay, cells were placed in a 37° bath, buffered with 50 mM HEPES, pH 7.4, and equilibrated for 60 min. Agonist or vehicle was added for the indicated times, and the incubations were terminated by placing the plate in an ice bath for 5 min. Cells were fixed without permeabilization for 10 min in 2% paraformaldehyde freshly prepared in PBS, pH 7.4. After repeated washes with HBSS (pH 7.4, containing 1.25 mM CaCl₂ and 0.81 mM MgSO₄), nonspecific protein sites were blocked using HEPES-buffered MEM, pH 7.4, containing 10% heat-inactivated normal goat serum (blocking buffer). Monoclonal anti-HA antibody HA0.11 (BAhCO) was incubated with the cells at a 1:500 dilution in blocking buffer for 30 min. After several washes in HBSS, Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was added at a 1:800 dilution in blocking buffer for 30 min. After washing, coverslips were mounted onto microscope slides using Vectashield (Vector Laboratories, Burlingame, CA).

Cells were viewed by confocal microscopy with a Zeiss 110 confocal scanning laser microscope with a 63 \times Zeiss objective, 1.4 NA, using an argon laser at 514 nm with a rhodamine filter. Optical sections were 0.5 μ m thick. The average thickness of 1321N1 cells was \sim 2.5 μ m, and the range of thickness measured was 1–5 μ m. Images were digitized using constant contrast and brightness parameters to facilitate direct comparison of fluorescence intensities. Immunofluorescence was quantified using NIH Image 1.57 after subtracting equal background levels from each image.

ELISA detection of surface immunoreactivity. Cells were seeded in 24-well plates and cultured for 4–5 days in DMEM. On the day of the assay, cells were placed in a 37° bath, buffered with 50 mM HEPES, pH 7.4, and equilibrated for 60 min. Agonist or vehicle was added in 50 μ l for the indicated times, and incubations were terminated by placing the plate in an ice bath for 5 min. Cells were fixed with paraformaldehyde as described above. Cells were washed, blocked, and incubated with anti-HA antibody (1:500) for 30 min. After several washes in HBSS, cells were incubated with HRP-conjugated goat anti-mouse Fab (Sigma) at a 1:3000 dilution in blocking buffer for 30 min. Cells were washed repeatedly with HBSS and then quickly rinsed with 0.1 M sodium citrate, pH 4.5 (substrate buffer), to reduce background. The substrate *O*-phenylenediamine was added at 1 mg/ml in substrate buffer, and 1 μ l 30% H₂O₂ was added per 10 ml substrate buffer. Product formation proceeded for 10–45 min, and 100- μ l aliquots were transferred to a 96-well plate and read at an absorbance of 450 nm on an EL340 Bio Kinetics microplate reader (Bio Tek Instruments, Winooski, VT). Product formation was linear at all time points tested and was directly proportional to the number of P2Y₂-HA receptor-expressing cells in the culture (data not shown).

Indirect immunofluorescence microscopy with antibody-prelabeled cells. Intact cells on glass coverslips were prelabeled with anti-HA antibody by incubation in an ice bath for 5 min, addition of blocking buffer at 4° for 15 min, and incubation with anti-HA antibody (1:300) for 45 min to form receptor/antibody complexes. Cells were repeatedly washed at 4°, quickly warmed to 37° in HEPES-buffered MEM, and incubated with agonist or vehicle for the indicated times. After incubation, cells were again placed in an ice bath for 5 min and fixed with paraformaldehyde as above. Cell surface receptor/antibody complexes were detected by incubation

with Cy3-conjugated anti-mouse IgG secondary antibody (1:800) for 30 min, followed by repeated washes and mounting as above.

To detect only intracellular primary antibody, the primary antibody remaining on the cell surface was bound with unconjugated goat anti-mouse IgG (1:25, Jackson ImmunoResearch) after paraformaldehyde fixation. Cells were repeatedly washed to remove the unbound unconjugated antibody and permeabilized with 0.05% saponin in blocking solution. Cells were incubated with Cy3-conjugated secondary antibody in the presence of saponin for 30 min to bind intracellular primary antibody, washed in the presence of saponin, and mounted.

Statistical analysis of surface receptor immunoreactivities. Significant differences between measurements were calculated using GraphPAD InStat (ver. 2.05a; San Diego, CA). Agonist-induced surface receptor changes measured by ELISA were expressed as a percentage of the absorbance quantified with cells that were incubated with vehicle alone. Data obtained by immunofluorescence quantification were expressed as fluorescence per unit area and then converted to a percentage based on the values obtained for cells that were incubated with vehicle alone. One-way analysis of variance and Dunnett's multiple comparison test were used to compare values obtained at different times of agonist incubation within a single experimental method (i.e., results obtained by ELISA were compared with results obtained by ELISA). The unpaired *t* test was used to compare values obtained for the same times of agonist incubation across experimental methods (i.e., results obtained by ELISA were compared with results obtained by immunofluorescence quantification). Statistical significance was set at *p* < 0.01.

Results

No direct studies of the P2Y₂ receptor have been reported due to the unavailability of radioligands for this receptor. Therefore, the P2Y₂ receptor was tagged with the HA-epitope and stably expressed in 1321N1 human astrocytoma cells as described in Materials and Methods. We have previously shown that these cells do not natively express P2Y₂ or other G protein-coupled P2Y receptors (Filtz *et al.*, 1994; Schachter *et al.*, 1996). Initial experiments using the P2Y₂ receptor agonists ATP and UTP confirmed the heterologous expression of P2Y₂ and P2Y₂-HA receptors in 1321N1 cells. Concentration-effect curves were generated to assess whether incorporation of the HA tag altered the pharmacological selectivity of the receptor using inositol phosphate accumulation as a measure of agonist potency and efficacy. The order of agonist potency (UTP ≥ ATP > ATPγS > App(NH)p) observed for stimulation of inositol phosphate accumulation in 1321N1 cells stably expressing the P2Y₂-HA receptor was essentially identical to that observed after expression of wild-type P2Y₂ receptors (Fig. 1) and with P2Y₂ receptors endogenously expressed in CF/T43 human airway epithelial cells (Brown *et al.*, 1991; Parr *et al.*, 1994). Although the relative level of expression of P2Y₂-HA and P2Y₂ receptors could not be compared after expression in 1321N1 cells, the maximal effect observed with P2Y₂ receptor agonists was very similar between the two engineered cell lines (data not shown). Furthermore, untagged and HA-tagged P2Y₂ receptors exhibited similar properties of agonist-induced desensitization (data not shown and see below). Thus, the signaling properties and pharmacological selectivity of the P2Y₂-HA receptor were indistinguishable from the wild-type P2Y₂ receptor.

Agonist-promoted desensitization of P2Y₂-HA receptors was examined by analyzing the time course of ATPγS-promoted inositol phosphate accumulation in the presence of

LiCl (Fig. 2). [³H]Inositol phosphates accumulated rapidly, achieving $12.1 \pm 1.2\%$ of the maximal accumulation within 10 sec of the addition of ATPγS. This initial rate of accumulation decreased with time of agonist incubation and plateaued within 30 min. The possibility that homologous desensitization of the P2Y₂-HA receptor was responsible for the decreased rate of inositol phosphate accumulation was examined (Fig. 3). Cells were incubated for 25 min with vehicle or 30 μM ATPγS. The cells then were challenged for 5 min with LiCl alone or in combination with either additional ATPγS (30 μM, final concentration) or with 1 mM carbachol and 2 units/ml thrombin, which activate natively expressed receptors in 1321N1 cells. Although preincubation with ATPγS for 25 min resulted in an almost complete loss of capacity of ATPγS to promote inositol phosphate accumulation, ATPγS pretreatment had no effect on carbachol/thrombin-stimulated inositol phosphate accumulation. Thus, neither inositol lipid substrate depletion nor modification of some other component in the signaling pathway is responsible for the decreased rate of inositol phosphate accumulation in the presence of ATPγS. These results suggest that the time-dependent decrease in ATPγS-promoted inositol phosphate accumulation (Fig. 2) represents a measure of modification of the function of the P2Y₂ receptor *per se*.

The potential role of an agonist-promoted change in the cellular distribution of P2Y₂ receptors in loss of receptor function was examined by developing an ELISA (see Materials and Methods) to measure quantitatively changes in surface P2Y₂-HA receptor levels. Cells expressing P2Y₂-HA

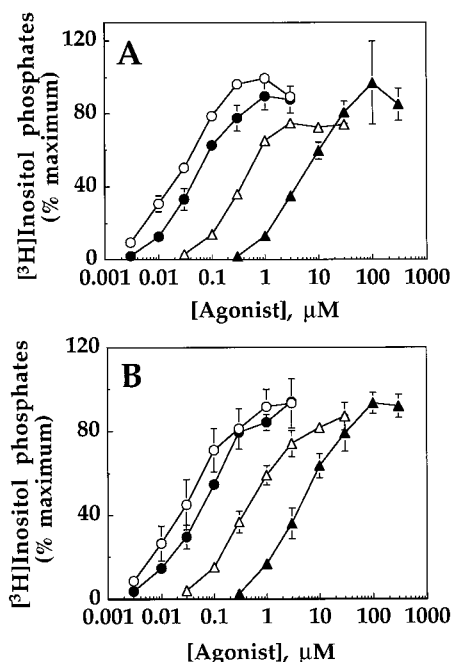


Fig. 1. Agonist selectivity of P2Y₂ receptor and P2Y₂-HA receptor. A, Data for 1321N1 cells expressing the wild-type P2Y₂ receptor. B, Data for 1321N1 cells expressing the HA-epitope tagged P2Y₂ receptor. [³H]Inositol-labeled cells were incubated with increasing concentrations of the P2Y₂ receptor agonists UTP (○), ATP (●), ATPγS (△), or App(NH)p (▲) in the presence of 10 mM LiCl for 15 min. Reactions were terminated and [³H]inositol phosphates were quantified as described in Materials and Methods. Data represent the averages ± standard error for three experiments carried out in triplicate. The data are plotted as percent of the maximal accumulation (~15,000 cpm) of inositol phosphates for the wild-type P2Y₂ receptor in the presence of UTP.

receptors were incubated with 100 μM UTP or ATP γS for the indicated times, and cell surface immunoreactivities were quantified (Fig. 4). A time-dependent decrease in surface immunoreactivity was observed with agonist incubation, and an apparent steady state 50–60% loss was observed after 30–60 min of agonist incubation ($t_{1/2}$ of decrease ~ 15 min).

To demonstrate that this decrease in surface immunoreactivity was related to occupancy of the P2Y $_2$ -HA receptor, concentration-effect curves were generated for UTP and ATP γS (Fig. 5). Loss of immunoreactivity occurred in a concentration-dependent manner for both agonists, and the agonist selectivity for promotion of this loss was consistent with that of a P2Y $_2$ receptor. The EC_{50} values for induction of receptor loss were 0.7 μM for UTP and 2.1 μM for ATP γS , values that were 7- and 20-fold greater, respectively, than the corresponding EC_{50} values for activation of phospholipase C (see Fig. 1). Thus, agonists promote a time- and

agonist concentration-dependent decrease in surface P2Y $_2$ -HA receptors.

Immunofluorescence confocal microscopy methodology was developed to visualize directly the agonist-promoted loss of surface P2Y $_2$ receptors. The paraformaldehyde fixation procedure used for these studies did not render cells generally permeant to antibodies; that is, only 1% of the paraformaldehyde-fixed cells exhibited immunofluorescence with antibody against the cytoskeletal protein vimentin. In contrast, permeabilization with 0.05% saponin resulted in anti-vimentin antibody immunofluorescence in 100% of the cells. Cells expressing P2Y $_2$ -HA receptors, but not those expressing

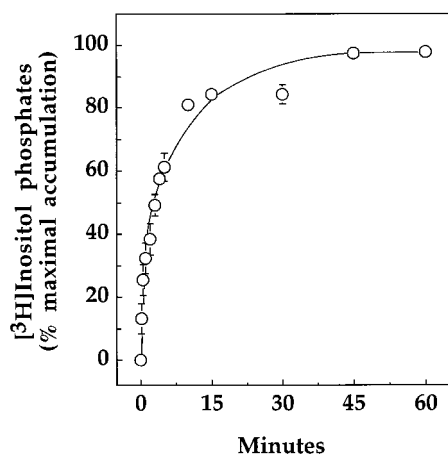


Fig. 2. Agonist-promoted accumulation of [^3H]inositol phosphates. [^3H]Inositol-labeled P2Y $_2$ -HA receptor-expressing cells were incubated with 15 mM LiCl for 10 min before the addition of 250 μl of ATP γS (30 μM , final concentration) for the indicated times. [^3H]Inositol phosphates were quantified as described. Data represent the averages \pm standard error from three experiments completed in triplicate. Maximal accumulations were 22,544, 21,756, and 25,404 cpm for three individual experiments.

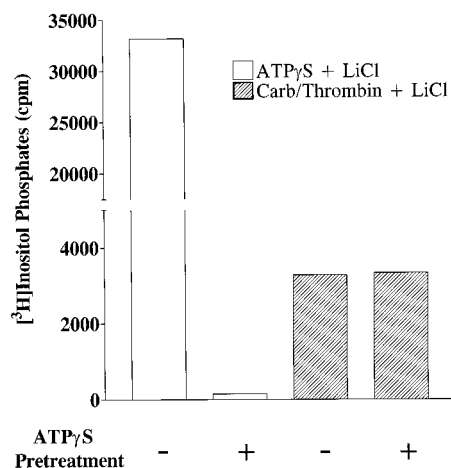


Fig. 3. P2Y $_2$ receptor-specific desensitization induced by ATP γS . [^3H]Inositol-labeled P2Y $_2$ -HA receptor-expressing cells were pretreated for 25 min with or without ATP γS (30 μM , final concentration). Cells then were challenged for 5 min with 15 mM LiCl and 30 μM ATP γS (open bars) or 15 mM LiCl, 1 mM carbachol, and 2 units/ml thrombin (hatched bars) as described in Materials and Methods. Values shown are the mean values from one of three representative experiments.

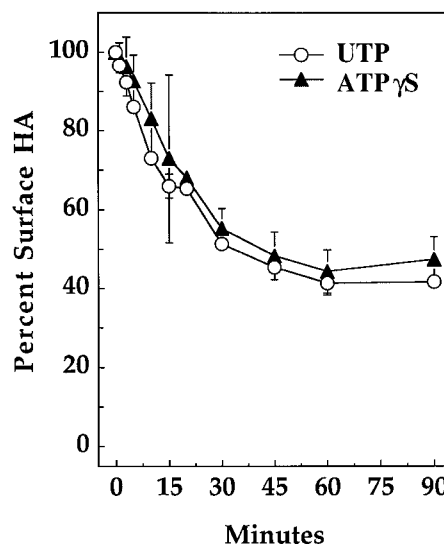


Fig. 4. P2Y $_2$ receptor agonist-promoted loss of surface immunoreactivity. P2Y $_2$ -HA receptor-expressing cells were incubated with 100 μM UTP (\circ) or ATP γS (\blacktriangle). Cell surface receptor expression was measured at the indicated times by an ELISA using an anti-HA antibody as described in Materials and Methods. Data represent the mean \pm standard error for triplicate values obtained from three experiments.

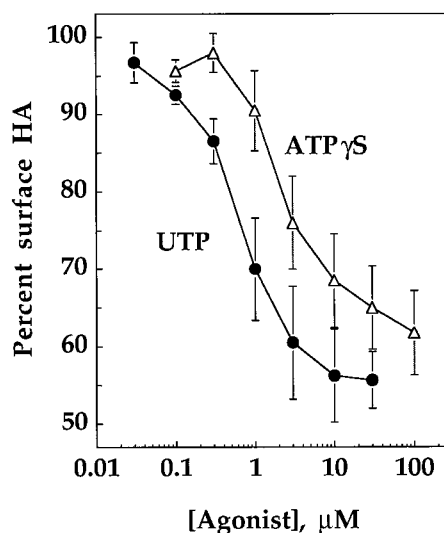


Fig. 5. Concentration dependence of agonists for induction of loss of surface immunoreactivity. Cells expressing the P2Y $_2$ -HA receptor were incubated with the indicated concentrations of UTP (\bullet) or ATP γS (\triangle) for 15 min. Reactions were terminated by placing the cells on ice, and the cells were fixed and processed for ELISA as described in Materials and Methods. The data represent the mean \pm standard error of four experiments completed in triplicate.

wild-type P2Y₂ receptors, exhibited marked cell surface immunofluorescence (Fig. 6A). This immunofluorescence exhibited a uniform, punctate pattern in agonist-naïve cells, and a similar pattern of cell surface immunofluorescence was observed with multiple clonal lines of P2Y₂-HA receptor expressing 1321N1 cells. Incubation of cells with ATP γ S for 15 and 30 min promoted a decrease in surface immunofluorescence intensity. Surface immunofluorescence after 30 min of agonist incubation was $51.2 \pm 4.9\%$ of control (Fig. 6B). This decrease was similar to results obtained in ELISA analyses (see Fig. 3).

Time-dependent decreases in surface P2Y₂ receptor levels were consistent with the occurrence of agonist-induced internalization of P2Y₂ receptors. To establish unambiguously that P2Y₂ receptor internalization occurred, experiments were carried out to demonstrate that receptors originating at the cell surface translocated to the cytosol as a consequence of agonist incubation. Thus, surface P2Y₂-HA receptors were labeled with anti-HA antibody to form receptor/antibody complexes, and these antibody-prelabeled cells then were incubated with agonist and fixed. Detection of surface receptors was accomplished by incubation with Cy3-conjugated secondary antibody. Intracellular receptors were visualized

using the Cy3 secondary antibody in a parallel set of cells that were permeabilized after blockade of surface P2Y₂ receptor/primary antibody complexes with unconjugated secondary antibody. Control experiments using antibody-prelabeled P2Y₂-HA receptors demonstrated that antibody binding neither activated phospholipase C nor modified P2Y₂ receptor agonist-promoted responses (data not shown). Preincubation of cells with antibody alone also did not induce the appearance of intracellular immunoreactivity and therefore did not induce P2Y₂ receptor internalization. In contrast, the immunofluorescence observed at the cell surface of antibody-prelabeled cells decreased with increasing time of ATP γ S incubation (Fig. 7), consistent with results described above using ELISA and immunofluorescence methodologies. Moreover, the loss of surface P2Y₂ receptors detected with increasing time of agonist incubation was accompanied by a parallel increase in intracellular immunofluorescence. These observations directly demonstrate agonist-promoted movement of P2Y₂-HA receptors from the cell surface to an internalized compartment. Precise quantification of the movement of receptors from a surface to intracellular location was not made with confocal microscopy since receptors located in subplas-

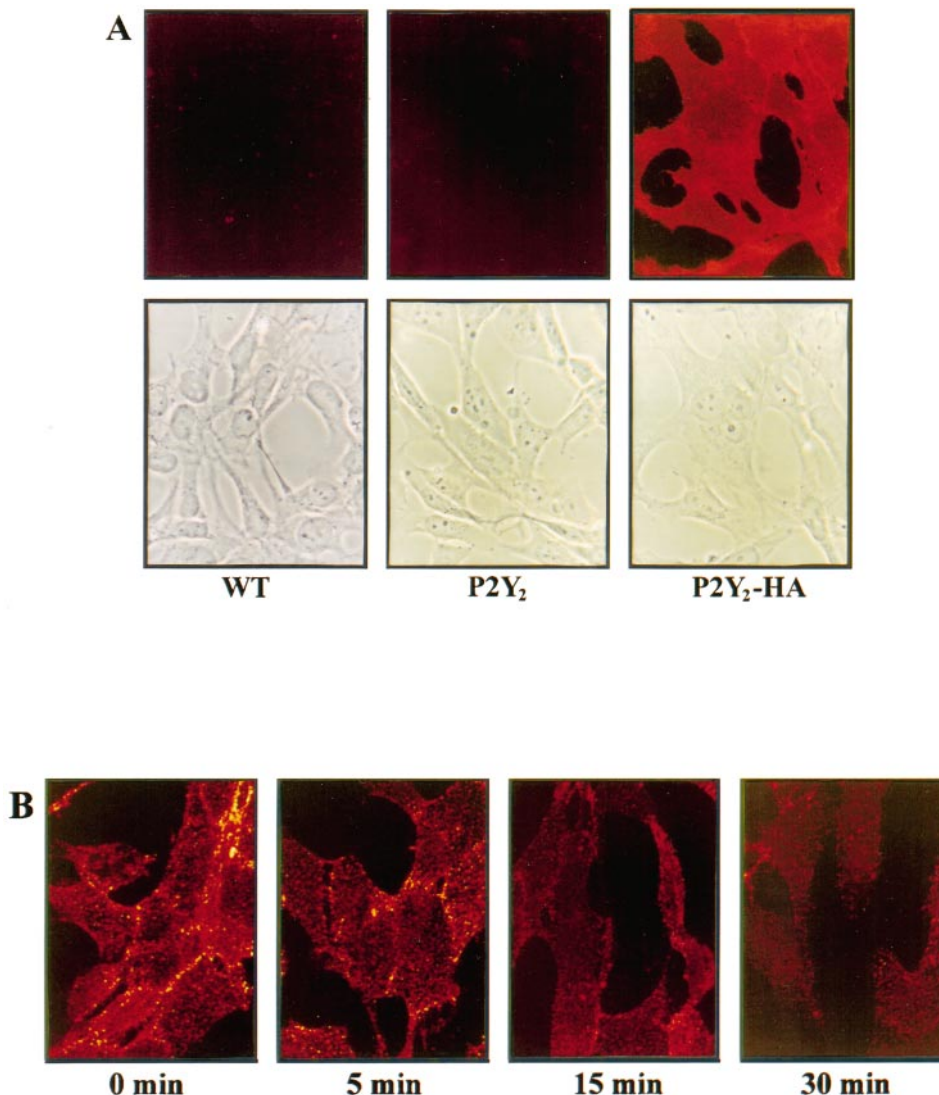


Fig. 6. Immunofluorescent visualization of cell surface P2Y₂ receptors. **A**, Wild-type (WT) cells (*left*) and cells expressing the P2Y₂ receptor (*middle*) or P2Y₂-HA receptor (*right*) were cultured and fixed. After blocking nonspecific protein-binding sites, cells were incubated with anti-HA primary and Cy3-conjugated secondary antibodies as described in Materials and Methods. *Top*, immunofluorescence micrographs. *Bottom*, bright-field micrographs. Photographs are representative of at least four individual experiments. **B**, P2Y₂-HA receptor-expressing cells were equilibrated with 50 mM DMEM-containing HEPES, pH 7.4, for 60 min at 37°. ATP γ S (30 μ M, final concentration) was added for the indicated times. Reactions were terminated, and cells were fixed and processed for detection of surface immunofluorescence as described in Materials and Methods. Confocal images of reconstructed surface views are shown (magnification, 2500 \times). Photographs are representative of five or six experiments.

malemmal structures may contribute to the signal that is considered surface related.

Analyses of the reversibility of the observed agonist-induced changes in the P2Y₂ receptor are made problematic by the lack of an effective competitive antagonist for this receptor and by the propensity of 1321N1 cells to release both ATP (Lazarowski *et al.*, 1995) and UTP (Lazarowski *et al.*, 1997) on mechanical stress, including simple changes of medium. To partially circumvent these concerns, we adapted a protocol in which the nucleotide-hydrolyzing enzyme apyrase was added to the medium at a concentration (3 units/ml) that completely hydrolyzed 30 μ M [³H]ATP in the bulk solution within 1 min under the conditions of these assays. Thus, cells were incubated with 30 μ M ATP for 15 min, apyrase was added to hydrolyze the ATP, and the incubation was continued for an additional 5–120 min. After a delay of ~15 min, P2Y₂ receptor levels measured by ELISA analyses returned to control levels within 90 min (Fig. 8). This recovery occurred in the presence of a concentration of cycloheximide (5 μ g/ml) that inhibits protein synthesis (data not shown). Moreover, receptors that originated at the cell surface and internalized as a consequence of agonist-incubation could be shown (using the HA-antibody prelabeling approach described above) to return to the cell surface subsequent to removal of agonist. Measurement of recovery of P2Y₂ receptor responsiveness was complicated by the problems mentioned above. However, in experiments using apyrase to re-

move ATP from the medium, responsiveness of the cells to ATP γ S recovered with roughly the same time course as observed for recovery of surface immunoreactivity; that is, the response (5-min assay) to ATP γ S 0, 15, 60, and 120 min after the addition of apyrase to ATP-pretreated cells was 40.6%, 52.8%, 90.9%, and 89.8% (two experiments in quadruplicate) of the response observed with control cells.

The results illustrated thus far indicate that P2Y₂ receptors desensitize and internalize as a consequence of agonist incubation. Comparison of the time courses of these events suggested that agonist-induced desensitization preceded the loss of surface receptors. However, such a comparison does not address whether these are independent or functionally related events. Therefore, agonist incubations were carried out at reduced temperature to inhibit internalization and to determine whether the capacity of agonists to promote P2Y₂ receptor desensitization was reduced.

A time course of inositol phosphate accumulation was determined during challenge at 16° with 30 μ M ATP γ S (Fig. 9). As was the case with agonist incubation at 37°, a plateau of ATP γ S-promoted inositol phosphate accumulation was reached within ~30 min of agonist incubation. Total inositol phosphate formation after 25 min in the presence of ATP γ S at 16° was 50–60% of that observed at 37° [21, 466 \pm 1,046 cpm (mean \pm standard error, three experiments) at 16° and 36,655 \pm 3,407 cpm (mean \pm standard error, three experiments) at 37°]. The time-dependent decrease in the rate of

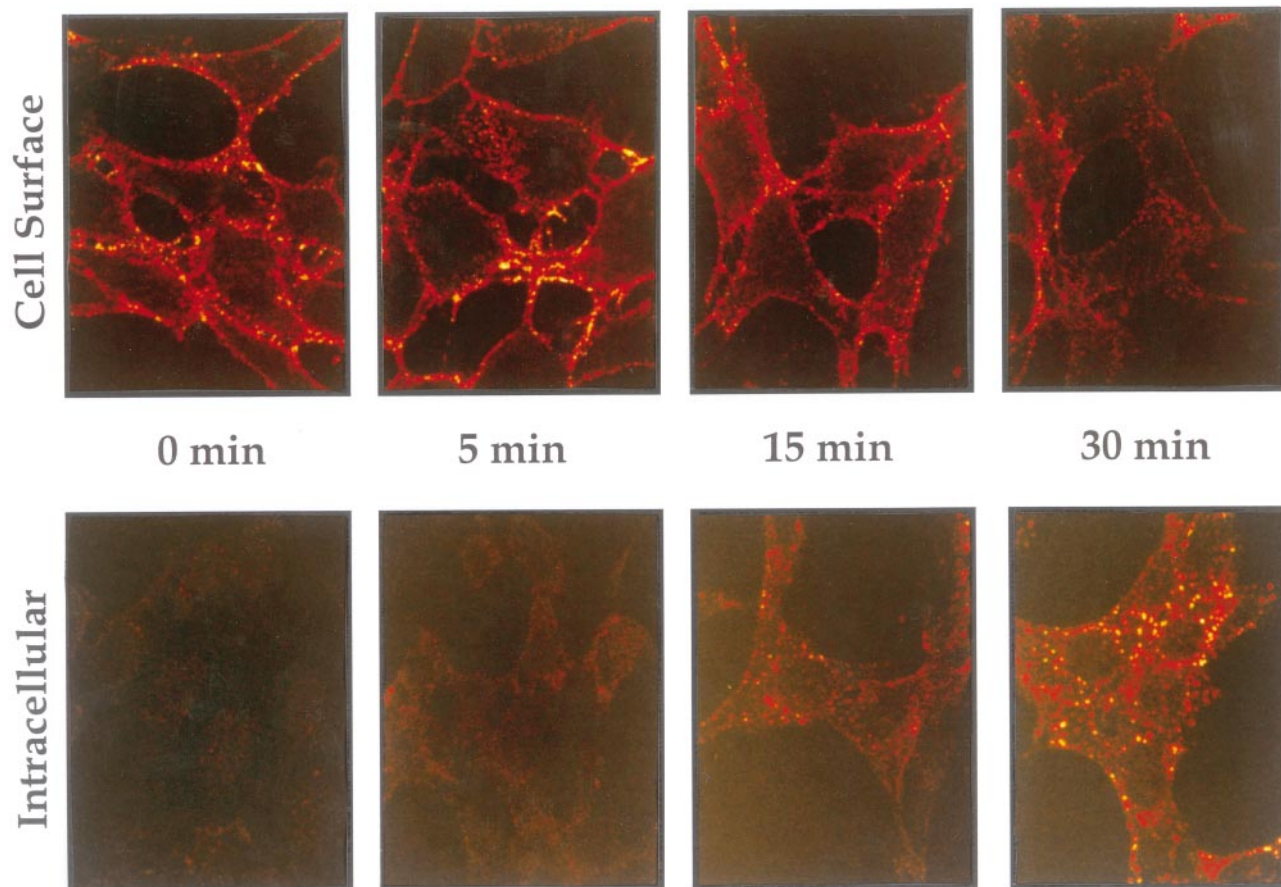


Fig. 7. Direct demonstration of agonist-induced internalization of the P2Y₂ receptor. Intact P2Y₂-HA receptor-expressing cells were prelabelled at 4° with anti-HA primary antibody as described in Materials and Methods. Cells were rapidly warmed to 37° and incubated with 30 μ M ATP γ S for the indicated times. Reactions were terminated, and cells were fixed and processed for detection of surface or intracellular immunofluorescence as described. Single optical sections obtained by confocal microscopy are shown. Results are representative of four individual experiments.

ATP γ S-promoted inositol phosphate accumulation in the presence of LiCl at 16° was due to desensitization of P2Y₂ receptors because no ATP γ S-induced change occurred in the level of carbachol/thrombin-promoted inositol phosphate accumulation (data not shown). Although agonist-induced desensitization of P2Y₂ receptors occurred at 16°, no agonist-induced loss of surface immunoreactivity associated with P2Y₂-HA receptors could be measured by ELISA analysis (Fig. 10). To demonstrate directly that reduced temperature inhibited receptor internalization, antibody-prelabeled cells were incubated with 30 μ M ATP γ S for 0–30 min at 16°, and cells were analyzed by immunofluorescence confocal microscopy for P2Y₂ receptor redistribution (Fig. 11). No change in the cell surface distribution of P2Y₂ receptors was observed in the presence of ATP γ S at 16°. Likewise, no accumulation

of intracellular immunofluorescence was observed over the same period. Therefore, agonist-induced receptor internalization did not occur at 16°, a condition under which desensitization of P2Y₂-HA receptors could be readily demonstrated. Incubation of cells in 0.45 M sucrose at 37° also prevented ATP γ S-promoted P2Y₂-HA receptor internalization without affecting the occurrence of agonist-induced desensitization (data not shown).

Discussion

In face of the lack of a radioligand binding assay for quantification of P2Y₂ receptors, an epitope-tagged P2Y₂ receptor construct was prepared. The stably expressed recombinant HA-tagged receptors exhibited pharmacological and second messenger signaling selectivities identical to their native correlate. P2Y₂-HA receptors also exhibited properties of agonist-induced desensitization analogous to the wild-type P2Y₂ receptor and characteristic of those reported for other G_q/phospholipase C-linked receptors. The epitope tag has allowed us to quantify the relative level of cell surface P2Y₂ receptors during incubation of cells with adenine nucleotides, and the results demonstrate for the first time that a P2Y receptor is sequestered away from the cell surface to an extent related to occupancy of the receptor by agonist. Although agonist-induced loss of P2Y₂ receptor responsiveness apparently occurred at the level of the receptor rather than at the level of G_q, phospholipase C- β , or the lipid substrate, both ELISAs and immunofluorescence confocal microscopy directly confirmed that loss of receptors from the cell surface does not account for desensitization of the nucleotide response.

The availability of an epitope-tagged P2Y₂ receptor and an antibody that very tightly binds the HA sequence (Field *et al.*, 1988) also allowed us to determine directly whether receptors at the cell surface were stimulated by agonists to traverse to an intracellular compartment in response to receptor activation. As observed in previous studies of the adenylyl cyclase-linked β_2 -adrenergic receptor (von Zastrow

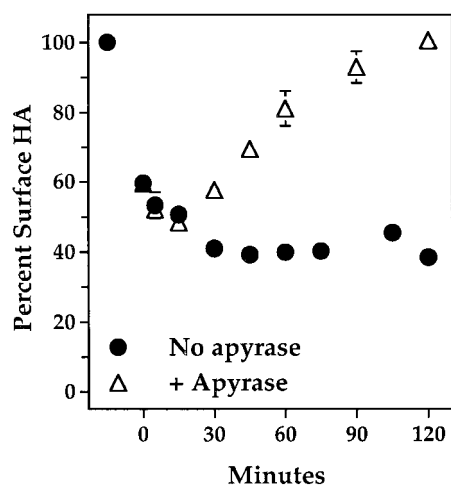


Fig. 8. Reversibility of agonist-promoted loss of surface immunoreactivity. Cells were incubated with 30 μ M ATP for 15 min. The cells then received vehicle (●) or 0.75 unit of apyrase (○) to hydrolyze the ATP, and the incubation was continued for the indicated times. Reactions were terminated by placing the cells on ice. Cells were fixed with paraformaldehyde and processed for ELISA determination of surface immunoreactivity. Data represent the average \pm standard error for three to five experiments completed in triplicate.

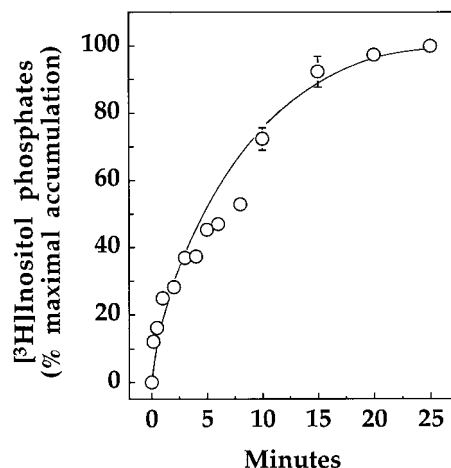


Fig. 9. Agonist-promoted accumulation of [3 H]inositol phosphates at 16°. [3 H]Inositol-labeled P2Y₂-HA receptor-expressing cells were incubated at 16° with 15 mM LiCl for 10 min before the addition of ATP γ S (final concentration, 30 μ M) for the indicated times. [3 H]Inositol phosphates were quantified as described in Materials and Methods. Data represent the mean \pm standard error for three experiments completed in triplicate.

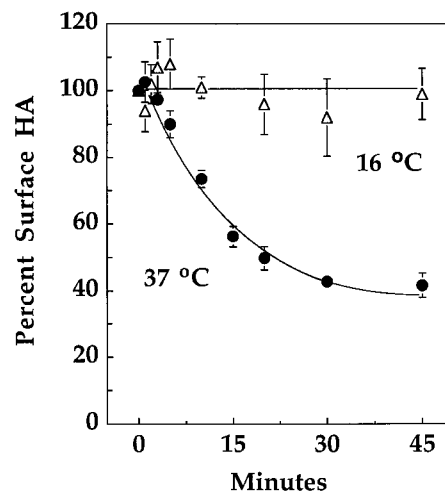


Fig. 10. Agonist-promoted change in surface immunoreactivity at 37° and 16°. Cells were incubated at 37° or 16° with 30 μ M ATP γ S for the indicated times. Reactions were terminated by placing the cells on ice. Cells were fixed and processed for ELISA determination of surface immunoreactivity levels. Data represent the mean \pm standard error for three or four experiments with assays carried out in triplicate.

and Kobilka, 1994; Morrison *et al.*, 1996), binding of antibody to the amino terminus of the P2Y₂ receptor resulted in neither activation of phospholipase C nor a change in the capacity of agonists to promote activation through this receptor. Moreover, agonists induced a directly measured internalization of the antibody-tagged P2Y₂ receptor with a time course and to an extent that were consistent with those observed for the decrease of cell surface P2Y₂ receptors measured by either ELISA or quantification of cell surface immunofluorescence. Thus, within the limitations of our studies, antibody-tagged P2Y₂ receptors can be used to follow the fate of functionally relevant P2Y receptors originating at the cell surface. In preliminary studies, internalized antibody-prelabeled P2Y₂ receptors relocated to the cell surface after removal of agonist from the medium with the same time course and extent of recovery as quantified with the ELISA (Lee JW, Sromek SM, and Harden TK, unpublished observations). Whether internalization/externalization is a dynamic process that continues to occur in the presence of agonist will be addressed in future experiments.

A punctate pattern of P2Y₂ receptor immunofluorescence was observed at the cell surface in both the absence and presence of agonist. This observation contrasts with results reported for epitope-tagged β_2 -adrenergic receptors (von Zastrow and Kobilka, 1994), which undergo an agonist-promoted change from a uniform to a punctate distribution before the

occurrence of receptor internalization. Whether these results indicate a fundamental difference in cell surface distribution of P2Y₂ receptors versus β_2 -adrenergic receptors is unclear. One possible explanation lies in our previous observations of the release of both cellular ATP (Lazarowski *et al.*, 1995) and UTP (Lazarowski *et al.*, 1997) by 1321N1 cells. However, addition of the ATP diphosphohydrolase apyrase to the medium failed to convert the P2Y₂ receptors to a nonpunctate, more uniformly distributed population of receptors (Sromek, 1997). Although control experiments indicated that this concentration of apyrase degraded ATP added to the medium, such results do not rule out the possibility that ATP or UTP, or both, is released constitutively from 1321N1 cells in amounts that are sufficient at the cell surface to activate P2Y₂ receptors and promote movement to a concentrated (i.e., punctate) surface localization. The elevated basal level of inositol phosphates in P2Y₂ receptor-expressing cells, which is not completely reversed by apyrase treatment, clearly indicates that autocrine activation of receptors occurs. Conversely, the marked inositol phosphate response observed after the addition of P2Y₂ receptor agonists to the medium of resting cells indicates that the majority of the receptors are not likely to be basally desensitized. More work is needed to define whether P2Y₂ receptors distribute in domains on 1321N1 cells that are not shared, at least in the resting state, with other G protein-coupled receptors.

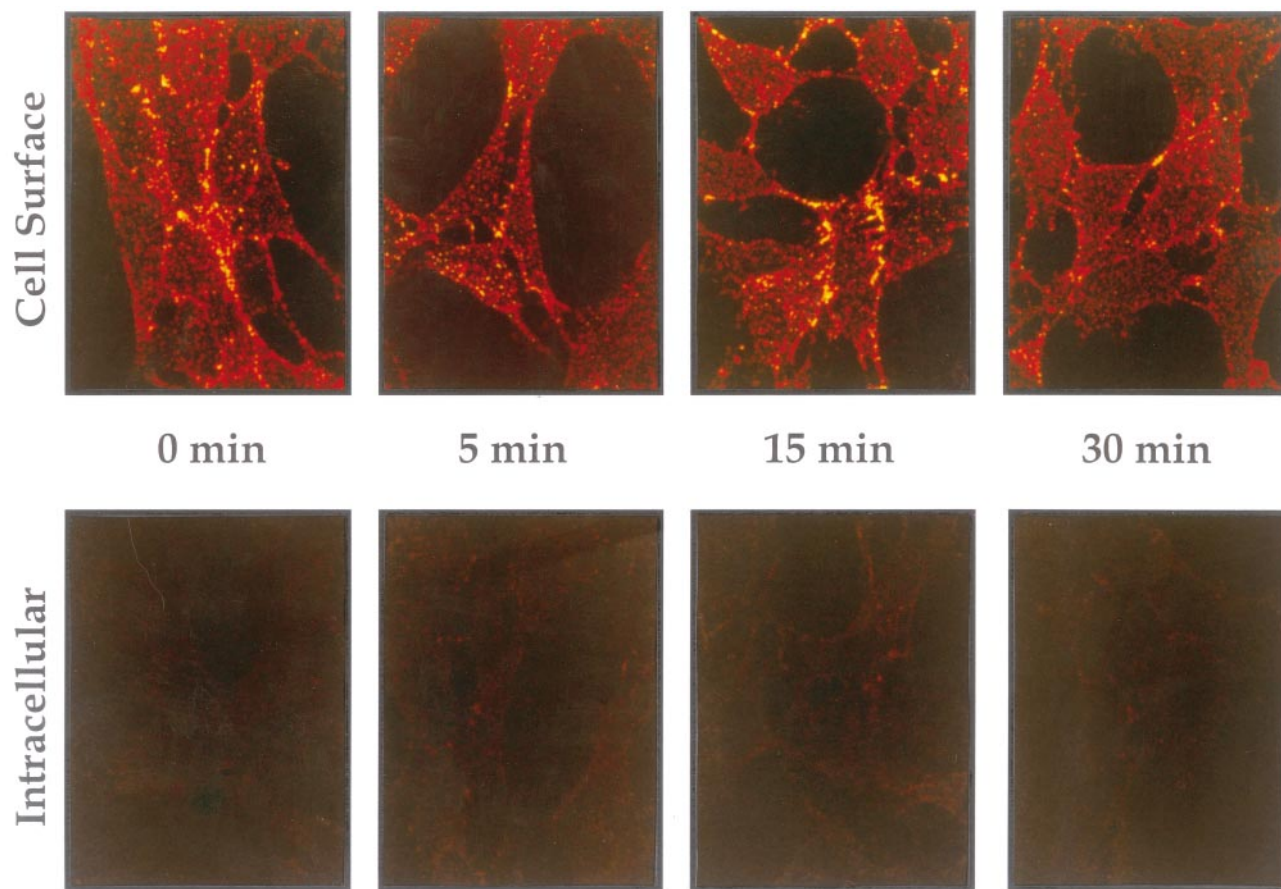


Fig. 11. Lack of agonist-induced internalization of P2Y₂ receptors at 16°. P2Y₂ receptor-expressing cells were prelabeled with anti-HA primary antibody as described in Materials and Methods. Cells were incubated at 16° with 30 μ M ATP γ S for the indicated times. Reactions were terminated, and cells were fixed and processed for surface or intracellular immunofluorescence as described. Immunofluorescence was analyzed by confocal microscopy. Cell surface images were reconstructed from consecutive optical sections. Intracellular images were from single optical sections. Results shown are representative of three individual experiments.

Studies of agonist-induced desensitization of a heterologously expressed G protein-coupled receptor should be interpreted cautiously. We do not know the level of expression of the recombinant P2Y₂ receptor relative to that of natively expressed P2Y₂ receptors, although we anticipate that the recombinant receptor is present at a high level. For example, the EC₅₀ values of all P2Y₂ receptor agonists determined with the cells used in this study were ~10-fold lower than their respective EC₅₀ values observed at natively expressed P2Y₂ receptors (Cowen *et al.*, 1989; Brown *et al.*, 1991; Lazarowski *et al.*, 1995). We also do not know whether receptor-specific proteins involved in, for example, regulation of the intracellular movement of receptors, are differentially expressed by different cell types. Different G protein-coupled receptors have been shown to undergo markedly different intracellular sorting, even when coexpressed in the same cell (von Zastrow *et al.*, 1993). Despite the above concerns, there are results that support the physiological relevance of our analyses. Foremost among these is the observation that the properties of desensitization of the epitope-tagged heterologously expressed P2Y₂ receptor are remarkably similar to the properties of desensitization of the P2Y₂ receptor natively expressed in, for example, human airway epithelial (Brown *et al.*, 1991) and bovine aortic (Wilkinson *et al.*, 1994) cells. Epitope-tagged heterologously expressed β_2 - and α_2 -adrenergic receptors also undergo agonist-induced desensitization and internalization with properties in accordance with the characteristics of the widely studied natively expressed adrenergic receptors (von Zastrow and Kobilka, 1992, 1994; von Zastrow *et al.*, 1993).

Although our experiments illustrate that a member of the P2Y receptor family of G protein-coupled receptors undergoes a change in cellular distribution as a consequence of receptor activation, they do not establish the functional consequence of this event or events. Internalization may serve as one of the initial steps involved in delivery of receptors to lysosomes and to eventual receptor degradation (Perkins *et al.*, 1991; Lohse, 1993). Our studies indicate that after short-term treatment of P2Y₂ receptor-expressing cells with agonist, the transfer of cells to an agonist-free medium results in full recovery of cell surface receptors within 1 hr and in recovery of receptor responsiveness. However, we have not yet established the role that receptor internalization and subsequent externalization may play in receptor resensitization. In contrast, after extended incubation of these cells with agonist (e.g., 24 hr), full recovery of cell surface P2Y₂ receptors required ≥ 24 hr in agonist-free medium, and this process was dependent on new protein synthesis (Lee JW, Sromek SM, and Harden TK, unpublished observations). It will be important to design experiments that attempt to dissociate agonist-promoted P2Y₂ internalization from agonist-induced down-regulation of P2Y₂ receptors.

The experiments at a reduced temperature clearly dissociate receptor internalization from the occurrence of agonist-induced desensitization. Although unambiguous data are not yet available, internalization of some G protein-coupled receptors apparently provides a mechanism for delivery of phosphorylated receptors to phosphatases in the intracellular compartment. Externalization of dephosphorylated receptor then ostensibly restores cell surface receptors to a state that fully couples to and activates the appropriate G protein and its associated signaling pathway. Data supporting phos-

phorylation \rightarrow internalization \rightarrow dephosphorylation \rightarrow externalization \rightarrow resensitization as a sequence of events in the G_q-activated inositol lipid signaling pathway are not extensive, and no data are available addressing whether phosphorylation/dephosphorylation reactions are involved in the regulation of P2Y receptor activity. The methodology introduced in this study should allow us to address these questions further, and the observation of agonist-induced internalization of the P2Y₂ receptor reported here provides direct evidence for at least one step in such a hypothetical regulatory pathway.

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