Agonist-Induced Internalization of the P2Y2 Receptor

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ABSTRACT

The Gq/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 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 quantitate 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°C 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 and exhibit a broad tissue distribution (Parr et al., 1997). The P2Y1, P2Y4, P2Y6, and P2Y11 receptors are specifically activated by either adenosine or uridine nucleotides (Communi et al., 1997; Friedholm et al., 1997). The P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors are specifically activated by either adenine or uridine nucleotides (Communi et al., 1997; Friedholm et al., 1997; Harden et al., 1998). The P2Y2 receptor is equipotently activated by both ATP and UTP (Lustig et al., 1993; Lazarowski et al., 1995). Activation of P2Y2 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°C 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.

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 adenyl cyclase-coupled receptors, such as the β2-adrenergic receptor, indicate that agonist-promoted receptor phosphorylation is at least partially responsible for decreased responsiveness (Leffkowitz, 1993; Lohse, 1993). However, the cellular and molecular mechanisms underlying agonist-induced desensitization of Gq/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...
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 CTT 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. Ligation 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 (Sequence 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 Rossman, 1989). Purified plasmids were used to transfect the murine fibroblast packaging cell line PA317 by calcium phosphate precipitation for production of retroviruses (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 1640 and equilibrated for 60 min. ATP or vehicle was added to the culture medium at a concentration of 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 with 30% v/v ethanol and analyzed for inositol phosphate accumulation using a microplate reader (Bio Tek Instruments, Winooski, VT). Inositol phosphate accumulation 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).

**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 μl for the indicated times, and the 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 (1:200) for 30 min. After several washes in HEPES, Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) was added at a 1:500 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× Zeiss objective, 1.4 NA, using an argon laser at 514 nm with a rhodamine filter. Optical sections were 0.5 μm thick. The average thickness of 1321N1 cells was 2.5 μm, and the range of thickness measured was 1.5–2.5 μm. 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.

**Indirect immunofluorescence microscopy with antibody-postlabeled cells.** Cells were seeded onto poly-d-lysine-coated (10 μg/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 with paraformaldehyde freshly prepared in PBS, pH 7.4. After repeated washes with HEPES-buffered MEM, 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 (BAbCO) was incubated with the cells at a 1:500 dilution in blocking buffer for 30 min. After several washes in HEPES, 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).

**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 μl for the indicated times, and the 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 HEPES, 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 HEPES and then quickly rinsed with 0.1 M sodium citrate, pH 4.5 (substrate buffer), to reduce background. The substrate 3,3′-diaminobenzidine was added at 1 mg/ml in substrate buffer, and 1 μl 30% H2O2 was added per 10 ml substrate buffer. Product formation proceeded for 10–45 min, and 100-μl 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

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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 immunoreactivity.** 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 P2Y$_2$ receptor have been reported due to the unavailability of radioligands for this receptor. Therefore, the P2Y$_2$ 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 P2Y$_2$ or other G protein-coupled P2Y receptors (Filtz et al., 1994; Schachter et al., 1996). Initial experiments using the P2Y$_2$ receptor agonists ATP and UTP confirmed the heterologous expression of P2Y$_2$ and P2Y$_2$-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 $\geq$ ATP $>$ ATP$_S$ $>$ Apn(NH)p) observed for stimulation of inositol phosphate accumulation in 1321N1 cells stably expressing the P2Y$_2$-HA receptor was essentially identical to that observed after expression of wild-type P2Y$_2$ receptors (Fig. 1) and with P2Y$_2$ 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 P2Y$_2$-HA and P2Y$_2$ receptors could not be compared after expression in 1321N1 cells, the maximal effect observed with P2Y$_2$ receptor agonists was very similar between the two engineered cell lines (data not shown). Furthermore, untagged and HA-tagged P2Y$_2$ receptors exhibited similar properties of agonist-induced desensitization (data not shown and see below). Thus, the signaling properties and pharmacological selectivity of the P2Y$_2$-HA receptor were indistinguishable from the wild-type P2Y$_2$ receptor.

Agonist-promoted desensitization of P2Y$_2$-HA receptors was examined by analyzing the time course of ATP$_S$-promoted inositol phosphate accumulation in the presence of LiCl (Fig. 2). [H]$^3$H]inositol phosphates accumulated rapidly, achieving $12.1 \pm 1.2\%$ of the maximal accumulation within 10 sec of the addition of ATP$_S$. This initial rate of accumulation decreased with time of agonist incubation and plateaued within 30 min. The possibility that homologous desensitization of the P2Y$_2$-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 $\mu$M ATP$_S$. The cells then were challenged for 5 min with LiCl alone or in combination with either additional ATP$_S$ (30 $\mu$M, final concentration) or with 1 mM carbachol and 2 units/ml thrombin, which activate natively expressed receptors in 1321N1 cells. Although preincubation with ATP$_S$ for 25 min resulted in an almost complete loss of capacity of ATP$_S$ to promote inositol phosphate accumulation, ATP$_S$ 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 ATP$_S$. These results suggest that the time-dependent decrease in ATP$_S$-promoted inositol phosphate accumulation (Fig. 2) represents a measure of modification of the function of the P2Y$_2$ receptor per se.

The potential role of an agonist-promoted change in the cellular distribution of P2Y$_2$ receptors in loss of receptor function was examined by developing an ELISA (see Materials and Methods) to measure quantitatively changes in surface P2Y$_2$-HA receptor levels. Cells expressing P2Y$_2$-HA

![Fig. 1. Agonist selectivity of P2Y$_2$ receptor and P2Y$_2$-HA receptor.](image-url)
receptors were incubated with 100 μM UTP or ATPγS 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 P2Y₂-receptor, concentration-effect curves were generated for UTP and ATPγS (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 P2Y₂ receptor. The EC$_{50}$ values for induction of receptor loss were 0.7 μM for UTP and 2.1 μM for ATPγS, 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 P2Y₂-HA receptors.

Immunofluorescence confocal microscopy methodology was developed to visualize directly the agonist-promoted loss of surface P2Y₂ 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 P2Y₂-HA receptors, but not those expressing

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**Fig. 2.** Agonist-promoted accumulation of $[^{3}H]$inositol phosphates. $[^{3}H]$Inositol-labeled P2Y₂-HA receptor-expressing cells were incubated with 15 mM LiCl for 10 min before the addition of 250 μl of ATPγS (30 μM, final concentration) for the indicated times. $[^{3}H]$Inositol phosphates were quantified as described. Data represent the averages ± 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.** P2Y₂ receptor-specific desensitization induced by ATPγS. $[^{3}H]$Inositol-labeled P2Y₂-HA receptor-expressing cells were pretreated for 25 min with or without ATPγS (30 μM, final concentration). Cells then were challenged for 5 min with 15 mM LiCl and 30 μM ATPγS (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.** P2Y₂ receptor agonist-promoted loss of surface immunoreactivity. P2Y₂-HA receptor-expressing cells were incubated with 100 μM UTP (○) or ATPγS (●). 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 ± 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 P2Y₂-HA receptor were incubated with the indicated concentrations of UTP (○) or ATPγS (●) 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 ± 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-naive 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 for 15 and 30 min promoted a decrease in surface immunofluorescence intensity. Surface immunofluorescence after 30 min of agonist incubation was 51.2 ± 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 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°C. ATP (30 μM, 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×). Photographs are representative of five or six experiments.
Malemml 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 μM [3H]ATP in the bulk solution within 1 min under the conditions of these assays. Thus, cells were incubated with 30 μM 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 μg/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 remove ATP from the medium, responsiveness of the cells to ATPγS recovered with roughly the same time course as observed for recovery of surface immunoreactivity; that is, the response (5-min assay) to ATPγS 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 μM ATPγS (Fig. 9). As was the case with agonist incubation at 37°, a plateau of ATPγS-promoted inositol phosphate accumulation was reached within ~30 min of agonist incubation. Total inositol phosphate formation after 25 min in the presence of ATPγS at 16° was 50–60% of that observed at 37° [21, 466 ± 1,046 cpm (mean ± standard error, three experiments) at 16° and 36,655 ± 3,407 cpm (mean ± 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 prelabeled at 4° with anti-HA primary antibody as described in Materials and Methods. Cells were rapidly warmed to 37° and incubated with 30 μM ATPγS 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.
ATPγS-promoted inositol phosphate accumulation in the presence of LiCl at 16°C was due to desensitization of P2Y2 receptors because no ATPγS-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°C, 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 μM ATPγS for 0–30 min at 16°C, 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 ATPγS at 16°C. Likewise, no accumulation of intracellular immunofluorescence was observed over the same period. Therefore, agonist-induced receptor internalization did not occur at 16°C, a condition under which desensitization of P2Y2-HA receptors could be readily demonstrated. Incubation of cells in 0.45 M sucrose at 37°C also prevented ATPγS-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 Gq/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 Gq, 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 adenyl cyclase-linked β2-adrenergic receptor (von Zastrow...
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 relocalized 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 β2-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 β2-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.

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**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 μM ATPγS 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 immunofluorescence sections. Intracellular images were from single optical sections. Results shown are representative of three individual experiments.
Studies of agonist-induced desensitization of a heterologically 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 (Cowan et al., 1989; Brown et al., 1991; Lazarski 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 heterologically expressed P2Y2 receptor are remarkably similar to the properties of desensitization of the P2Y2 receptor natively expressed, for example, human airway epithelial (Brown et al., 1991) and bovine aortic (Wilkinson et al., 1994) cells. Epitope-tagged heterologously expressed β2 and α2-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 P2Y2 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 phosphorylation → internalization → dephosphorylation → externalization → resensitization as a sequence of events in the Gα-activated inositol lipid signaling pathway are not extensive, and no data are available addressing whether phosphorylation/dephosphorylation reactions are involved in the regulation of P2Y2 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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References


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