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A Fusion Protein of the Human $P2Y_1$ Receptor and NTPDase1 Exhibits Functional Activities of the Native Receptor and Ectoenzyme and Reduced Signaling Responses to Endogenously Released Nucleotides

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ABSTRACT

To begin to address the functional interactions between constitutively released nucleotides, ectonucleotidase activity, and P2Y receptor-promoted signaling responses, we engineered the human $P2Y_1$ receptor in a fusion protein with a member of the ectonucleoside triphosphate diphosphohydrolase family, NTPDase1. Membranes prepared from Chinese hamster ovary (CHO)-K1 cells stably expressing either wild-type NTPDase1 or the $P2Y_1$ receptor-NTPDase1 fusion protein exhibited nucleotide-hydrolytic activities that were over 300-fold greater than activity measured in membranes from empty vector-transfected cells. The molecular ratio for nucleoside triphosphate versus diphosphate hydrolysis was approximately 1:0.4 for both the wild-type NTPDase1 and $P2Y_1$-NTPDase1 fusion protein. Stable expression of the $P2Y_1$-NTPDase1 fusion protein conferred an ADP and 2MeSADP-promoted Ca^{2+} response to CHO-K1 cells. Moreover, the maximal capacity of the nonhydrolyzable agonist ADPβS to stimulate inositol phosphate accumulation was similar, and the EC_{50} of ADPβS was lower in the fusion protein than the wild-type receptor. In contrast, the substantial nucleotide-hydrolyzing activity of the fusion protein resulted in a greater than 50-fold shift to the right of the concentration-effect curve of ADP for activation of phospholipase C compared with the wild-type receptor. Heterologous expression of the $P2Y_1$ and other P2Y receptors results in marked increases in basal inositol phosphate levels. Given the high nucleotidase activity and apparently normal receptor signaling activity of the $P2Y_1$ receptor-NTPDase1 fusion protein, we quantitated basal inositol phosphate accumulation in cells stably expressing either the wild-type $P2Y_1$ receptor or the fusion protein. Although marked elevation of inositol phosphate levels occurred with wild-type $P2Y_1$ receptor expression, levels in cells expressing the fusion protein were not different from those in wild-type CHO-K1 cells.

The regulated release of cellular nucleotides and nucleosides results in myriad physiological responses through approximately 20 different G protein-coupled and ligand-gated ion channel receptors (Harden et al., 1998a; Ralevic and Burnstock, 1998; Khakh et al., 2000). Hormone and neurotransmitter signaling pathways require effective mechanisms for removing or metabolizing extracellular signaling molecules, and in the case of extracellular nucleotide signaling, a broad range of nucleotide-degrading and interconverting ecto- or extracellular enzymes have been identified (Zimmermann, 1996, 2000). These include the ectonucleoside triphosphate diphosphohydrolase family, which is composed of 1) ecto-ATP diphosphohydrolase (NTPDase1; also known as ecto-ATPDase, apyrase, or CD39), which hydrolyzes nucleoside tri- and diphosphates with similar rates; 2) ecto-ATPase (NTPDase2; also known as CD39L1), which hydrolyzes nucleoside triphosphates with high selectivity over diphosphates; and 3) NTPDase3 (also known as CD39L3), which hydrolyzes nucleoside triphosphates with some selectivity over nucleoside diphosphates. Other enzymes involved in the degradation and interconversion of nucleotides include ectonucleotide pyrophosphatases/phosphodiesterases, nucleoside diphosphokinase, adenylate kinase, ecto-5′-nucleotidase, and other enzymes (Zimmermann, 1996, 2000).

The physiological significance of the NTPDase family and other ectoenzymes is not fully understood. For example, no specific high-affinity inhibitors of molecularly defined ectonucleotidases are available, and very few studies have di-

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ABBREVIATIONS: NTPDase, ectonucleoside triphosphate diphosphohydrolase; 2MeSADP, 2-methylthio-ADP; ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); PCR, polymerase chain reaction; CHO, Chinese hamster ovary.

rectly considered the role of metabolizing enzymes in regulation of P2 receptor-mediated responses. Nonetheless, ubiquitous distribution and apparent colocalization of metabolizing enzymes with nucleotide receptors suggest a primary role of these enzymes in extracellular nucleotide signaling. Targeted disruption of the NTPDase1 gene resulted in severe alterations of hemostasis, underscoring an important role previously proposed for P2 receptor signaling in platelet aggregation (Enjyoji et al., 1999).

Marked transient elevation of extracellular nucleotides occurs as a consequence of regulated release from both excitatory and nonexcitatory cells (Dubyak and El-Moatassim, 1993; Lazarowski et al., 1995, 1997; Schlosser et al., 1996; Grygorczyk and Hanrahan, 1997). In addition, basal constitutive release of nucleotides occurs from most if not all cell types (Lazarowski et al., 2000; Ostrom et al., 2000). This constitutive nucleotide release is counterbalanced by ectonucleotidase-catalyzed degradation, and resting steady-state levels of extracellular nucleotides range from 5 to 50 nM depending on the nucleotide and cell type studied (Lazarowski and Harden, 1999; Lazarowski et al., 2000). Evidence has accrued for an important role of these resting levels of extracellular nucleotides in regulation of cellular function (Mitchell et al., 1998; Ostrom et al., 2000; Braunstein et al., 2001; Fleischhauer et al., 2001).

Most signaling pathways are probably efficiently organized as multiprotein complexes in specific membrane microdomains. NTPDase1 and possibly other ectoenzymes are associated with caveolae (Kittel et al., 1999; Koziak et al., 2000), which suggests a plausible means by which these metabolic enzymes might functionally associate with signaling proteins involved in cellular responses to extracellular nucleotides.

To begin to directly address the functional relationship between nucleotide-metabolizing enzymes and P2Y receptor-mediated signaling, we have fused the human $P2Y_1$ receptor in one-to-one stoichiometry with human NTPDase1. The stably expressed fusion protein recapitulates the surface membrane localization and functional characteristics of both the receptor and ectonucleotidase. Moreover, the receptor exhibits greater than 50-fold loss of sensitivity to exogenous ADP in assays of inositol phosphate accumulation, and in contradistinction to the wild-type $P2Y_1$ receptor, expression of the fusion protein does not result in elevation of basal levels of inositol phosphates. Given that the fusion protein retains full responsiveness to hydrolysis-resistant nucleotides, this engineered protein should prove valuable in biochemical and pharmacological investigation of the functional relationships between constitutive and regulated nucleotide release, ectonucleotidase activity, and P2Y receptor-mediated signaling responses. To our knowledge, the $P2Y_1$ receptor-NTPDase1 fusion protein represents the first example of a G protein-coupled receptor fused in a fully functional state with an enzyme that metabolizes its activating agonist.

Materials and Methods

Materials. Potato apyrase (EC 3.6.1.5) grade 1, ATP, ADP, and 2MeSADP were obtained from Sigma-Aldrich (St. Louis, MO); ADPβS was purchased from Calbiochem-Novabiochem (San Diego, CA); and hygromycin B was obtained from Roche Diagnostics (Indianapolis, IN). The pcDNA4/myc-His mammalian expression vector and zeocin were purchased from Invitrogen (Carlsbad, CA); anti–penta-his monoclonal antibody was obtained from QIAGEN (Valencia, CA); anti-mouse horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent substrate were obtained from Pierce Chemical (Rockford, IL); LipofectAMINE Plus Reagent was purchased from Invitrogen; myo-[^{3}H]inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO); γ-[^{32}P]ATP was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA); and Fluo-3-acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). All tissue culture reagents were obtained from the Lineberger Comprehensive Cancer Center tissue culture facility at the University of North Carolina.

Construction of $P2Y_1$ Receptor, Human NTPDase1, and $P2Y_1$-NTPDase1 Expression Plasmids. The human $P2Y_1$ receptor was prepared as described previously (Schachter et al., 1996). The construction of the $P2Y_1$ receptor with a hexahistidine tag in the N terminus was generated by PCR amplification using as forward primer 5′-GAGA**GAATTC**TGGCCATGGGGGGTTCTCATCATCATCATCATCATACCGAGGTGCTGTGGCCGGCT-3′ and reverse primer 5′- GAGA**GCGGCCGC**TCACAGGCTTGTATCTCCATTCTGCTTG-3′. The forward primer includes an *Eco*RI site, a start codon, and an in-frame hexahistidine sequence added upstream of the second codon of the $P2Y_1$ receptor gene (the enzyme restriction sites are in bold). The reverse primer added a *Not*I restriction site after the stop codon. The PCR amplification product was ligated into the retroviral vector pLXPIH previously digested with *Eco*RI and *Not*I. The plasmid encoding the NTPDase1 was obtained by PCR from the pLXPIH-CD39. The forward primer 5′-GAGA**GAATTC**CGAGGAAAGAGGAGGAAAACAAAAGCTGC-3′ was used to add an *Eco*RI site. The reverse primer 5′-GAGA**TCTAGA**ACCATATCTTTCCAGAAATATGAAGG-3′ was used to remove the stop codon, add a *Xba*I site, and maintain the reading frame with the myc-his6 tags of the pcDNA4 vector (Invitrogen). The plasmid encoding the $P2Y_1$-NTPDase1 was constructed by PCR amplification of the $P2Y_1$ receptor and NTPDase1 vectors described above. The $P2Y_1$ receptor gene was amplified with the forward primer 5′-GAGA**GAATTC**TGGCCATGGGGGGTTCTCATCATCATCATCATACCGAGGTGCTGTGGCCGGCT -3′ and reverse primer 5′-TCTC**GTCGAC**CAGGCTTGTATCTCCATTCTGCTTGA-3′. The forward primer added an *Eco*RI site upstream of the start codon, and the reverse primer omitted the stop codon and added a *Sal*I site. The NTPDase 1 was amplified from the pcDNA4-NTPDase1 with the forward primer 5′-GAGA**GTCGAC**ATGGAAGATACAAAGGAGTCTAACGTG-3′ and reverse primer 5′-TCTC**GGATCC**TCAATGGTGATGGTGATGATGACC-3′. The forward primer added a *Sal*I site upstream of the start codon, and the reverse primer added a *Bam*HI site after the stop codon of the pcDNA4 myc-his6. The PCR amplification products were digested with the corresponding restriction enzymes and cloned by a three-way ligation into the retroviral vector pLXPIH previously digested with *Eco*RI and *Bam*HI. This construct resulted in an in-frame sequence of the $P2Y_1$ receptor and NTPDase1 genes. The resulting plasmids were amplified, purified, and sequenced at the University of North Carolina-Chapel Hill automated DNA sequencing facility.

Generation of Stably Transfected Cell Lines. Purified plasmid DNA containing the $P2Y_1$ receptor, NTPDase1, or $P2Y_1$-NTPDase1 genes were stably transfected into CHO-K1 cells using LipofectAMINE 2000 (Invitrogen). Briefly, 8 μg of plasmid DNA were combined with 20 μl of LipofectAMINE 2000 in a final volume of 1 ml of OPTI-MEM reagent (Invitrogen). After incubation for 20 min at room temperature, the mixture was added to a 90% confluent monolayer of CHO-K1 cells in a 60-mm dish containing 5 ml of Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum. After incubation for 12 h at 37°C, the medium was replaced with 5 ml of fresh F12-medium with 10% fetal bovine serum. Cell populations stably expressing these genes were obtained by selection with 0.4 mg/ml hygromycin B (Roche Diagnostics) for $P2Y_1$ receptor and $P2Y_1$-NTPDase1 and 0.4 mg/ml of zeocin (Invitrogen) for NTPDase1.

Clonal cell lines were isolated, and P2Y$_1$ receptor and NTPDase1 expression were evaluated by Western blot analysis.

Ectonucleotidase Activities. CHO-K1 cells stably expressing vector control, NTPDase1, or P2Y$_1$-NTPDase1 fusion protein were seeded into 48-well plates at 4×10^4 cells per well and assayed after cells reached confluence. Briefly, the cells were washed once with 500 μl of phosphate-free saline solution consisting of 125 mM NaCl, 5.2 mM KCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5 mM D-glucose and incubated at 37°C in a 200-μl final volume of the same medium containing the indicated concentrations of nucleotide. Incubations were terminated by transferring 170 μl of the cell-free supernatants to a new plate containing 170 μl of 20 mM EDTA at 4°C. Ectonucleotidase activity was measured as the release of inorganic phosphate from the substrates ATP or ADP. Inorganic phosphate was determined colorimetrically using a modification of the malachite green-based assay (Lanzetta et al., 1979). Cell supernatants (30 μl) were combined with 100 μl of malachite green reagent, mixed, and the absorbance at 590 nm was determined in a plate reader. In some experiments, ATP hydrolysis was measured as the release of $^{32}P_i$ from γ-[^{32}P]ATP, using activated charcoal for separation.

P2Y$_1$ Receptor-Promoted Phospholipase C Activity. Agonist-stimulated inositol phosphate production was measured in CHO-K1 cells stably expressing the P2Y$_1$ receptor, the P2Y$_1$-NTPDase1 fusion protein, or in vector control cells. Cells were grown to confluence on 48-well plates. The inositol lipid pool was radiolabeled by incubation for 24 h before the assay in 200 μl of serum-free inositol-free Dulbecco's modified Eagle's medium supplemented with myo-[^{3}H]inositol (2 μCi/ml) at 37°C in a 5% CO_2 atmosphere. The cells were challenged with receptor agonists (ADP or ADPβS) in assay buffer (10 mM LiCl, 20 mM HEPES, pH 7.4, and Hank's buffered saline solution) for 15 min, and incubations were terminated by aspiration of the drug-containing medium and addition of 450 μl of 50 mM ice-cold formic acid. After 15 min at 4°C, samples were neutralized with 150 μl of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by anion exchange chromatography by transferring 600 μl of sample to Dowex AG 1-X8 (200–400 mesh) columns (Bio-Rad, Hercules, CA) containing 10 ml of H_2O. Columns were washed with 10 ml of 50 mM ammonium formate, and the eluate was discarded. [^{3}H]Inositol phosphates were eluted with 2 ml of 1.2 M ammonium formate and 100 mM formic acid and quantitated by liquid scintillation spectrometry.

Intracellular Calcium Mobilization. CHO-K1 cells stably expressing the P2Y$_1$ receptor and the P2Y$_1$-NTPDase1 fusion protein were seeded in 96-well black wall/clear-bottomed culture plates (Corning Glassworks, Corning, NY), and assays were conducted 2 days later with the cells at confluence. On the day of the assay, the growth medium was aspirated, replaced with medium containing 2.5 μM Fluo-3-acetoxymethyl ester, and incubated for 1 h at 37°C before replacing the dye with assay buffer (10 mM KCl, 118 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.4). Intracellular Ca^{2+} levels were monitored as changes in fluorescence intensity using a fluorometric imaging plate reader from Molecular Devices Corp. (Sunnyvale, CA).

Immunoblot Analysis. CHO-K1 cells (3×10^6) expressing the human NTPDase1, P2Y$_1$ receptor, or P2Y$_1$-NTPDase1 fusion protein were seeded and grown on 60-mm dishes. After 2 days in culture, the medium was aspirated, and reducing Laemmli's sample buffer was added to the cells. The samples then were subjected to SDS-polyacrylamide gel electrophoresis on an 8% polyacrylamide gel, and the resolved proteins were transferred to nitrocellulose membranes. Proteins were identified using a monoclonal mouse anti–penta-His IgG (Invitrogen) in 5% milk in Tris-buffered saline, followed by visualization by chemiluminescence after incubation with horseradish peroxidase-conjugated goat anti-mouse IgG using SuperSignal West Pico chemiluminescent substrate (Pierce Chemicals) according to the manufacturer's instructions.

Results

Expression of the P2Y$_1$ Receptor-NTPDase1 Fusion Protein. Wild-type CHO-K1 cells were infected with pLXPIH vector containing either the human P2Y$_1$ receptor gene or a recombinant gene combining the P2Y$_1$ receptor and NTPDase1 genes, which resulted in expression of the P2Y$_1$ receptor fused at its carboxy terminus with the amino terminus of NTPDase1 (Fig. 1). CHO-K1 cells stably expressing wild-type NTPDase1 were obtained after transfection of a pcDNA4 vector containing the NTPDase1 gene. A hexahistidine tag was included at the amino terminus of the P2Y$_1$ receptor, and Myc and hexahistidine tags were included at the carboxy terminus of NTPDase1 and the P2Y$_1$-NTPDase1 fusion protein. The presence of these tags had no effect on the expression, targeting to the plasma membrane, or biochemical or pharmacological properties of the P2Y$_1$ receptor or NTPDase1 (data not shown; see below). Western blots of CHO-K1 cells stably expressing these proteins revealed species migrating at approximately 40, 100, and 135 kDa (Fig. 2), corresponding to the expected sizes for the P2Y$_1$ receptor, NTPDase1, and the P2Y$_1$-NTPDase1 fusion protein, respectively. No immunoreactivity was observed in cells transfected with empty vector alone.

Ectonucleotidase Activity of the P2Y$_1$-NTPDase1 Fusion Protein. To assess whether ecto-ATPase and ecto-ADPase activities were retained in the P2Y$_1$-NTPDase1 fusion protein, membrane preparations from intact CHO-K1 cells expressing wild-type NTPDase1 or P2Y$_1$-NTPDase1 fusion protein were prepared and incubated with exogenous ADP or ATP as described under *Materials and Methods*. Relatively little hydrolytic activity against ADP (5 nmol P_i/min/mg) was observed under these assay conditions in membranes prepared from wild-type CHO-K1 cells. We anticipate that even this low activity comes primarily from intracellular nucleotidases in the membrane preparation. In contrast, hydrolytic activity against ADP in membranes from P2Y$_1$-NTPDase1 expressing cells was 381 ± 79 nmol of P_i/min/mg (mean ± S.D.; $n = 4$), whereas the wild-type NTPDase1-expressing cells exhibited an activity of 398 ± 24 nmol of P_i/min/mg ($n =$

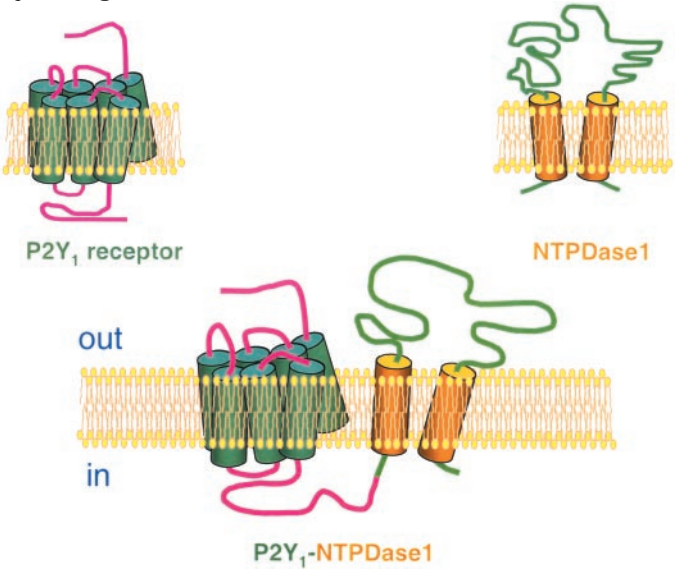

Fig. 1. Schematic representation of the P2Y$_1$ receptor, NTPDase1, and P2Y$_1$-NTPDase fusion protein.

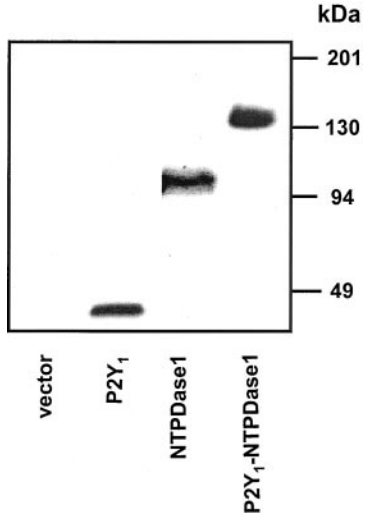

Fig. 2. Expression of hexahistidine-tagged $P2Y_1$ receptor, NTPDase1, and $P2Y_1$-NTPDase1 fusion protein. Immunoblots are presented of CHO-K1 cells stably transfected with the $P2Y_1$ receptor, NTPDase1, or $P2Y_1$-NTPDase1 fusion protein. The positions of molecular mass standards are indicated on the right. Comparable results were observed in three separate analyses.

4). Similarly, whereas little hydrolytic activity was observed under these assay conditions in membranes from wild-type cells, large hydrolytic activities against ATP were observed in membranes from $P2Y_1$-NTPDase1 fusion protein-expressing (951 ± 76 nmol/min/mg; $n = 4$) and wild-type NTPDase1-expressing (1015 ± 48 nmol/min/mg; $n = 4$) cells (Fig. 3). The substrate selectivity of the ectonucleotidase fused to the $P2Y_1$ receptor was essentially identical to that observed in the wild-type NTPDase1, with both proteins hydrolyzing nucleoside triphosphates and nucleoside diphosphates with a 1:0.4 molecular ratio (Table 1).

Similar results were obtained in measurements of ectonucleotidase activity in intact cells. That is, expression of the $P2Y_1$-NTPDase1 fusion protein markedly increased the capacity of CHO-K1 cells to hydrolyze extracellular ATP and ADP, and a slight preference for ATP over ADP was observed (Fig. 4). Therefore, the $P2Y_1$-NTPDase1 fusion protein is targeted to the plasma membrane, and ectonucleotidase activity and substrate selectivity are fully retained in the fusion protein.

Signaling Properties of the $P2Y_1$ Receptor Fused to NTPDase1. To determine whether $P2Y_1$ receptor signaling properties were retained in the $P2Y_1$-NTPDase1 fusion protein, we measured intracellular Ca^{2+} responses in intact CHO-K1 cells stably expressing the fusion protein. Although carbachol, acting through an endogenous muscarinic receptor, promoted a marked Ca^{2+} response in empty vector-transfected cells, no response to ADP or 2MeSADP was observed. In contrast, both diphosphate agonists produced robust responses in $P2Y_1$ receptor- and $P2Y_1$-NTPDase1 fusion protein-expressing cells (Fig. 5). Thus, the G protein-coupled receptor component of the fusion protein is appropriately inserted into the plasma membrane, and the observed responses to adenine diphosphates are representative of those observed for activation of the wild-type $P2Y_1$ receptor observed in many other systems. These results also are consistent with those obtained with this (Vöhringer et al., 2000) and other G protein-coupled receptors fused to different proteins, such as green fluorescent protein (Kallal and Benovic, 2000).

Relationship Between Ectonucleotidase Activity and $P2Y_1$ Receptor Activation in the $P2Y_1$-NTPDase1 Fusion Protein. To determine whether the catalytic presence of NTPDase1 in the fusion protein altered the capacity of agonists to activate the $P2Y_1$ receptor, we carried out a detailed comparison of the activities of ADP and the nonhydrolyzable ADP analog, ADPβS, using activation of phospholipase C as a proximal measure of receptor activity. As illustrated in Fig. 6, the capacity of ADPβS to maximally promote inositol phosphate accumulation was similar between cells expressing the wild-type $P2Y_1$ receptor and cells expressing the $P2Y_1$ receptor fused to NTPDase1. The EC_{50} of ADPβS for activation of the $P2Y_1$-NTPDase1 fusion protein was 150 ± 28 nM ($n = 4$ experiments), whereas the EC_{50} of ADPβS for stimulation of the wild-type $P2Y_1$ receptor was 530 ± 198 nM ($n = 4$ experiments). Thus, the $P2Y_1$ receptor fused to NTPDase1 was at least as effective as the wild-type receptor for activation of downstream signaling responses. In contrast to the results obtained with the nonhydrolyzable analog, the natural agonist ADP was 60-fold less potent in

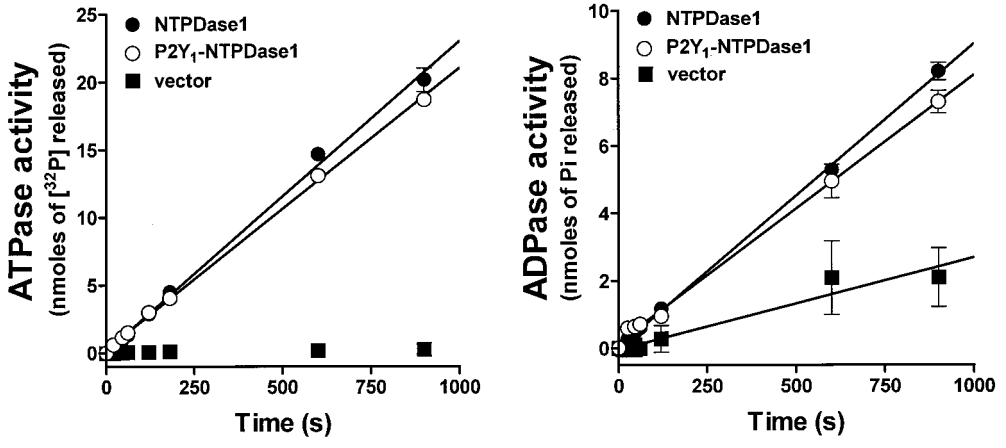

Fig. 3. Nucleotidase activities of NTPDase1 and the $P2Y_1$-NTPDase1 fusion protein in membrane preparations. Membrane preparations from CHO-K1 cells expressing wild-type NTPDase1, the $P2Y_1$-NTPDase1 fusion protein, or the empty vector were incubated in the presence of 2 mM ATP (left) or ADP (right), and nucleotide hydrolysis was quantitated as described under *Materials and Methods*. Values are the mean ± S.D. of three experiments, each carried out in duplicate assays.

TABLE 1

Nucleotidase activities in membranes from CHO-K1 cells expressing wild-type NTPDase1 or the P2Y$_1$-NTPDase1 fusion protein. ATPase and ADPase activities were measured as described under *Materials and Methods* in 10 min with 2 mM nucleotide substrate. Values are the mean ± S.D. of three experiments, each carried out in duplicate.

Substrate	Specific Activity	
	wt-NTPDase1	P2Y$_1$-NTPDase1
	nmol/min/mg	
ATP	1034 ± 13	921 ± 31
ADP	372 ± 1	348 ± 34
ATPase/ADPase ratio	1:0.36	1:0.38

wt, wild-type.

cells expressing the P2Y$_1$-NTPDase1 fusion protein (EC_{50} = 10 ± 4 μM; n = 4 experiments) than in cells expressing the wild-type P2Y$_1$ receptor (EC_{50} = 0.17 ± 0.06 μM; n = 4 experiments) (Fig. 6). Thus, nucleotide-hydrolytic activity contributed by the ADPase activity of NTPDase1 in the fusion protein has remarkable functional consequences on the stimulatory activity of the cognate agonist of the P2Y$_1$ receptor.

Loss of Basal Signaling Activity in the P2Y1-NTPDase1 Fusion Protein. Heterologous expression of the P2Y$_1$ receptor (Filtz et al., 1994; Schachter et al., 1996) or other P2Y receptors (Lazarowski et al., 1995; Boyer et al., 1997) typically results in an increase in basal inositol phosphate levels. Although such a result could follow from constitutive phospholipase C-stimulating activity of the expressed receptor, a more plausible explanation follows from the observation that basal or constitutive release of nucleotides occurs from many cell types (Lazarowski et al., 2000, 2001; Ostrom et al., 2000). A role for released nucleotides in the cellular response to expressed P2Y receptors is supported by the observations from several studies (Lazarowski et al., 1995, 1997; Boyer et al., 1997; Watt et al., 1998; Ostrom et al., 2000) that addition of apyrase to the medium reduces, but does not eliminate, the elevation of inositol phosphate levels after receptor expression. A major reason for the construction of a fusion protein of the P2Y$_1$ receptor and NTPDase1 was to address more directly the role of released nucleotide in the elevation of inositol phosphates that occurs during heterologous expression of the P2Y$_1$ receptor in the absence of added agonist. As illustrated above, the response to the nonhydrolyzable nucleotide, ADPβS, of the P2Y$_1$-NTPDase1 fusion protein was at least as great as that of the wild-type receptor, whereas the concentration-effect curve of ADP for stimulation of inositol phosphate accumulation was shifted to the right by greater than 50-fold. Thus, the P2Y$_1$ receptor fusion protein seems to be similarly active to the wild-type receptor, but the tethering of nucleotidase activity to the receptor makes it much less responsive to ADP added to the bulk medium. Given these results, we compared basal levels of inositol phosphates in cells expressing the P2Y$_1$-NTPDase1 fusion protein versus cells expressing the wild-type P2Y$_1$ receptor. Similar amounts of immunoreactive protein and similar maximal levels of 2MeSADP-stimulated inositol phosphate accumulation were observed with the wild-type receptor versus the P2Y$_1$ receptor/NTPDase1 fusion protein, suggesting that equivalent levels of active receptor were expressed under each condition. As illustrated in Fig. 7, the basal accumulation of [^{3}H]inositol phosphates in cells expressing the wild-type P2Y$_1$ receptor was markedly higher than accumulation in cells expressing the P2Y$_1$-NTPDase1 fusion protein. These elevated levels of inositol phosphates in

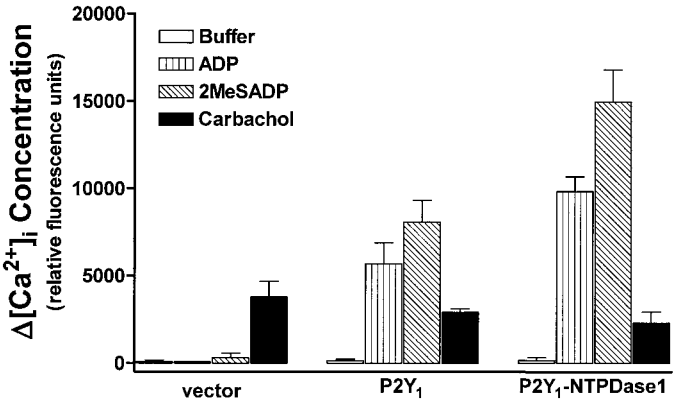

Fig. 5. Agonist-stimulated elevation of Ca^{2+} in CHO-K1 cells expressing the P2Y$_1$ receptor or P2Y$_1$-NTPDase1 fusion protein. Cells were incubated with either 100 nM 2MeSADP or 100 nM ADP, and intracellular Ca^{2+} levels were quantitated by a fluorometric imaging plate reader calcium assay system as described under *Materials and Methods*. Data are mean ± S.E.M. of duplicate determinations and are representative of at least three independent experiments.

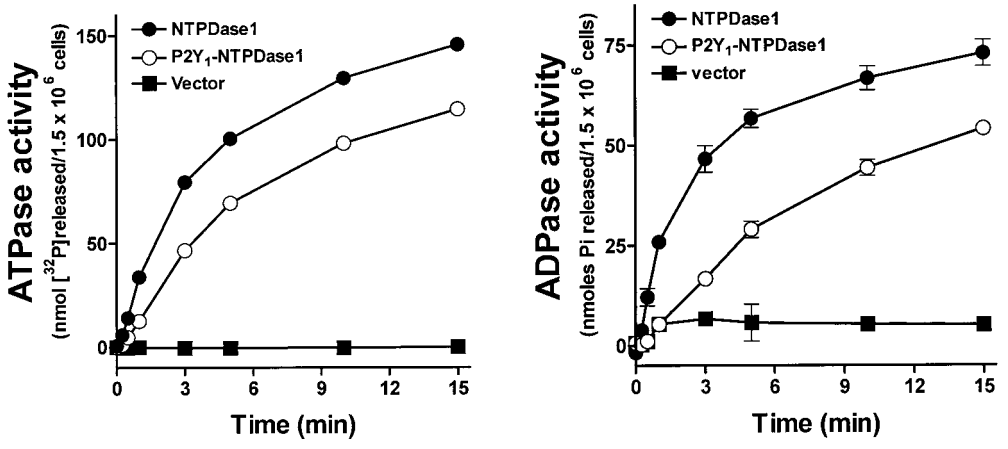

Fig. 4. Ectonucleotidase activities in CHO-K1 cells stably expressing NTPDase1 or P2Y$_1$-NTPDase1. The hydrolysis of extracellular ATP (left) or ADP (right) at 1 mM final concentration was measured as described under *Materials and Methods* in intact CHO-K1 cells stably expressing either NTPDase1, the P2Y$_1$-NTPDase1 fusion protein, or empty vector. Data shown are mean ± S.D. of triplicate assays from an experiment representative of three similar experiments.

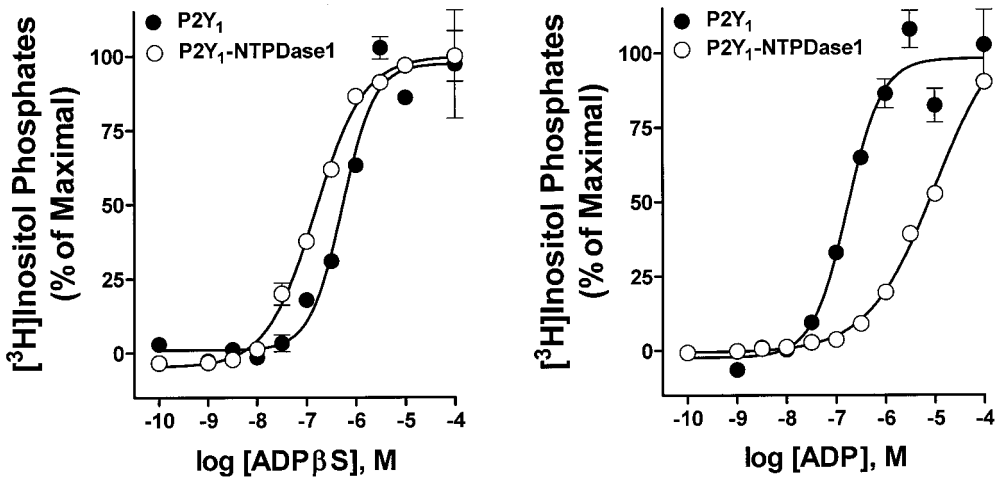

Fig. 6. ADP- and ADPβS-promoted inositol phosphate accumulation in CHO-K1 cells expressing the $P2Y_1$ receptor or $P2Y_1$-NTPDase1 fusion protein. Cells expressing either the $P2Y_1$ receptor or $P2Y_1$-NTPDase1 fusion protein were incubated with the indicated concentrations of ADPβS (left) or ADP (right) for 15 min at 37°C. The accumulation of total [^{3}H]inositol phosphates was determined as described under *Materials and Methods*, and values were normalized to the maximal response produced by ADP. Data are mean ± S.E.M. of triplicate determinations and are representative of at least three independent experiments.

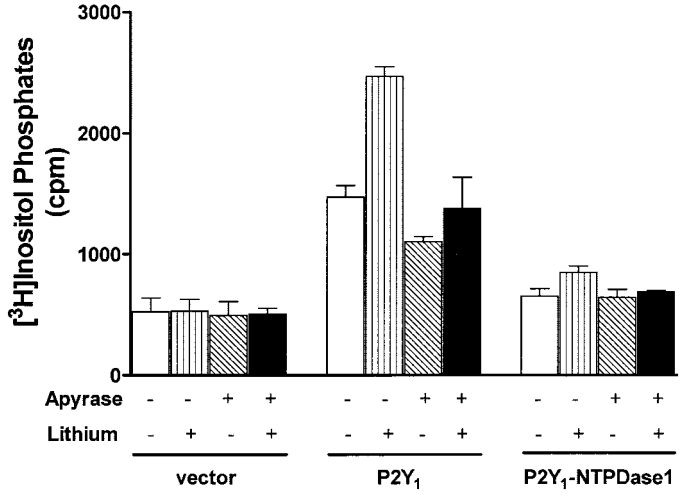

Fig. 7. Basal receptor activation by constitutively released extracellular nucleotides in CHO-K1 cells expressing the $P2Y_1$ receptor or the $P2Y_1$-NTPDase1 fusion protein. CHO-K1 cells were radiolabeled overnight with 0.4 μCi [^{3}H]inositol per well and subsequently treated with or without apyrase (1 U) for 15 min at 37°C in the presence or absence of 10 mM of LiCl. The accumulation of total [^{3}H]inositol phosphates was determined as described under *Materials and Methods*. Data are mean ± S.E.M. of triplicate determinations and are representative of at least three independent experiments.

cells expressing wild-type $P2Y_1$ receptor were even higher (by approximately 2-fold) if 10 mM LiCl was added to the medium for 20 min to inhibit inositol phosphate breakdown. Although addition of a maximally effective concentration of apyrase partially reduced the elevated levels of [^{3}H]inositol phosphates in $P2Y_1$ receptor-expressing cells, levels remained significantly elevated above that observed in wild-type CHO-KI cells. In contrast to the results obtained with expression of the wild-type $P2Y_1$ receptor, expression of the $P2Y_1$-NTPDase1 fusion protein did not result in elevated [^{3}H]inositol phosphate levels compared with wild-type CHO-KI cells, and addition of apyrase had no effect. Furthermore, addition of 10 mM LiCl to the medium did not result in a reproducible increase in [^{3}H]inositol phosphate levels in $P2Y_1$-NTPDase1–expressing cells (Fig. 7). Taken together, these observations strongly support the idea that basally released nucleotide accounts for the increase in basal activation of phospholipase C in P2Y receptor-expressing cells. Moreover, they indicate that no basal signaling responses occur with expression of the $P2Y_1$-NTPDase1 fusion protein.

Discussion

The $P2Y_1$-NTPDase1 fusion protein studied here retained the signaling properties of the native $P2Y_1$ receptor and the catalytic properties of the native ectoenzyme. Cellular expression of this $P2Y_1$ receptor in a form tethered with a nucleotide-hydrolyzing enzyme reduced the apparent potency of ADP for stimulation of phospholipase C by over 50-fold, without reducing the potency of a nonhydrolyzable agonist. Moreover, no basal signaling responses occurred with expression of the $P2Y_1$-NTPDase1 fusion protein. These results indicate that we have engineered a modified P2Y receptor that, when expressed in a cellular context, is activated less favorably by hydrolyzable nucleotides but retains full responsiveness to exogenous agonists that are resistant to the action of the nucleotidase.

The P2Y receptor family of signaling proteins is essentially ubiquitously expressed on nonexcitatory cells, such as fibroblasts and hepatocytes, as well as endothelial, epithelial, glial, and smooth muscle cells (Dubyak and El-Moatassim, 1993; Harden et al., 1995; Ralevic and Burnstock, 1998). The source of nucleotide regulating these receptors has not been unambiguously established. Although ATP is released in a Ca^{2+}-dependent manner from neurons and other excitatory cells, paracrine/autocrine stimulation of P2Y receptors by nucleotide released from nonexcitable cells probably provides the predominant form of regulation of these receptors. Many if not most cell types release nucleotides as a consequence of mechanical stimulation [e.g., shear stress, hypotonic swelling, stretch, or other physical stimuli (Dubyak and El-Moatassim, 1993; Lazarowski et al., 1995, 1997, 2001; Schlosser et al., 1996; Grygorczyk and Hanrahan, 1997; Lazarowski and Harden, 1999)].

Extracellular ATP and other nucleotides also are charac-

teristically present under resting cell conditions. Indeed, basal or "constitutive" release of ATP has been illustrated to occur with several cell types (Lazarowski et al., 2000, 2001; Ostrom et al., 2000), and extracellular levels of nucleotides at rest reflect a steady state in which the extent of basal release is balanced by the extent of nucleotide hydrolysis. Nucleotide release occurring as a consequence of mechanical stimulation (e.g., a change of medium) has been shown to contribute to the elevated inositol phosphate levels found associated with overexpression of several different P2Y receptor subtypes. Similarly, signaling activities of P2Y receptors observed under conditions that were carefully controlled to reduce any contribution of stimulated release of nucleotide may involve contribution of receptor activation occurring as a consequence of basally released nucleotide. For example, addition of apyrase or hexokinase to resting P2Y receptor-expressing 1321N1 cells resulted in a decrease in the basal level of inositol phosphates (Lazarowski et al., 1995, 1997; Boyer et al., 1997; Watt et al., 1998; Ostrom et al., 2000), suggesting that local nucleotide levels were sufficient to promote a basal activity of these receptors. Indeed, these and other studies suggest that quantification of the bulk concentration of nucleotides considerably underestimates the nucleotide mass that accumulates transiently at the level of the P2Y receptor on the cell surface. Dubyak and colleagues (Beigi et al., 1999) used cell surface-bound luciferase to illustrate that ATP concentrations in the bulk medium of thrombin-stimulated platelets underestimated concentration at the cell surface by at least 10-fold.

Results obtained with the $P2Y_1$ receptor-NTPDase1 fusion protein studied here illustrate that, by fixing a P2Y receptor in one-to-one apposition with its related agonist-metabolizing nucleotidase, basal activation of phospholipase C no longer occurs, although the receptor remains fully responsive to added nonhydrolyzable agonists. Thus, we conclude that the enhanced basal activity previously observed with overexpression of the $P2Y_1$ receptor followed from constitutive or mechanically induced release of nucleotide rather than through any constitutive activity of the receptor per se. Interestingly, we have not observed any elevation of inositol phosphate levels by expression of the $P2Y_1$ receptor/NTPDase1 fusion protein to high levels under a number of conditions. In contrast, expression of the wild-type $P2Y_1$ receptor resulted in elevation of inositol phosphates under all of the conditions we have studied. Moreover, although addition of apyrase resulted in a decrease in inositol phosphate levels in $P2Y_1$ receptor-expressing cells, conditions have not been identified under which addition of a maximally effective concentration of apyrase consistently reversed elevated levels completely to basal levels. These observations lead us to speculate that nucleotide is released in close apposition to the $P2Y_1$ receptor such that high concentrations of apyrase in the bulk medium fail to access the nucleotide, and therefore, fail to fully prevent receptor activation.

The large differences in ADP concentration-effect curves for the wild-type $P2Y_1$ receptor versus $P2Y_1$ receptor fusion protein were observed in 15-min assays of inositol phosphate accumulation. In contrast, in preliminary experiments, little difference in ADP concentration-effect curves was observed between the wild-type $P2Y_1$ receptor and $P2Y_1$ fusion protein in rapid (i.e., 1–2 s) measurements of ADP-promoted Ca^{2+} mobilization. The physiological significance of rapid Ca^{2+} measurements after addition of agonist to the bulk solution of a cultured cell is uncertain, because endogenous release of nucleotides occurs into a limited pericellular space in predictable close proximity to nucleotide-hydrolyzing enzymes, which may have dramatic effects on agonist concentration. Thus, our results are of clear relevance to physiological responses (i.e., cell growth and proliferation) that occur downstream to sustained release of nucleotides, and given the realities of a small pericellular space, also are probably relevant to acute responses to nucleotides in vivo.

The cellular architecture of the proteins responsible for signaling responses promoted by extracellular nucleotides has not been established. The P2Y receptors individually exhibit strict agonist selectivity among nucleotides, with some of these receptors activated by triphosphates and others by diphosphates, and with only the $P2Y_2$ receptor not exhibiting absolute specificity for either adenine or uridine nucleotides (Harden et al., 1998b). Thus, ectonucleotidases, nucleoside diphosphokinase, and potentially, other ectoenzymes carry out enzymatic reactions that inactivate the agonist for certain P2Y receptors while coincidentally producing the cognate agonist for another P2Y receptor. We envision that the cellular levels of individual P2Y receptors are tightly coordinated with the expression of the enzymes important for nucleotide metabolism and interconversion. Similarly, although the mechanism(s) of basal and regulated release of nucleotides from nonexcitable cells remains undefined, structures responsible for this process also may lie in close apposition to the receptors responding to the released nucleotides as well as to the important metabolic enzymes (Huang et al., 2001). NTPDdase1 is associated with caveolae (Kittel et al., 1999; Koziak et al., 2000), and thus caveolae, lipid rafts, or similar domain-defining macromolecular structures may play critical roles in organizing nucleotide-promoted signaling processes. The $P2Y_1$ receptor contains a strong PDZ domain-binding motif at its carboxy terminus, and multiprotein scaffolding of the receptor with other components of the nucleotide signaling apparatus can be envisioned.

An ectoenzyme on a target cell potentially could regulate an associated P2Y receptor through a receptor-mediated process. For example, ecto-ATPase converts ATP to ADP, the cognate agonist of the $P2Y_1$ receptor. Thus, constitutive release of ATP in the presence of ecto-ATPase may result in extracellular ADP concentrations that both activate the $P2Y_1$ receptor as well as cause a time-dependent down-regulation of the receptor. In turn, a relatively constant basal signaling response might occur, whereas the maximally attainable response to ADP is blunted by receptor desensitization. In a cell lacking ecto-ATPase, the $P2Y_1$ receptor would not promote a "basal" signal upon release of cellular ATP but would respond more robustly to increases in extracellular ADP. We speculate that a similar phenomenon accounts for the 3-fold decrease in EC_{50} observed for ADPβS at the $P2Y_1$-NTPDase fusion protein relative to the wild-type $P2Y_1$ receptor. That is, the presence of the ectoapyrase in one-to-one stoichiometry with the receptor in the fusion protein substantially reduces the basal concentration of ADP at the level of the receptor. Thus, whereas basal down-regulation of the wild-type $P2Y_1$ receptor occurs, this is not the case (or is less so) for the receptor in the fusion protein, which is "protected" from ADP by its metabolic enzyme. It will be important to

establish whether the opposite holds true for a $P2Y_1$ receptor fused with NTPDase2 (ecto-ATPase) rather than with NTPDase1. That is, we hypothesize that fusion with ecto-ATPase will result in substantial production of the cognate agonist (i.e., ADP) for the $P2Y_1$ receptor. Construction of such a $P2Y_1$ receptor-ATPase fusion protein is underway to test this idea.

In summary, we have introduced a fusion protein that places the $P2Y_1$ receptor in intimate association with an enzyme that hydrolyzes its activating agonist. This protein apparently retains the full function of both component proteins and was used to illustrate that the basal activity previously observed upon expression of the $P2Y_1$ receptor, and probably other P2Y receptors, occurred as a consequence of released cellular nucleotide. Thus, the $P2Y_1$ receptor-NTPDase1 should prove highly useful for studying the biology of the $P2Y_1$ receptor under conditions where its activated state can be precisely regulated by exogenous addition of nonhydrolyzable agonists.

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