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Regulation of P2Y$_1$ receptor-mediated signaling by the ecto-nucleoside triphosphate diphosphohydrolase isozymes NTPDase1 and NTPDase2

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Running title: NTPDase1 and NTPDase2 in $P2Y_1$ receptor-mediated signaling

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Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; 2MeSADP, 2'-methylthioadenosine 5'-diphosphate; ADPβS, adenosine 5'-O-(2-thiodiphosphate); UTP, uridine 5'-triphosphate; UDP, uridine 5'-diphosphate; UDP-glucose, uridine 5-diphosphoglucose; MRS2179, 2'-deoxy-N^6-methyl-adenosine 3',5'-diphosphate; Pi, inorganic phosphate; CD39, lymphoid cell activation antigen 39 (ecto-apyrase, NTPDase1); GPCR, G protein-coupled receptor; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Abstract

Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) control the concentration of released extracellular nucleotides, but the precise physiological roles played by these isozymes in modulation of P2 receptor signaling remain unclear. Activation of the human $P2Y_1$ receptor was studied in the presence of NTPDase1 or NTPDase2 expressed either in the same cell as the receptor or in $P2Y_1$ receptor-expressing cells cocultured with NTPDase-expressing cells. Coexpression of NTPDase1 with the $P2Y_1$ receptor resulted in increases in the EC_{50} for 2MeSADP (12-fold), ADP (50-fold), and ATP (10-fold) for activation of phospholipase C. Similar effects were observed when the $P2Y_1$ receptor and NTPDase1 were expressed on different cells. These results are explained by the capacity of NTPDase1 to hydrolyze both nucleoside triphosphates and diphosphates. NTPDase2 preferentially hydrolyzes nucleoside triphosphates, and the presence of NTPDase2 under either coexpression or coculture conditions did not change the EC_{50} of 2MeSADP, ADP, and ADPβS for activation of the $P2Y_1$ receptor. However, the EC_{50} for ATP was 15-fold lower in the presence of NTPDase2 than in cells expressing the $P2Y_1$ receptor alone. Whereas expression of NTPDase1 decreased basal activity of the $P2Y_1$ receptor, the presence of the NTPDase2 resulted in $P2Y_1$ receptor-dependent increases in basal activity. These results suggest that basal activity of the $P2Y_1$ receptor is maintained by paracrine or autocrine release of receptor agonists, and that the biological and/or pharmacological response mediated by P2Y receptors in target tissues is highly dependent on the types of ectonucleotidases expressed in the vicinity of the receptor.

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Introduction

The concept that adenine and uridine nucleotides function as extracellular signaling molecules has expanded markedly in the last decade. At least fifteen nucleotide-activated cell surface receptors exist in mammals, and remarkably broad physiological responses occur downstream of nucleotide receptor activation (Abbracchio and Burnstock, 1998; Ralevic and Burnstock, 1998). The significance of extracellular nucleotides also is underscored by ubiquitous distribution of several large classes of ectoenzymes that catalyze breakdown and interconversion of extracellular nucleotides (Zimmermann, 2000). Purinergic signaling was initially proposed on the basis of smooth muscle responses to autonomic nerve stimulation that were not blocked by adrenergic and cholinergic receptor antagonists (Burnstock, 1972). However, observation of responses to nucleotides in essentially all peripheral tissues, including those not significantly innervated by the autonomic nervous system, indicates that extracellular nucleotides arising from non-neuronal sources underlie many important physiological processes.

The actions of extracellular nucleotides are mediated by two distinct families of cell surface receptors. P2X receptors are ligand-gated ion channels that conduct extracellular cations in response to ATP. Seven receptors ($P2X_1$ through $P2X_7$) comprise this family found largely, but not exclusively, on excitatory tissues (Khakh, et al., 2000). P2Y receptors are a group of eight molecularly defined GPCR that exist both in the central and autonomic nervous systems as well as on most non-excitatory cells (Abbracchio, et al., 2003). Like P2X receptors, which are activated by ATP (but not ADP), the sparsely expressed $P2Y_{11}$ receptor is the only P2Y receptor that is activated

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selectively by ATP (Ralevic and Burnstock, 1998). The $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors are selectively activated by ADP, the $P2Y_2$ receptor is activated equipotently by UTP and ATP, the $P2Y_4$ receptor is selectively activated by UTP, the $P2Y_6$ receptor is selectively activated by UDP, and the $P2Y_{14}$ receptor is activated by nucleotide sugars, e.g. UDP-glucose and UDP-galactose (Ralevic and Burnstock, 1998; Abbracchio et al., 2003).

The concentration of extracellular nucleotides is regulated by a variety of surface-located enzymes known as ectonucleotidases (Zimmermann, 2000). In addition to their potential role in termination of purinergic signaling, the ectonucleotidases may prevent P2 receptor desensitization (Enjyoji, et al.,1999) and control the availability of ligands for either nucleotide or adenosine receptors (Bonan et al., 2001, Sévigny et al., 2002). The most prominent of these ectoenzymes are members of the ecto-nucleoside 5'-triphosphate diphosphohydrolase (NTPDase) family (Zimmermann, 2000). Although seven NTPDases (1,2,3,4,5,6 and 8) (Zimmermann, 2000; 2001; Bigonnesse et al., 2004) have been extensively studied at the biochemical and molecular level, their cellular actions remain to be defined. The most widely expressed NTPDases, NTPDase1 and NTPDase2 exhibit tissue distributions, e.g. in neural, vascular, and secretory tissues, that roughly correspond with reported distributions of several P2X and P2Y receptors (Vlajkovic et al., 2002a,b). The substrate selectivities of these isozymes suggest they may play markedly different roles in regulation of P2Y receptor-mediated signaling since NTPDase1 (also known as ecto-ATPDase, CD39, apyrase) hydrolyzes both nucleoside tri- and diphosphates to their corresponding monophosphates (Kaczmarek et al., 1996; Heine et al., 1999), whereas NTPDase2 (ecto-ATPase, CD39L1) selectively hydrolyzes

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nucleoside triphosphates to their corresponding diphosphates (Kegel, 1997; Kirley, 1997; Mateo, 1999).

To begin to address the physiological roles of NTPDase isozymes in P2Y receptor-mediated signaling, we engineered 1321N1 human astrocytoma cells to stably coexpress the $P2Y_1$ receptor with either NTPDase1 or NTPDase2. Given the selectivity of activation of the human $P2Y_1$ receptor by ADP, we addressed whether NTPDase2 versus NTPDase1 exhibit different functional relationships in $P2Y_1$ receptor-mediated signaling with the former producing the cognate $P2Y_1$ receptor agonist from ATP, and the latter ectoenzyme reducing the agonist action of both ATP and ADP at the $P2Y_1$ receptor. The functional consequence of proximity of NTPDases to the P2Y receptor also was examined in experiments in which the $P2Y_1$ receptor was coexpressed on the same cell as the ectoenzymes versus studies in which the $P2Y_1$ receptor and ectoenzymes were present on different cells. Finally, we addressed the underlying contributions of nucleotide release and ectoenzyme activity to basal $P2Y_1$ receptor signaling in cocultures of cells expressing the $P2Y_1$ receptor with cells expressing NTPDases.

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Materials and Methods

Materials

ADP, 2MeSADP, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). ATP was from Amersham Biosciences UK limited (Buckinghamshire, England) and ADPβS was from Calbiochem-Novabiochem (La Jolla, CA). The selective $P2Y_1$ receptor antagonist MRS2179 (tetra-ammonium salt) was obtained from Tocris (Ballwin, MO). Hygromycin B was from Roche Diagnostics Corporation (Indianapolis, IN). G-418 was obtained from Gibco (Grand Island, NY). Plasmid purification kits were purchased from QIAGEN (Valencia, CA). Myo-[^{3}H]inositol, 20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]MRS2279 (89 Ci /mmol) was synthesized as described by Waldo et. al., 2002. All tissue culture reagents were from the Lineberger Comprehensive Cancer Center tissue culture facility at the University of North Carolina. We are grateful to Dr. John Olsen (Department of Medicine, University of North Carolina) for his kind gifts of PA317 cells and pLXSN and pLXPIH vectors.

Cell culture

The murine packaging cell line PA317 and 1321N1 human astrocytoma cells were grown in Dulbecco's modified Eagle's medium high-glucose (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37^{o}C in a humidified atmosphere of 95% air/ 5% CO_2. 1321N1 cells stably expressing NTPDase1 or NTPDase2 and pLXPIH vector control cells were grown in DMEM supplemented with 5 % FBS and 600 μg/ml hygromycin B, while 1321N1 cells stably expressing the human $P2Y_1$ receptor

(Schachter et al., 1996) were grown in 600 µg/ml G418-containing medium. In some experiments cells were cocultured such that human $P2Y_1$ receptor expressing cells were grown in the presence of an equivalent number of cells expressing either NTPDase1 or NTPDase2.

Stable expression of the human NTPDase1, NTPDase 2, and $P2Y_1$ receptor in 1321N1 human astrocytoma cells.

Retrovirus harboring the human NTPDase1 or NTPDase2 coding sequences or the control retrovirus were produced as previously described (Comstock et al., 1997). Briefly, complementary DNAs encoding the human NTPDase1 or NTPDase2 were cloned into the retroviral expression vector pLXPIH (Mateo, 1999), then recombinant retroviral particles were produced by calcium phosphate-mediated transfection of PA317 murine packaging cells (Comstock et al., 1997). Transfected cells were incubated for 48 h at 32ºC in the presence of 5 µM sodium butyrate, and the cell supernatant containing packaged retroviruses was collected, filtered, and used to infect wild type 1321N1 human astrocytoma cells. Infection was carried out for 2 h in the presence of 8 µg/ml polybrene. After 48 h of culture, positive clones were selected in culture medium containing 600 µg/ml hygromycin B for NTPDase-expressing cells. To examine the functional consequences of proximity of NTPDases to the human $P2Y_1$ receptor, both proteins were expressed in the same cell by reinfection of clonal human $P2Y_1$ receptor expressing cells with either NTPDase1 or NTPDase2 recombinant retroviruses.

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Assay of ecto-nucleotidase activity

The pLXPIH vector control cells and 1321N1 cells expressing human $P2Y_1$ receptor or the NTDPases were seeded into 48-well plates at 4 x 10^4 cells per well and assayed after cells reached confluence. Briefly, the cells were washed twice with 500 µL 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^{o}C in a water bath. Fifty µL of ATP and ADP were added to each well (final volume, 200 µL) and cells were incubated for 5 min. The incubation was terminated by transferring 170 µL of the cell-free supernatants to a new plate containing 170 µL of 20 mM EDTA at 4^{o}C. Ectonucleotidase activity was measured as the release of inorganic phosphate from the nucleotides. Inorganic phosphate was determined colorimetrically using a modification of the malachite green-based assay (Lanzetta et al., 1979). Usually, 30 µL of cell supernatants were combined with 100 µL of malachite green reagent and mixed, and absorbance was determined at 590 nm in a plate reader.

$P2Y_1$ receptor-stimulated phospholipase C activity.

Agonist-induced inositol phosphate production was measured in 1321N1 cells grown to confluence on 48-well plates. Twelve hours before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 200 µL of serum-free, inositol-free Dulbecco's modified Eagle's medium, containing 0.4 µCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. On the day of the assay, cells were challenged with 50 µL of the five-fold concentrated solution of

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receptor agonists (ATP, ADP, 2MeSADP or ADPβS) in 200 mM Hepes, pH 7.3, containing 50 mM LiCl for 20 min at 37^{o}C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μL of ice-cold 50 mM formic acid. After 15 min at 4^{o}C, samples were neutralized with 150 μL of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by ion exchange chromatography on Dowex AG 1-X8 columns as previously described (Filtz, et al., 1994)

Radioligand Binding Assay

Control cells and 1321N1 cells expressing human $P2Y_1$ receptor alone or coexpressed with either NTPDase1 or NTPDase2 were seeded into 12-well plates at 4 x 10^5 cells per well and assayed after cells reached confluence. On the day of assay, the cells were incubated at 4°C with 9 nM [^{3}H]MRS2279 (approximately 30,000 cpm) in 20 mM Tris, pH 7.5 at 4°C, 145 mM NaCl, and 5 mM $MgCl_2$ in a volume of 400 µl. Specific binding was usually defined as total [^{3}H]MRS2279 binding minus binding occurring in the presence of a 10 µM concentration of the $P2Y_1$ receptor-specific antagonist MRS2179. Incubations were for 30 min in an ice-water bath. Binding reactions were terminated by aspiration of the radioligand-containing medium followed by a quick single wash of 1 ml of binding buffer and addition of 1 ml of 0.1 M NaOH. After neutralizing each sample with 0.1 M HCL, the radioactivity was quantified by liquid scintillation spectrometry.

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Data analysis

Agonist potencies from concentration-response curves were obtained by non-linear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, C.A.). All experiments were performed in triplicate assays and repeated at least three times.

Results

The human $P2Y_1$ receptor is selectively activated by ADP (Schachter et al., 1996; Leon et al., 1997; Palmer et al., 1998). For example, the purified human $P2Y_1$ receptor binds ADP with 20-fold higher affinity than ATP, and ATP is a relatively weak partial agonist compared to ADP at the purified receptor (Waldo and Harden, 2004). Thus, ATP is very unlikely to be a physiological agonist of the $P2Y_1$ receptor. However, given the large basal and mechanically induced release of ATP that occurs from most if not all cells, extracellular ATP potentially serves as the major source for $P2Y_1$ receptor activating ADP.

With the goal of studying the influence of ecto-NTPDases on P2Y receptor-mediated signaling we generated six different stable lines of 1321N1 human astrocytoma cells. A control 1321N1 cell line was isolated from cells infected with empty pLXPIH retrovirus. Stable cell lines expressing either the human $P2Y_1$ receptor, NTPDase1, or NTPDase2 alone were selected after infection of 1321N1 cells with retroviruses harboring the corresponding gene, and stable lines co-expressing the $P2Y_1$ receptor with either NTPDase1 or NTPDase2 also were selected.

Hydrolysis of ATP and ADP was quantified in the extracellular medium as a measure of the ectoenzyme activity present on the 1321N1 cell lines expressing NTPDase1, NTPDase2, NTPDase1-$P2Y_1$ receptor, or NTPDase2-$P2Y_1$ receptor. As illustrated in **Fig. 1**, the capacity of NTPDase1- and NTPDase2-expressing cells to hydrolyze adenine nucleotides was markedly increased relative to the very low hydrolysis observed under these conditions in vector-infected cells. The initial rate of ATP and ADP

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hydrolysis was quantified in all six cell lines (**Table 1**). Whereas hydrolysis was near background in vector and $P2Y_1$ receptor-expressing cells, robust and similar rates of hydrolysis of ATP were observed between NTPDase1- and $P2Y_1$ receptor-NTPDase1-expressing cells and between NTPDase2- and $P2Y_1$ receptor-NTPDase2-expressing cells. Thus, stable cell lines expressing NTPDases alone and NTPDases coexpressed with the $P2Y_1$ receptor were produced such that similar amounts of functional ectoenzyme activities were observed. As has been previously established for these molecularly defined enzymes, NTPDase1 hydrolyzed ATP and ADP similarly whereas NTPDase2 showed selectivity for ATP over ADP (**Table 1**).

P2Y receptors are not natively expressed by 1321N1 human astrocytoma cells, but stable expression of the human $P2Y_1$ receptor conferred [^{3}H]inositol phosphate responses to ADP and to adenine diphosphate analogues. Concentration effect curves to ADPβS were generated to determine whether the apparent potency of this nonhydrolyzable ADP analogue was altered when the $P2Y_1$ receptor was coexpressed (in the same cell) with NTPDase1 versus NTPDase2, or when $P2Y_1$ receptor-expressing cells were cocultured with 1321N1 cells stably expressing either NTPDase1 or NTPDase2. As illustrated in **Fig. 2**, the EC_{50} value for ADPβS was approximately 1 μM in cells expressing $P2Y_1$ receptor alone or in $P2Y_1$-expressing cells cocultured with NTPDase1 or NTPDase2. Interestingly, the EC_{50} of ADPβS was approximately 20-fold lower in cells coexpressing the $P2Y_1$ receptor with NTPDase1 but was only marginally lower in cells coexpressing the $P2Y_1$ receptor with NTPDase2, suggesting different functional interactions between the $P2Y_1$ receptor and NTPDase1 versus NTPDase2. Furthermore, the molecular proximity between the receptor and the ecto-enzyme apparently is critical

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since, in contrast to the result obtained under coexpression conditions, the EC_{50} of ADPβS did not change when $P2Y_1$ receptor-expressing cells were cocultured with NTPDase1-expressing cells.

NTPDase1 catalyzes conversion of both tri- and diphosphates to their corresponding nucleoside monophosphate. Therefore, functional apposition of this ecotenzyme with the $P2Y_1$ receptor might be expected to reduce the capacity of both nucleoside triphosphates and nucleoside diphosphates to activate their cognate P2Y receptors. This was observed to be the case for both ADP (**Fig. 3**) and ATP (**Fig. 4**) for activation of the $P2Y_1$ receptor. Importantly, this inhibitory action of NTPDase1 was observed irrespective of whether the ectoenzyme was coexpressed on the same cell as the $P2Y_1$ receptor or was expressed at similar levels on an independent cell that was co-cultured at 1:1 ratio with the $P2Y_1$ receptor-expressing cells. Thus, the capacity of adenine nucleotides to promote $P2Y_1$ receptor-mediated signaling in response to adenine nucleotides added to the bulk medium is similarly attenuated by NTPDase1 expressed in close apposition with the $P2Y_1$ receptor or expressed at a distance in another cell.

NTPDase2 selectively hydrolyzes nucleoside triphosphates over nucleoside diphosphates (**Table 1**). Since the ADP-activated $P2Y_1$ receptor is only weakly activated by ATP, we hypothesized that ATP would be converted to ADP in the presence of NTPDase2, and therefore, the apparent agonist action of ATP might be augmented at $P2Y_1$ receptors expressed in the presence of NTPDase2. Conversely, since NTPDase2 exhibits high selectivity for hydrolysis of nucleoside triphosphates over nucleoside diphosphates, we hypothesized that the effect of NTPDase2 would be negligible on the capacity of ADP to promote $P2Y_1$ receptor-mediated signaling. These ideas were

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supported by the results presented in **Fig. 3** and **Fig. 4**. Thus, neither coexpression of NTPDase2 with the $P2Y_1$ receptor nor coculture of NTPDase2-expressing cells with $P2Y_1$ receptor-expressing cells altered the concentration effect curve of ADP for stimulation of [^{3}H]inositol phosphate accumulation by the $P2Y_1$ receptor. In contrast, the concentration effect curve of ATP for stimulation of [^{3}H]inositol phosphate accumulation was shifted to the left by approximately 15-fold by the presence of NTPDase2. This effect was similarly observed irrespective of whether the ectoenzyme was expressed on the same cell as the $P2Y_1$ receptor or was expressed on a neighboring cell.

2MeSADP is a hydrolyzable ADP analogue that, similar to ADP, is metabolized by NTPDase1 and to a much less extent by NTPDase2 (data not shown). The EC_{50} value of 2MeSADP for stimulation of [^{3}H]inositol phosphate accumulation by the $P2Y_1$ receptor was approximately 10-fold higher in cells coexpressing the $P2Y_1$ receptor with NTPDase1 or in cocultures of NTPDase1-expressing cells with $P2Y_1$ receptor-expressing cells. As for ADP, neither coexpression of NTPDase2 with the $P2Y_1$ receptor nor coculture of NTPDase2-expressing cells with $P2Y_1$ receptor-expressing cells, caused a significant change in the concentration-effect curve of 2MeSADP for $P2Y_1$ receptor activation (**Table 2**). 2MeSADP is a more potent agonist for the human $P2Y_1$ receptor than is ADP (Schachter et al., 1996; Waldo and Harden, 2004). Thus, the extent to which the apparent potency of 2MeSADP and ADP for $P2Y_1$ receptor activation was diminished in the presence of NTPDase1 could be due to a combination between: 1) differences in the Kd values of these agonists for the $P2Y_1$ receptor and 2) differences in the Km values of NTPDase1 for these substrates. NTPDase1 likely hydrolyzes 2MeSADP and ADP with similar Km values. Therefore, we speculate that the smaller shift to the right of the

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concentration-effect curve for 2MeSADP compared to ADP for $P2Y_1$ receptor activation occurs because 2MeSADP activates the $P2Y_1$ receptor at lower concentrations that are less affected by NTPDase1 activity than the higher concentrations of ADP necessary to activate the receptor.

Heterologous expression of P2Y receptors has been widely reported to result in elevation of "basal" [^{3}H]inositol phosphate accumulation (Filtz et al., 1994; Parr et al., 1994; Lazarowski et al., 1995; Alvarado-Castillo et al., 2002). Whereas constitutive activity has been shown previously to account for elevated responses of some overexpressed recombinant GPCR (Seifert and Wenzel-Seifert, 2002), the most parsimonious interpretation of data for P2Y receptors to date is that endogenous nucleotides either constitutively released or released after mechanical stimulation of cells accounts for most if not all of these "basal" activities of expressed P2Y receptors. This conclusion largely was based on observation of release of endogenous nucleotide into the extracellular medium at levels capable of P2Y receptor activation (Lazarowski et al., 1995, 2000, Ostrom et al., 2000; Joseph et al., 2003; Lazarowski et al., 2003) as well as on observation of lowered [^{3}H]inositol phosphate accumulation in P2Y receptor-expressing cells after addition of soluble apyrase to the medium (Parr et al., 1994; Ostrom et al., 2000). The availability of cell lines engineered to coexpress the $P2Y_1$ receptor with either NTPDase1 or NTPDase2 as well as the availability of means to generate cocultures in which $P2Y_1$ receptor-expressing cells are in the presence of NTPDase1- or NTPDase2-expressing cells, provide two different cell systems to more rigorously address the influence of ectoenzymes on the receptor-stimulating activities of cellular nucleotides released into the extracellular medium.

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Basal [^{3}H]inositol phosphate accumulation was measured in cells expressing the $P2Y_1$ receptor alone or in coculture with cells expressing either NTPDase1 or NTPDase2. As illustrated in **Fig. 5**, expression of the $P2Y_1$ receptor alone resulted in a marked increase in [^{3}H]inositol phosphate accumulation. This increase largely was inhibited by the presence of the $P2Y_1$ receptor antagonist MRS2179 during the incubation suggesting that basally released extracellular nucleotide activates the receptor in a manner prevented by the addition of exogenous receptor antagonist. This contention is supported by the observation that coculture of NTPDase1-expressing cells with $P2Y_1$ receptor-expressing cells almost completely inhibited $P2Y_1$ receptor-dependent increases in [^{3}H]inositol phosphate accumulation. In contrast, coculture of NTPDase2-expressing cells with $P2Y_1$ receptor-expressing cells resulted in a marked increase in [^{3}H]inositol phosphate accumulation by a mechanism that also was inhibited by the presence of the $P2Y_1$ receptor antagonist MRS2179. The most parsimonious interpretation of these results is that cellular ATP is released into the extracellular medium, and the presence of NTPDase2 results in the formation of ADP; ADP in turn activates the $P2Y_1$ receptor more effectively than does ATP. Again, the inhibitory effect of MRS2179 supports the idea of autocrine regulation of the overexpressed receptor.

Basally released nucleotides also potentially induce down-regulation of the $P2Y_1$ receptor, and therefore, the presence of ectoenzymes that metabolize nucleotides might be expected to differentially regulate receptor levels. Indeed, quantification of $P2Y_1$ receptors using [^{3}H]MRS2279 revealed approximately five-fold higher receptor levels in cells coexpressing NTPDase1 compared to cells expressing $P2Y_1$ receptor alone

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(Table 3). In contrast, $P2Y_1$ receptor levels in cells coexpressing NTPDase2 were similar to levels in cells expressing $P2Y_1$ receptor alone.

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Discussion

The results reported here illustrate marked NTPDase-dependent alteration of P2Y receptor-promoted signaling. The molecular form of the NTPDase, and therefore, the relative selectivity for metabolism of extracellular ATP versus ADP, determines whether the effect of ectoenzyme is to enhance or to decrease the apparent potency of the extracellular nucleotide. Autocrine and paracrine regulation may account for much of the physiological responses mediated through the P2Y receptor family of signaling proteins, and the data illustrated here show a marked influence of NTPDases on the activation state of $P2Y_1$ receptors under both basal and agonist-stimulated conditions.

P2Y receptors are not natively expressed in 1321N1 human astrocytoma cells, and therefore, this cell line has been widely applied for heterologous expression of molecularly identified P2Y receptors. NTPDase activity also is relatively low in 1321N1 cells (Lazarowski et al, 2000), and as is illustrated here, approximately 50-fold increases in the hydrolysis rates of extracellular nucleotides are conferred in 1321N1 cells stably expressing either NTPDase1 or NTPDase2. The reported high selectivity of NTPDase2 for ATP over ADP and lack of selectivity of NTPDase1 for ATP versus ADP also was recapitulated in the cells overexpressing NTPDases. Thus, these engineered 1321N1 cell lines provide a useful system for examining the pharmacological interactions of NTPDase-catalyzed nucleotide metabolism and $P2Y_1$ receptor signaling. Moreover, the system has allowed us to address for the first time the influences rendered by NTPDases when present on the same cell as the receptor versus when NTPDases are present on a neighboring cell.

P2Y1 receptor-promoted activation of phospholipase C was quantified during 20 min incubations with nucleotide added to the bulk solution. Therefore, the observed EC50 values should be considered "averages" of the relative concentration dependence of agonists over time and under conditions in which NTPDase-catalyzed breakdown of nucleotide occurred at an indeterminate rate at the cell surface. Quantification of rapid Ca^{2+} responses to nucleotides potentially would provide acute measure of relative EC50 values, but the physiological significance of such measurements after addition of nucleotides to the bulk solution is unclear. Indeed, the results as presented here with bulk addition of agonists in assays quantified over longer periods of time more likely approximate physiological responses that may occur with sustained release of nucleotides. Given the architectural boundaries of small pericellular spaces relative to cell surface receptors and NTPDases, measurement of acute Ca^{2+} responses might provide relevant insight when the influence of NTPDases is studied under conditions in which the release of small boluses of cellular nucleotide are effected by, for example, mechanical stimulation of cells expressing both P2Y1 receptors and NTPDases.

The presence of overexpressed NTPDase2 did not modify the signaling response of the $P2Y_1$ receptor to the nonhydrolyzable agonist ADPβS. Whereas coculture of cells overexpressing NTPDase1 with 1321N1 cells overexpressing the $P2Y_1$ receptor also did not modify the concentration dependence of ADPβS for activation of phospholipase C, a reproducible 20-fold increase in the potency of ADPβS was observed in cells coexpressing the $P2Y_1$ receptor with NTPDase1. The molecular significance of NTPDase1-promoted increase in effectiveness of ADPβS is not known. However, we previously illustrated that basal release of ATP occurs in resting 1321N1 cells

(Lazarowski et al, 2000), and the elegant studies of Joseph and colleagues (Joseph et al., 2003) using cell surface-attached luciferase indicate that the concentration of ATP quantified in the medium of 1321N1 cells underestimates that at the cell surface by many fold. Since extracellular hydrolysis of ADPβS did not occur under the conditions of these experiments, one interpretation of the current data is that the presence of NTPDase1 on the same cell as the $P2Y_1$ receptor serves to metabolize released ATP (and ADP) preventing basal down regulation of the $P2Y_1$ receptor and/or its downstream signaling cohorts. Indeed, results from preliminary studies quantifying cell surface $P2Y_1$ receptor expression on intact cells were consistent with the idea that the presence of ectoenzymes modifies $P2Y_1$ receptor levels. Thus, $P2Y_1$ receptor density was near 5-fold higher in cells that coexpress the $P2Y_1$ receptor with NTPDase1 than in cells that express either the $P2Y_1$ receptor alone or with NTPDase2.

NTPDase1 exhibits similar catalytic activities against nucleoside triphosphates and diphosphates as substrates for conversion to AMP. Therefore, cellular expression of this ectoenzyme can be expected to terminate the action of the cognate agonist for nucleoside triphosphate-activated P2Y receptors, e.g. the $P2Y_2$, $P2Y_4$, and $P2Y_{11}$ receptors, as well as nucleoside diphosphate-activated P2Y receptors, e.g. the $P2Y_1$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{13}$ receptors. This was clearly observed for the $P2Y_1$ receptor, with 10 to 30-fold shifts to the right for activation curves for both ATP and ADP. The inhibitory action of NTPDase1 was similar irrespective of whether the ectoenzyme was coexpressed on the same cell as the $P2Y_1$ receptor or was expressed on a different cell in cocultures with $P2Y_1$ receptor-expressing cells. Similar results were observed previously with a fusion protein of the human $P2Y_1$ receptor and NTPDase1 (Alvarado-Castillo, et al

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2002). Addition of ATP or ADP to the bulk medium in these experiments may approximate the physiological situation where activating nucleotide is released from a distant cell.

In contrast to the action of NTPDase1, NTPDase2 exhibits high selectivity for nucleoside triphosphates over nucleoside diphosphates. Therefore, whereas NTPDase2 would be expected to have no effect on ADP levels in the medium, this ectoenzyme readily converts ATP to ADP. Indeed, NTPDase2 potentially could provide a major source for extracellular ADP (or UDP) since the release of cellular ADP (or UDP) has not been broadly described. Accordingly, whereas the concentration effect curve of ADP for activation of the $P2Y_1$ receptor was unchanged by the presence of NTPDase2, the concentration effect curve of ATP was remarkably shifted to the left in the presence of this ecotenzyme. This follows from the fact that ATP is a very weak agonist at the $P2Y_1$ receptor (Waldo and Harden, 2004), and NTPDase2-catalyzed conversion of ATP to ADP would produce the active cognate agonist of the receptor. Again, the fact that the influence of NTPDase2 on the response to added ATP was essentially the same irrespective of whether NTPDase2 was on the same cell as the $P2Y_1$ receptor or on a neighboring cell co-cultured with $P2Y_1$ receptor-expressing cells suggests that the concentrations of nucleotide in the bulk phase may largely approximate that at the level of the receptor when the $P2Y_1$ receptor agonist arises from a distal source.

Mechanically-induced release of ATP occurs by many if not most non-excitatory cells (Lazarowski et al., 2003), and constitutive release of nucleotide under resting conditions is increasingly appreciated to occur in many cell types (Lazarowski et al., 2000; Ostrom et al., 2000; Joseph et al, 2003). The experiments carried out here

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measuring $P2Y_1$ receptor-mediated signaling in 1321N1 cells maintained under resting conditions may approximate that occurring physiologically during autocrine/paracrine regulation of cell signaling. Thus, as we and others have observed with heterologous expression of most members of the P2Y receptor family (Lazarowski et al, 2003), expression of the human $P2Y_1$ receptor enhanced inositol lipid signaling. That this effect occurs as a consequence of constitutive nucleotide release was supported by the opposing action of NTPDase2 versus NTPDase1 on basal signaling. The substrate selectivity of NTPDase2 would be expected to result in conversion of released ATP into the activating agonist ADP, and as was illustrated in Fig. 5, basal $P2Y_1$ receptor signaling was doubled by NTPDase2 expression. Conversely, NTPDase1 metabolizes nucleoside tri-and diphosphates to the corresponding monophosphate, and NTPDase expression almost completely inhibited basal $P2Y_1$ receptor-dependent inositol lipid signaling. That these opposing NTPDase-promoted changes follow from metabolism of ATP also is supported by the observation that the $P2Y_1$ receptor selective antagonist MRS2179 inhibited by up to 80% both basal and NTPDase2-promoted elevation of [^{3}H]inositol phosphates.

The tissue distribution of NTPDase1 and NTPDase2 markedly differ in mammals. For example, NTPDase1 localized with microglia in brain, with the vasculature of brain and other tissues, and with activated lymphocytes (Enjyoji et al., 1999; Braun et al., 2000; Zimmermann, 2000), while NTPDase2 was expressed by astrocyte-like cells in the germinal zones of the adult rat brain (Braun et al., 2003), found in portal fibroblasts within the liver (Dranoff et al., 2002), associated with microvascular pericytes of the cardiac vasculature (Sévigny et al., 2002), and found in various compartments of the cochlea (Vlajkovic et al., 2002a,b). Recent studies also illustrated that whereas

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NTPDase1 was restricted to blood vessel walls, NTPDase2 was selectively associated with nonmyelinating glia and fibroblasts of the peripheral nervous system, where presumably NTPDase2 plays an important role in the control of nucleotide-mediated activation of peripheral neurons or glia and in the dialogue between these two cell types (Braun et al., 2004). Interestingly, a subpopulation of myenteric neurons in the murine colon expresses the ADP-activated $P2Y_1$ receptor (Giaroni et al., 2002), further implicating a role of NTPDase2 in ligand production. Functional and pharmacological studies indicate that P2Y receptors are widely and differentially distributed in mammalian tissues (Ralevic and Burnstock, 1998). However, quantification of the precise localization of these receptors has largely not been possible due to lack of reliable receptor-selective antibodies or radioligands. Thus, although it is clear that the $P2Y_1$ receptor is found in tissues that also contain NTPDase1 and/or NTPDase2, colocalization of the receptor with these or other nucleotide-metabolizing enzymes has yet to be established unambiguously.

The work described here provides a first step in understanding the relationship of NTPDase1 and NTPDase2 expression to regulation of $P2Y_1$ receptor-mediated signaling. It will be important to carry these studies to native tissues with the advent of improved molecular reagents for quantifying both the enzymes and P2Y receptors.

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Footnotes

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Figure Legends

Figure 1. Ecto-nucleotidase activities of NTPDase1- and NTPDase2-expressing 1321N1 cells. NTPDase1-expressing cells (left panel) or NTPDase2-expressing cells (right panel) were prepared as described in Methods. Cells were incubated with increasing concentrations of ATP, ADP or AMP for 3 min at 37^{o}C and nucleotide hydrolysis was measured as the release of inorganic phosphate. Data shown are mean ± S.E.M. of triplicate assays from a representative experiment repeated at least three times.

Figure 2. Effect of ecto-enzymes on the pharmacological activity of a non-hydrolyzable nucleotide analogue. P2Y$_1$ receptor- promoted [^{3}H]inositol phosphate accumulation was quantified after incubation of cells with the indicated concentrations of ADPβS for 20 min at 37^{o}C. Activity of the P2Y$_1$ receptor was studied in the presence of NTPDase1 or NTPDase2 coexpressed in the same cell (right panel) or after coculture of P2Y$_1$ receptor-expressing cells with either NTPDase1- or NTPDase2-expressing cells (left panel). The accumulation of [^{3}H]inositol phosphates was normalized to the maximal response produced by ADPβS. Data are mean ± S.E.M. of triplicate determinations and results shown are representative of at least three independent experiments.

Figure 3. Expression of NTPDase1 but not NTPDase2 markedly decreases the apparent potency of ADP. P2Y$_1$ receptor-promoted [^{3}H]inositol phosphate accumulation was studied in the presence of NTPPase1 or NTPDase2 either coexpressed with the receptor (right panel) or in P2Y$_1$ receptor-expressing cells cocultured with NTPDase1 or NTPDase2-expressing cells (left panel). The accumulation of [^{3}H]inositol

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phosphates was normalized to the maximal response produced by ADP. Data are mean ± S.E.M. of triplicate determinations, and results shown are representative of at least three independent experiments.

Figure 4. Effect of expression of NTPDase isozymes on P2Y$_1$ receptor-mediated responses to ATP. P2Y$_1$ receptor-promoted [^{3}H]inositol phosphate accumulation was studied in the presence of NTPDase1 or NTPDase2 either coexpressed with the receptor (right panel) or in P2Y$_1$ receptor-expressing cells cocultured with NTPDase1 or NTPDase2-expressing cells (left panel). The accumulation of [^{3}H]inositol phosphate was normalized to the maximal response produced by ATP. Data shown are the mean ± S.E.M. of triplicate determinations, and are representative of at least three independent experiments.

Figure 5. Differential influence of NTPDase1 and NTPDase2 on basal P2Y$_1$ receptor-mediated signaling. Wild type cells infected with empty-vector (vector), P2Y$_1$ receptor-expressing cells (P2Y$_1$), NTPDase1-expressing (NTPDase1) or NTPDase2-expressing (NTPDase2) cells were cocultured as indicated at a 1:1 cell ratio in 48-well plates as described in Methods. Twenty-four hours after seeding, cells were labeled with [^{3}H]inositol in inositol-free medium in the presence of 100 μM MRS2179 and 1mM LiCl. After 48 hours, the media was removed, and the basal levels of [^{3}H]inositol phosphates were measured as described in Methods. Data are mean ± S.E.M. of triplicate determinations and are representative of at least two independent experiments.

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Figure 6. Formation of the cognate agonist of the $P2Y_1$ receptor by NTPDase2 and inactivation of $P2Y_1$ receptor-activating nucleotides by NTPDase1. The scehmatic illustrates: (top panel) the release of cellular ATP and its conversion by NTPDase2 into the cognate agonist ADP for the $P2Y_1$ receptor and (bottom panel) the release of cellular ATP and the conversion of ATP (and ADP) to AMP, which is not an agonist of the $P2Y_1$ receptor.

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Table 1. Enzymatic activity of NTPDase-expressing cells. Hydrolysis of ATP and ADP was quantified in intact cells for 3 min with 500 μM nucleotide substrate as described in Methods. Values are the mean ± SD of three experiments, each performed in triplicate. Vector, cells infected with empty vector; P2Y$_1$, cells expressing P2Y$_1$ receptor alone; NTPDase1, cells expressing NTPDase1; NTPDase2, cells expressing NTPDase2; P2Y$_1$-NTPDase1, cells coexpressing the P2Y$_1$ receptor and NTPDase1; P2Y$_1$-NTPDase2, cells coexpressing the P2Y$_1$ receptor and NTPDase2.

	Rate of hydrolysis nmols of Pi x min^{-1}		
Cell line	ATP	ADP	ATP/ADP ratio
Vector	0.4 ± 0.2	0.5 ± 0.1	-
P2Y$_1$	0.5 ± 0.1	0.5 ± 0.2	-
NTPDase1	19.7 ± 0.4	8.9 ± 1.6	**1:0.45**
NTPDase2	15.1 ± 2.7	2.3 ± 0.4	**1:0.15**
P2Y$_1$-NTPDase1	12.7 ± 1.4	6.1 ± 0.9	**1:0.48**
P2Y$_1$-NTPDase2	12.5 ± 0.6	2.2 ± 0.0	**1:0.17**

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Table 2. Effect of NTPDase1 and NTPDase2 on the observed pharmacological selectivity of the $P2Y_1$ receptor. The human $P2Y_1$ receptor was coexpressed with either NTPDase1 or NTPDase2 in the same cell or $P2Y_1$ receptor-expressing cells were cocultured with cells expressing either NTPDase1 or NTPDase2. Agonist-stimulated [^{3}H]inositol phosphate accumulation was measured as described in Methods. The changes in apparent potency of agonists are expressed as fold-increases (+) or decreases (-) in the EC_{50} values compared with cells expressing the $P2Y_1$ receptor alone.

Agonist	$P2Y_1$ receptor EC_{50} (μM)	$P2Y_1$ receptor			
		NTPDase1		NTPDase2	
		Cocultured	Coexpressed	Cocultured	Coexpressed
2MeSADP	0.1 ± 0.0	12.3 (+)	11.0 (+)	1.7 (+)	1.4 (+)
ADP	1.0 ± 0.2	46.6 (+)	53.3 (+)	1.1 (+)	1.1 (+)
ATP	4.3 ± 0.6	7.4 (+)	11.3 (+)	16.4 (-)	18.6 (-)
ADPβS	1.0 ± 0.4	1.2 (+)	20.0 (-)	1.2 (-)	1.5 (-)

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Table 3. Effect of expression of NTPDase1 on cell surface expression of $P2Y_1$ receptors**.** $P2Y_1$ receptor levels were determined in 1321N1 cells expressing the human $P2Y_1$ receptor alone and in cells in which the $P2Y_1$ receptor was coexpressed with either NTPDase1 or NTPDase2 in the same cell. [^{3}H]MRS2279 binding assays were carried out on the surface of intact cells as described in Methods. The data are mean ± S.E.M. of triplicate determinations and results shown are representative of at least three independent experiments.

Cell line	**Cell surface $P2Y_1$ receptor expression** fmol/mg protein
Vector	3 ± 4
$P2Y_1$	125 ± 10
$P2Y_1$ + NTPDase1	568 ± 52
$P2Y_1$ + NTPDase2	102 ± 37

Fig 1

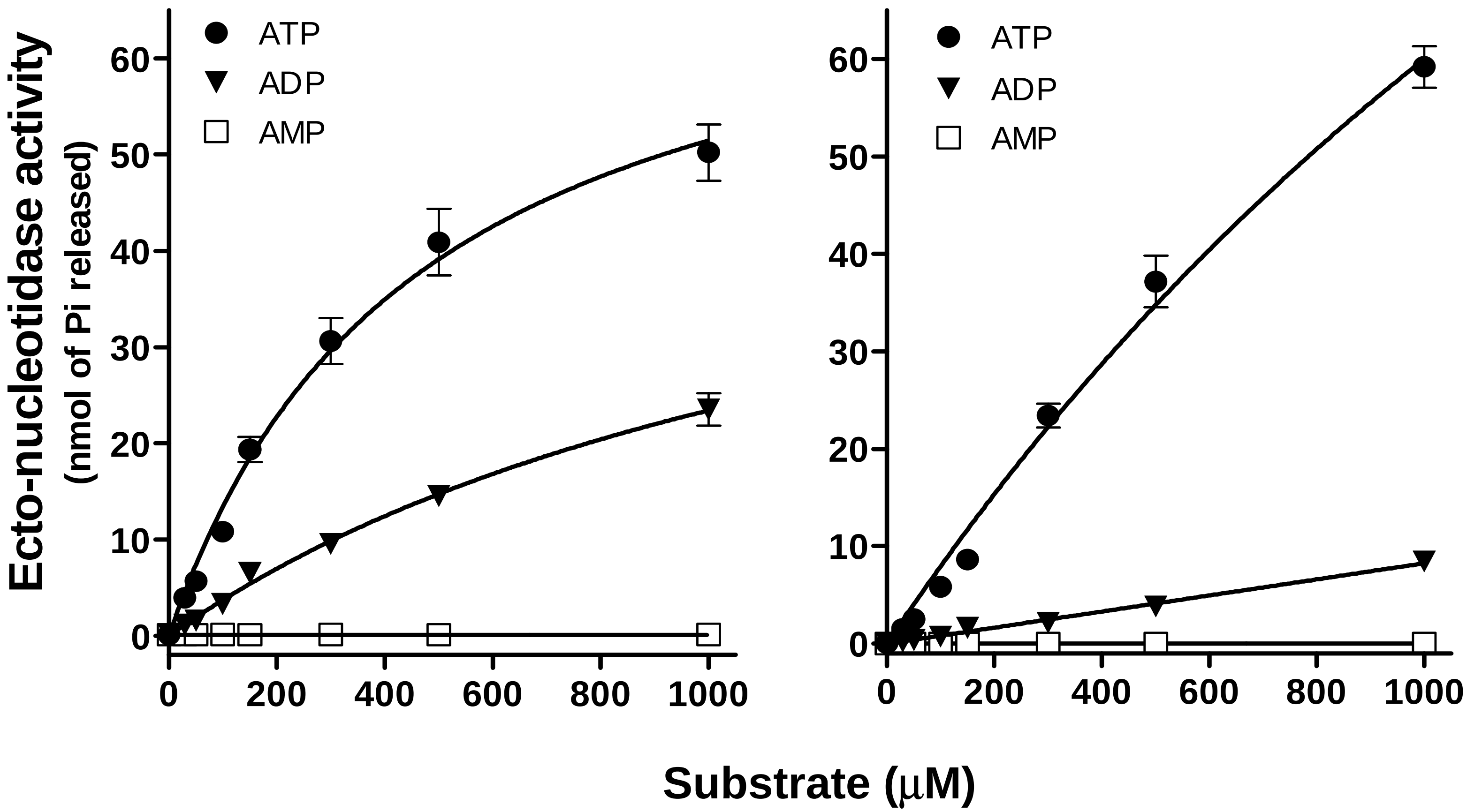
Ecto-nucleotidase activity
(nmol of Pi released)
ATP
ADP
AMP
0
10
20
30
40
50
60
0
200
400
600
800
1000
ATP
ADP
AMP
Substrate (μM)

Fig 2

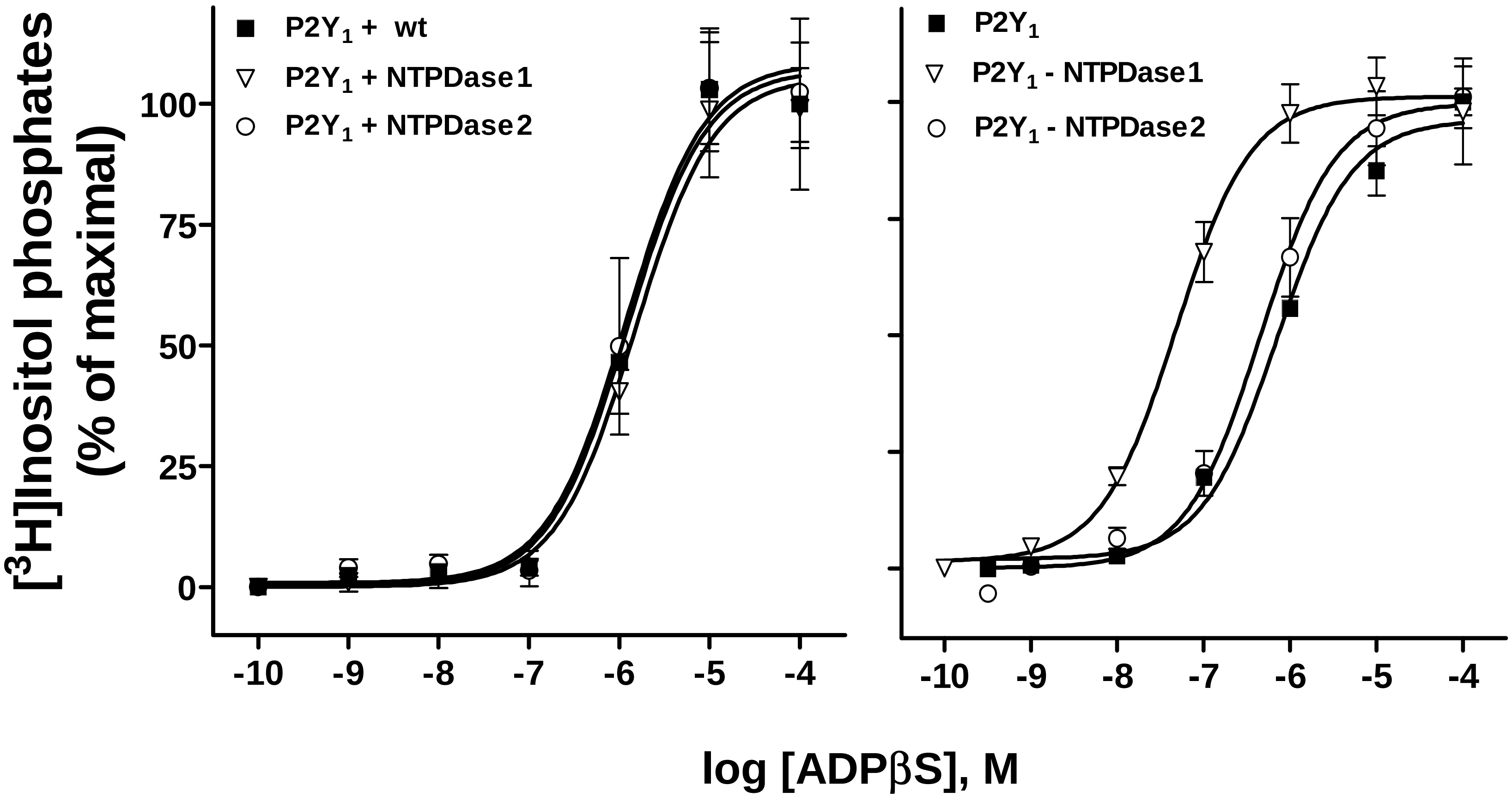

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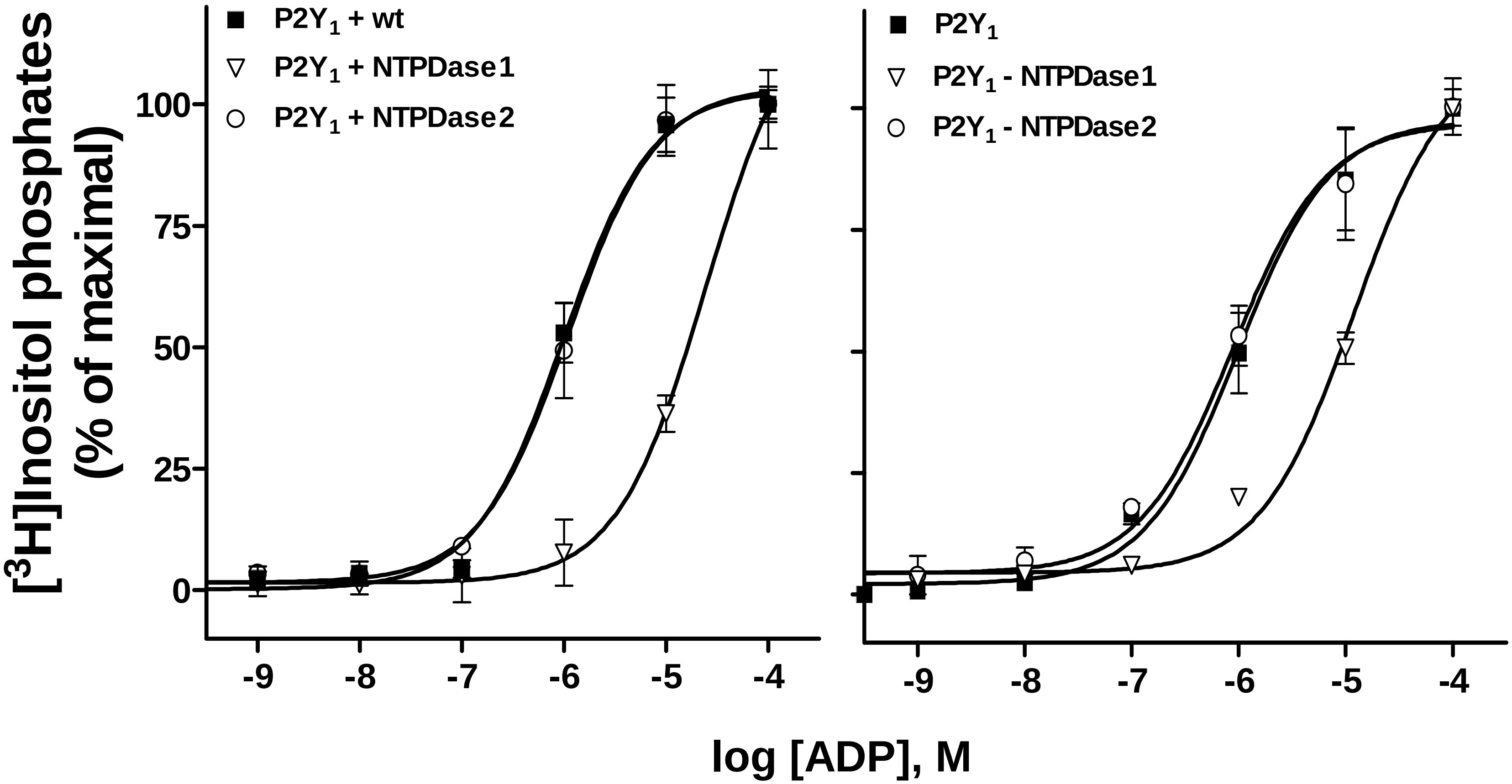
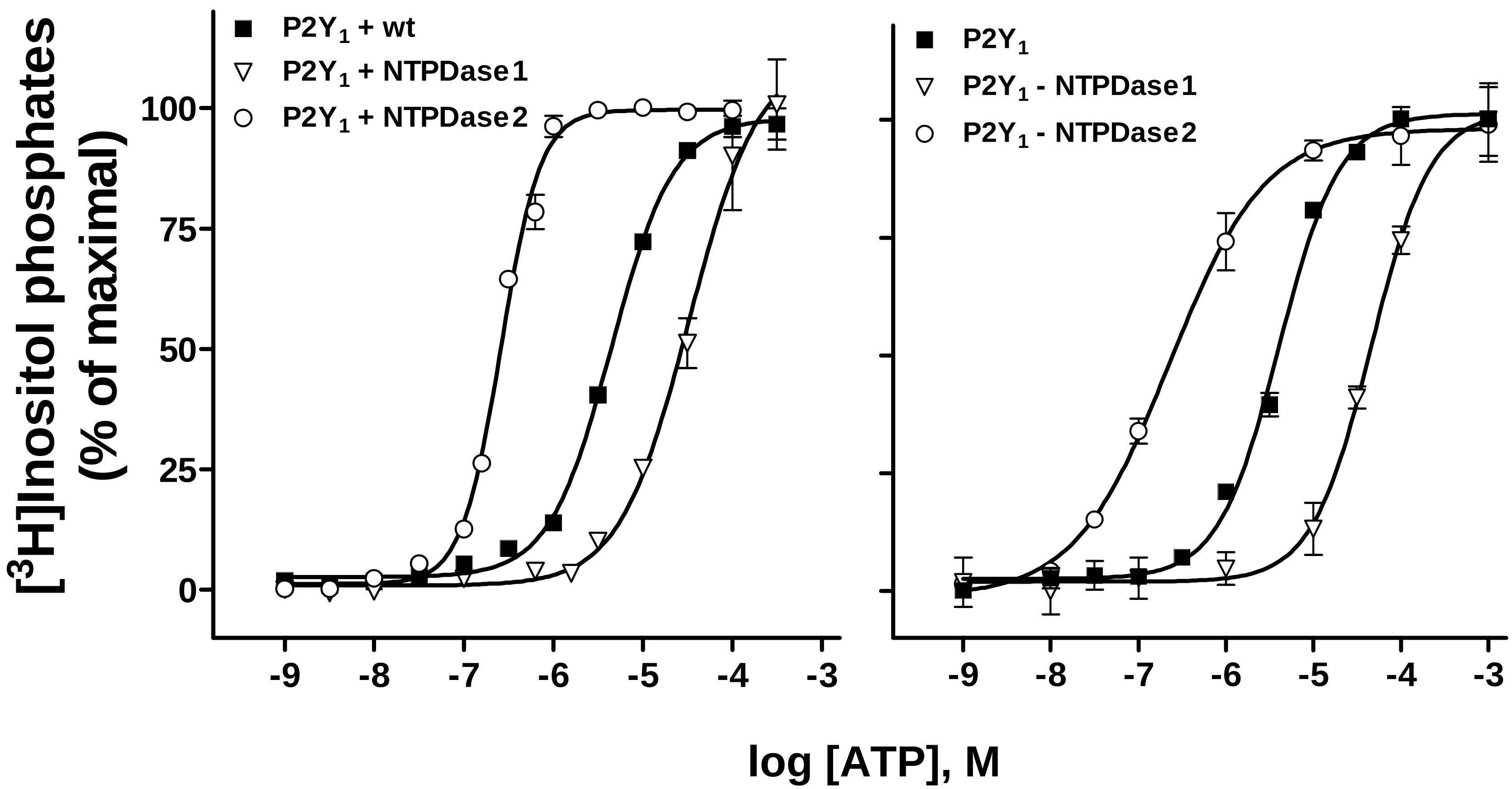
Fig 3



P2Y1 + wt
P2Y1 + NTPDase1
P2Y1 + NTPDase2
P2Y1
P2Y1 - NTPDase1
P2Y1 - NTPDase2
[3H]Inositol phosphates (% of maximal)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
log [ADP], M

Fig 4



P2Y1 + wt
P2Y1 + NTPDase1
P2Y1 + NTPDase2
[3H]Inositol phosphates (% of maximal)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
P2Y1
P2Y1 - NTPDase1
P2Y1 - NTPDase2
log [ATP], M

Fig 5

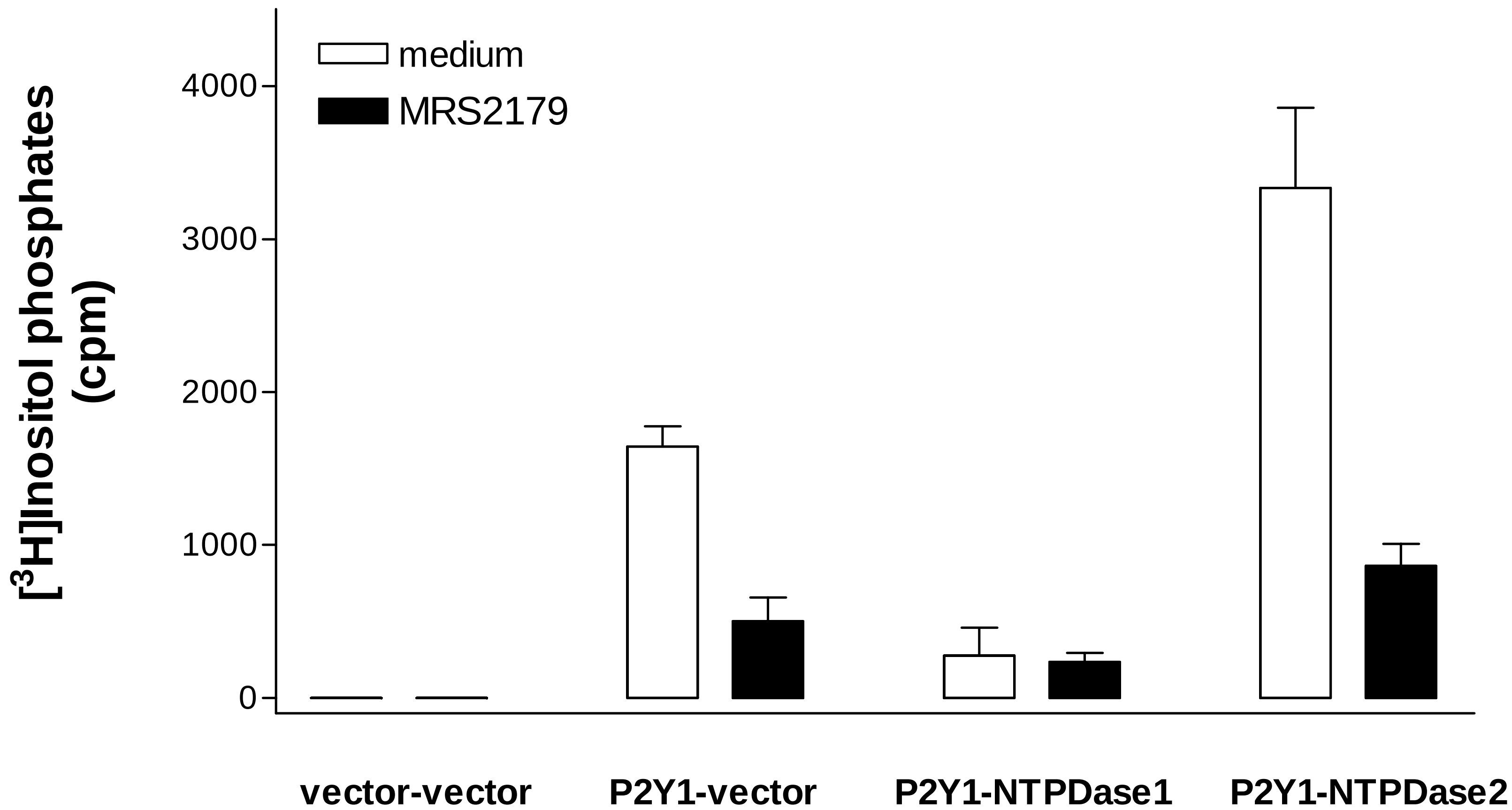
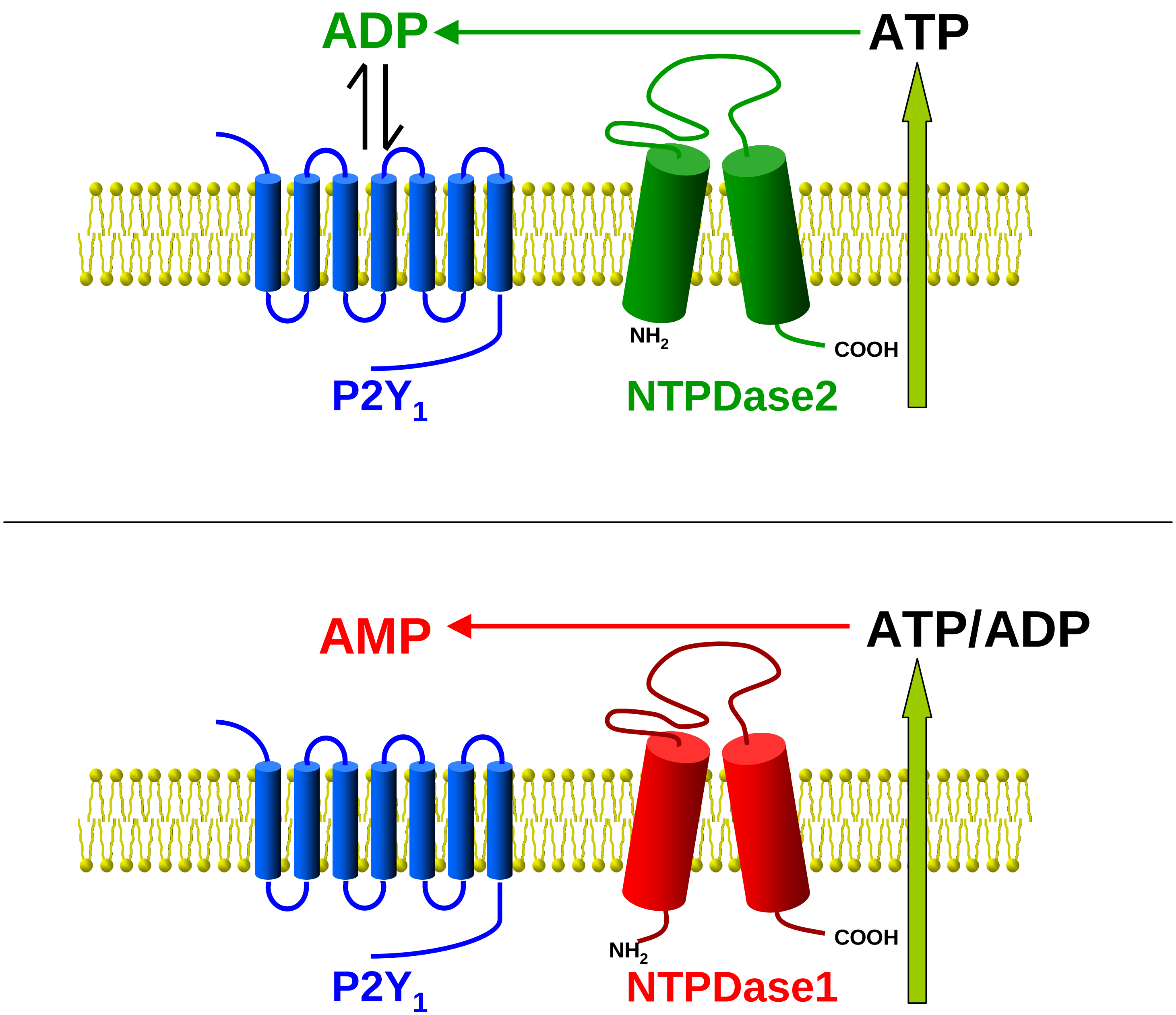

medium
MRS2179
4000
3000
2000
1000
0
[3H]Inositol phosphates (cpm)
vector-vector
P2Y1-vector
P2Y1-NTPDase1
P2Y1-NTPDase2

Fig 6



ADP
ATP
NH2
COOH
P2Y1
NTPDase2
AMP
ATP/ADP
NH2
COOH
P2Y1
NTPDase1