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Purification and Functional Reconstitution of the Human P2Y$_{12}$ Receptor

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ABSTRACT

The human P2Y$_{12}$ receptor (P2Y$_{12}$-R) is a member of the G protein coupled P2Y receptor family, which is intimately involved in platelet physiology. We describe here the purification and functional characterization of recombinant P2Y$_{12}$-R after high-level expression from a baculovirus in Sf9 insect cells. Purified P2Y$_{12}$-R, G$\beta_1\gamma_2$, and various Gα-subunits were reconstituted in lipid vesicles, and steady-state GTPase activity was quantified. GTP hydrolysis in proteoliposomes formed with purified P2Y$_{12}$-R and G$\alpha_{i2}\beta_1\gamma_2$ was stimulated by addition of either 2-methylthio-ADP (2MeSADP) or RGS4 and was markedly enhanced by their combined presence. 2MeSADP was the most potent agonist (EC$_{50}$ = 80 nM) examined, whereas ADP, the cognate agonist of the P2Y$_{12}$-R, was 3 orders of magnitude less potent. ATP had no effect alone but inhibited the action of 2MeSADP; therefore, ATP is a relatively low-affinity antagonist of the P2Y$_{12}$-R. The G protein selectivity of the P2Y$_{12}$-R was examined by reconstitution with various G protein α-subunits in heterotrimeric form with G$\beta_1\gamma_2$. The most robust coupling of the P2Y$_{12}$-R was to Gα_{i2}, but effective coupling also occurred to Gα_{i1} and Gα_{i3}. In contrast, little or no coupling occurred to Gα_o or Gα_q. These results illustrate that the signaling properties of the P2Y$_{12}$-R can be studied as a purified protein under conditions that circumvent the complications that occur in vivo because of nucleotide metabolism and interconversion as well as nucleotide release.

Extracellular nucleotides regulate a wide range of physiological responses in multifarious tissues (Harden et al., 1995; Ralevic and Burnstock, 1998). Both ionotropic (P2X) and G protein-coupled (P2Y) receptors are responsible for mediating these responses. For example, the P2X$_1$ receptor is highly expressed in smooth muscle tissue, such as the vas deferens and bladder, where it is stimulated by ATP acting as a primary sympathetic transmitter (Burnstock, 1972). In contrast, the G protein-coupled P2Y$_2$ receptor is activated by both ATP and UTP and controls mucociliary clearance in lung airways (Lazarowski and Boucher, 2001). Of the eight members of the P2Y receptor family identified so far, five couple to Gα_q to stimulate phospholipase C-β and three couple to yet-to-be-defined members of the Gi family to inhibit adenylyl cyclase or regulate ion channels and potentially promote other signaling responses (Fredholm et al., 1997; Harden et al., 1998; Dangelmaier et al., 2000; Communi et al., 2001; Hollopeter et al., 2001; Simon et al., 2002; Abbracchio et al., 2003; Resendiz et al., 2003). Two members of this family, the Gq-coupled P2Y$_1$ receptor (P2Y$_1$-R) and the recently cloned Gi-coupled P2Y$_{12}$-R (also known as P2T, P2Yac, HORK3, and SP1999) (Hollopeter et al., 2001; Takasaki et al., 2001; Zhang et al., 2001), are of particular interest because of their role in the platelet aggregation response to ADP (Daniel et al., 1998; Hechler et al., 1998a,b; Jin and Kunapuli, 1998; Savi et al., 1998). The concomitant activation of these two receptors results in the activation of $\alpha_{IIb}\beta_3$, and the subsequent aggregation of platelets.

Although they signal through different G protein pathways, the P2Y$_1$-R and the P2Y$_{12}$-R display similar agonist selectivity. ADP is the cognate agonist for both receptors and certain analogs of ADP (e.g., 2MeSADP) also are potent agonists of these two receptors (Boyer et al., 1993; Schachter et al., 1996; Hechler et al., 1998a). Molecular insight into the mechanisms of action of these and other P2Y receptors and delineation of their definitive pharmacological selectivities has proven difficult to establish because of problems inherent in: 1) release of cellular nucleotides, 2) metabolism and interconversion of extracellular nucleotides by a complex array of ectoenzymes, 3) lack of availability of selective agonists and antagonists, and 4) lack of reliable radioligand binding assays. We reasoned that many of these issues could be obviated by purification and functional reconstitution of the protein cohorts of P2Y receptor-regulated signaling pathways. Moreover, this approach can be used to delineate the

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ABBREVIATIONS: P2Y$_{12}$-R, P2Y$_{12}$ receptor; P2Y$_1$-R, P2Y$_1$ receptor; 2MeSADP, 2-methylthio-ADP; RGS, regulator of G protein signaling; Ni-NTA, nickel-nitrilotriacetic acid; FPLC, fast-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

mechanism of interaction of P2Y receptors with their signaling partners.

In this study, we have expressed the human P2Y$_{12}$-R to high levels and purified this important platelet protein to near homogeneity using a hexahistidine tag and Ni-NTA affinity column chromatography as well as ion exchange chromatography. Characterization of the purified receptor was carried out in a reconstituted assay system with Gα and Gβγ subunits and model phospholipid vesicles. The purified P2Y$_{12}$-R retains binding and signaling activities reminiscent of the natively expressed protein and the pharmacological and G protein selectivities of the purified receptor have been defined unambiguously.

Materials and Methods

Protein Purification. The human P2Y$_{12}$-R gene was subcloned into pFastbac Htb, which encodes a hexahistidine tag and TEV protease site in the 5′ direction from the subcloned gene. Baculovirus for P2Y$_{12}$-R was generated using the Invitrogen FastBac system (Invitrogen, Carlsbad, CA). Four liters of Sf9 insect cells (1.8–2.2 × 10^6 cells/ml) were infected with virus encoding the P2Y$_{12}$-R at a multiplicity of infection of 1. Forty-eight hours after infection, cells were collected by low-speed centrifugation and resuspended and lysed by nitrogen cavitation in 300 ml of lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 1 mM β-mercaptoethanol, 500 nM aprotinin, 10 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, and 10 μM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone). Unlysed cells and cellular debris were removed by low-speed centrifugation, and cell membranes were collected by centrifugation of the low speed supernatant at 100,000*g* for 35 min. The pelleted membranes were resuspended in extraction buffer (20 mM Tris, pH 8, 150 mM NaCl, 1 mM β-mercaptoethanol, 1% digitonin, and protease inhibitors) to a concentration of 5 mg of protein/ml, and extraction was carried out for 1 h at 4°C. Solubilized membrane proteins were recovered by collection of the supernatant after centrifugation at 100,000*g*. The soluble fraction was incubated in batch with 1 ml of Ni-NTA-agarose resin (QIAGEN, Valencia, CA) for 3 h at 4°C. The resin was loaded into a column and washed with 10 ml of high salt buffer (20 mM Tris, pH 8, 500 mM NaCl, 0.5% digitonin, and protease inhibitors). The P2Y$_{12}$-R was eluted with 2 ml of elution buffer (20 mM Tris, pH 8, 150 mM NaCl, 150 mM imidazole, pH 8, 0.1% digitonin, and protease inhibitors). Eluted receptor was diluted to 10 ml with buffer A (20 mM Tris, pH 8, 150 mM NaCl, and 0.1% digitonin) and loaded onto a 1-ml HighTrap metal chelate FPLC column (Amersham Biosciences, Piscataway, NJ) charged with Ni^{2+}. Fractions (0.5 ml) were collected over a 20-bed volume gradient from 0 to 100% buffer B (20 mM Tris, pH 8, 150 mM NaCl, 0.1% digitonin, and 500 mM imidazole, pH 8). In some preparations, an ion exchange step was included between the Ni-NTA and the charged metal chelate FPLC chromatography steps. Thus, the receptor fraction eluted from the Ni-NTA column was diluted to 10 ml with buffer AQ (20 mM Tris, pH 8, and 0.1% digitonin) and loaded onto a 1-ml HighTrap Q FPLC column (Amersham Biosciences, Piscataway, NJ). Fractions (0.5 ml) were collected over a 10-bed volume gradient from 0 to 100% buffer BQ (20 mM Tris, pH 8, 0.1% digitonin, and 1 M NaCl). Receptor-containing fractions were pooled and concentrated using a Centricon YM-30 centrifugal filter device (Millipore, Bedford, MA). The protein concentration of purified P2Y$_{12}$-R was determined by Coomassie staining relative to a bovine serum albumin standard curve resolved by SDS-PAGE. Yield was ~30 to 50 μg of purified receptor (Fig. 1) per 4 liters of Sf9 culture. Gα- and Gβγ-subunits (Kozasa and Gilman, 1995) and muscarinic receptors (Parker et al., 1991) were purified after expression from baculoviruses in Sf9 insect cells as described. Hexahistidine-tagged RGS4 was purified as described previously (Saugstad et al., 1998).

Vesicle Reconstitution and Characterization. Detergent/phospholipid mixed micelles were prepared by drying 110 μg of phosphatidylethanolamine, 70 μg of phosphatidylserine, and 8 nmol of cholesteryl hemisuccinate under argon and resuspended in detergent buffer (0.4% deoxycholate, 20 mM HEPES, 1 mM EDTA, 100 mM NaCl) via bath sonication. Fifty μl of this preparation was combined with 15 pmol of P2Y$_{12}$-R, 50 pmol of Gα, and 150 pmol of G$\beta_1\gamma_2$ and the volume was increased to 100 μl with G-50 buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 2 mM $MgCl_2$). The mixture was immediately loaded onto a G-50 buffer-equilibrated G-50-Sepharose column, and the vesicle-containing void volume was eluted and collected with G-50 buffer. Gα incorporation was assessed by incubating 5 μl of the vesicle preparation with 1 μM ^{35}S-labeled guanosine-5′-*O*-(3-thiotriphosphate) (~500,000 cpm) in the presence (to quantitate total Gα) or absence (to quantitate vesicle incorporated Gα) of 0.1% $C_{12}E_{10}$ detergent (total volume, 100 μl) at 30°C for 90 min. Samples labeled in the absence of $C_{12}E_{10}$ were filtered over GF/F filters (Millipore) and $C_{12}E_{10}$-containing samples were filtered over BA85 nitrocellulose filters (Protran; Schleicher and Schüll, Dassel, Germany).

Steady-State GTPase Assays. One to two microliters of the vesicle preparation was equilibrated on ice in the presence or absence of 100 nM (final) RGS4 and concentration ranges of various drugs. Assays were initiated by the addition of GTP mix (20 mM HEPES, pH 8, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 2 μM final GTP, and ~500,000 cpm [γ-^{32}P]GTP), brief vortex mixing, and incubation at 30°C for 15 min. The assay was quenched on ice with 975 μl of ice-cold 5% activated charcoal in 20 mM NaH_2PO_4. The charcoal was pelleted by centrifugation and a portion of the supernatant was added to scintillant for quantification of $^{32}P_i$.

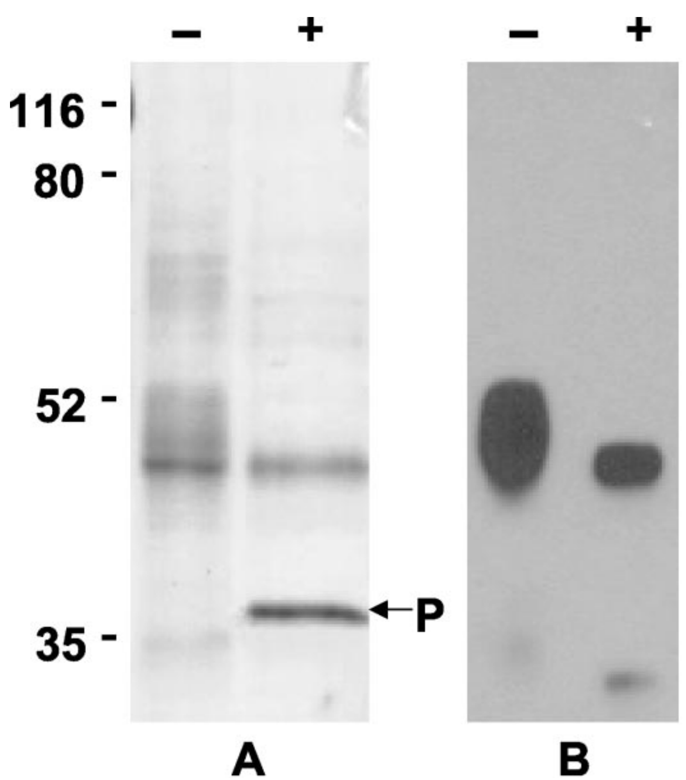

Fig. 1. SDS-PAGE and immunoblot analysis of purified recombinant human P2Y$_{12}$-R. Recombinant P2Y$_{12}$-R tagged with a His$_6$ epitope was purified from a digitonin extract of Sf9 insect cell plasma membranes as described under *Materials and Methods*. Purified P2Y$_{12}$-R treated with *N*-glycosidase F (PNGase F, denoted as P on figure) (+) or untreated P2Y$_{12}$-R (−) was subjected to SDS-PAGE analysis. The resulting gels were either stained with Coomassie blue (A) or transferred to nitrocellulose and immunoblotted with anti-His$_6$ antibody (B).

COS-7 Cell Transfection and Quantification of Receptor Activity. COS-7 cells were seeded at a density of 20,000 cells/cm^2 in 12-well culture dishes in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C. Plasmid DNA vectors containing either the human P2Y$_{12}$ receptor or the chimeric G protein G$\alpha_{q/i}$ were transfected using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN) transfection reagent according to the manufacturer's protocol. Approximately 24 h after the addition of the DNA and transfection reagent, the inositol lipids were radiolabeled by incubating the cells in inositol-free DMEM containing 1 μCi of [^{3}H]inositol/well. Twelve hours after labeling, cells were treated with the indicated drug concentration in the presence of LiCl (final concentration, 10 mM) to initiate the accumulation of [^{3}H]inositol phosphates. The assay was terminated after 60 min by aspirating the medium and adding 50 mM ice-cold formic acid. The samples were neutralized with 150 mM NH_4OH. [^{3}H]Inositol phosphates were quantified by Dowex chromatography followed by liquid scintillation counting as described previously (Boyer et al., 1993).

Results

The human P2Y$_{12}$-R was purified as described under *Materials and Methods* after expression from a recombinant baculovirus in Sf9 insect cells. A time course of immunoreactivity of P2Y$_{12}$-R expression revealed 48 h after infection to be the optimal time for receptor expression (data not shown). Approximately 50% of the receptor extracted with 1% digitonin. The extracted receptor bound efficiently to Ni-NTA resin, and after elution with imidazole, was further purified by a subsequent ion exchange chromatographic step. The purified P2Y$_{12}$-R migrated as a diffuse band on SDS-PAGE at approximately 42 to 52 kDa, as seen by both Coomassie staining and immunoblot analysis with anti-His antiserum (Fig. 1). *N*-glycosidase F treatment of the protein preparation resulted in a less diffuse, faster migrating receptor species (Fig. 1). The sharp band located at approximately 42 kDa in the untreated lane of the Coomassie stain is a comigrating contaminant that is consistently seen and is not immunoreactive to the anti-His antibody.

Functional activity and pharmacological properties of the purified P2Y$_{12}$-R were assessed after reconstitution in proteoliposomes with purified Gα_{i2} and G$\beta_1\gamma_2$ as described under *Materials and Methods*. Relatively low GTPase activity was observed in the vesicle preparation alone, and addition of the P2Y$_{12}$-R agonist 2MeSADP (10 μM) alone stimulated GTPase activity by approximately 5-fold over basal activity (Fig. 2). Addition of RGS4, which is known to be an effective GTPase activating protein for Gα_{i2}, produced a 10- to 15-fold stimulation of GTPase activity under these conditions. The combined presence of 100 nM RGS4 and 10 μM 2MeSADP resulted in 5 and 10-fold increases in GTP hydrolysis over that observed with either RGS4 or 2MeSADP, respectively. Thus, approximately 30-fold stimulation of GTPase activity over basal activity was observed in the combined presence of the agonist 2MeSADP and the GTPase-activating protein RGS4. GTP hydrolysis was linear over 15 min in either the absence or the presence of activators (Fig. 3). Therefore, steady-state GTP hydrolysis was observed under the conditions of these assays, and both guanine nucleotide exchange and nucleotide hydrolysis by the involved GTPase seem to be rate-limiting, given the markedly synergistic action of 2MeSADP and RGS4 compared with activation by either alone. These results indicate that the P2Y$_{12}$-R was purified in a form that retains both agonist binding and functional activity.

The use of purified components in a reconstituted system allows circumvention of problems associated with the study of nucleotide-activated receptors, which include hydrolysis and interconversion of added nucleotides as well as cellular release of nucleotides (Harden et al., 1997). Thus, we initially assessed the selectivity of the P2Y$_{12}$-R for ADP and its reportedly more potent analog 2MeSADP in steady state GTPase assays. A concentration-dependent increase in GTP hydrolysis was observed with both ADP and 2MeSADP (EC$_{50}$ = 30 μM and 16 nM, respectively) (Fig. 4A), and we observed a difference of nearly 3 orders of magnitude in the potencies of the two compounds. This result was somewhat surprising given the robust action of ADP observed in many studies of platelet function; therefore, we explored this question further in mammalian expression studies. The human P2Y$_{12}$-R construct was subcloned into a mammalian cell expression vector, and the receptor was transiently coexpressed in COS-7 cells with a chimeric (G$\alpha_{q/i}$) construct of Gα_q and Gα_i that enables Gi-linked receptors to activate phospholipase C-β. Therefore, P2Y$_{12}$-R-promoted hydrolysis of [^{3}H]labeled phosphatidylinositol 4,5-bisphosphate was assessed as an assay of agonist activity. Whereas the apparent potencies of ADP and 2MeSADP (EC$_{50}$ = 2.4 μM and 0.6 nM, respectively) observed in transfected COS-7 cells were both greater than those observed with the purified P2Y$_{12}$-R, the ~1,000-fold

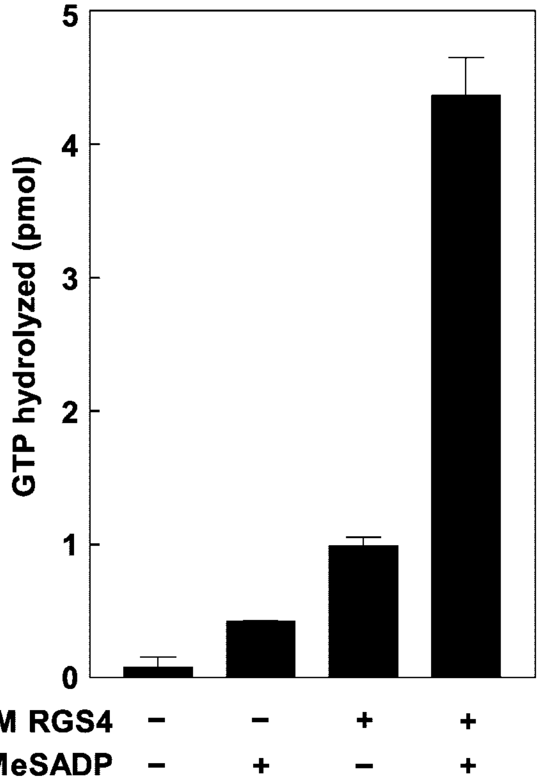

Fig. 2. Functional activity of purified P2Y$_{12}$-R reconstituted with G$\alpha_{i2}\beta_1\gamma_2$. Purified P2Y$_{12}$-R, G$\alpha_{i2}$, and G$\beta_1\gamma_2$, were reconstituted into proteoliposomes as described under *Materials and Methods*. Steady-state GTP hydrolysis was measured in proteoliposomes incubated in the absence or presence of either 100 nM RGS4 and/or 10 μM 2MeSADP. The data are presented as mean ± S.E.M. of an experiment representative of three independent experiments.

difference in potencies observed with the purified receptor was retained after mammalian expression (Fig. 4B).

The activity of the purified P2Y$_{12}$-R was further assessed in the presence of various adenine nucleotide derivatives as well as UDP and UTP (Fig. 5). Adenosine-5′-*O*-(2-thiodiphosphate) weakly stimulated the P2Y$_{12}$-R, whereas no activation was observed under these conditions with α,β-methylene ADP, β,γ-methylene ATP, or adenosine-5′-*O*-(3-thiotriphospate). In addition, neither UDP nor UTP activated the P2Y$_{12}$-R, and ATP was also inactive at 100 μM. The decrease in GTPase activity observed with UDP and UTP is most likely the result of a nonspecific effect. Similar results have been seen in our lab with other purified receptors in the same system (G. L. Waldo and T. K. Harden, unpublished observations). Furthermore, there is no precedence for any action by UDP or UTP at the P2Y$_{12}$-R.

Various laboratories have reported very different activities for ATP at the P2Y$_{12}$-R. For example, recent studies by Simon et al. (2002) and Takasaki et al. (2001) concluded that ATP is a full agonist at the P2Y$_{12}$-R, whereas Park and Hourani (1999) identified ATP as an antagonist at the P2Y$_{12}$-R in platelet studies. Thus, in light of our initial findings and these conflicting results in the literature, the action of ATP relative to 2MeSADP was further tested at the purified P2Y$_{12}$-R under conditions in which no breakdown or interconversion of ATP to other adenine nucleotides occurs. ATP hydrolysis was measured under the conditions of these assays and found to be less then 0.5% (data not shown). That is, more than 99% of the added ATP was recovered as unchanged nucleoside triphosphate after a 15-min incubation at 30°C. ATP did not activate the P2Y$_{12}$-R–mediated GTPase activity under the conditions of these assays, even at concentrations as high as 300 μM (Fig. 6A). The potential antagonist activity of ATP was compared with the known P2Y$_{12}$-R antagonist, 2-methylthio-AMP, which inhibited 2MeSADP-stimulated GTP hydrolysis in a concentration-dependent manner with an IC$_{50}$ value of approximately 20 μM in assays carried out in the presence of 1 μM 2MeSADP (Fig. 6B). ATP also apparently acts as an antagonist at the P2Y$_{12}$-R, albeit with a lower potency than that observed with 2-methylthio-AMP (Fig. 6B).

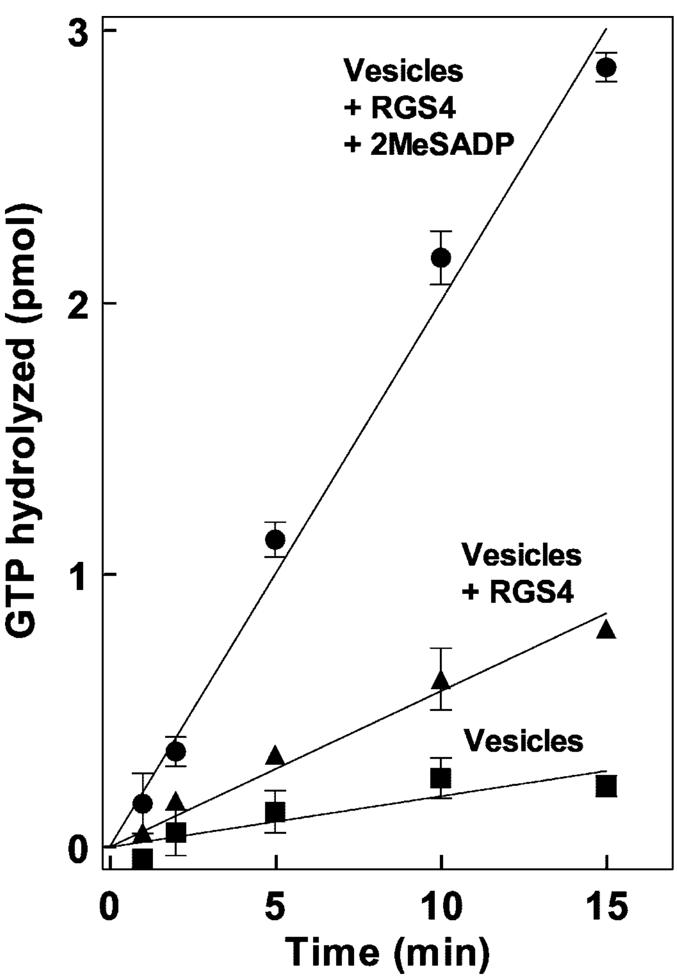

Fig. 3. Steady-state GTP hydrolysis in vesicles reconstituted with purified P2Y$_{12}$-R and G$\alpha_{i2}\beta_1\gamma_2$. Purified P2Y$_{12}$-R, G$\alpha_{i2}$, and G$\beta_1\gamma_2$ were reconstituted in phospholipid vesicles as described under *Materials and Methods*. GTP hydrolysis was quantified with vesicles alone (■), in the presence of 100 nM RGS4 (▲), or in the presence of 100 nM RGS4 and 10 μM 2MeSADP (●). The results are presented as mean ± S.E.M. of an experiment representative of three independent experiments.

Current interest in the P2Y$_{12}$-R rests primarily in its expression and physiological importance in platelets, where simultaneous stimulation of the P2Y$_1$-R and the P2Y$_{12}$-R by ADP leads to platelet aggregation. Although the contribution of the P2Y$_{12}$-R to this response has been shown to be through Gi-family members, coupling specificity of the P2Y$_{12}$-R to various Gα-subunits has yet to be definitively described. Availability of the purified receptor in a fully active form allows the assessment of the coupling efficiencies of the P2Y$_{12}$-R with various G proteins. Thus, Gα-subunit selectivity of the P2Y$_{12}$-R was determined through reconstitution of the purified receptor and G$\beta_1\gamma_2$ in combination with various Gα-subunits as described under *Materials and Methods*. Under these conditions, in the presence of 100 nM RGS4, no apparent coupling to either Gα_q or Gα_o occurred (Fig. 7). In contrast, 2MeSADP-stimulated GTPase activity was observed with P2Y$_{12}$-R reconstituted with all three Gα_i subunits. The EC$_{50}$ values of 2MeSADP for the P2Y$_{12}$-R when reconstituted with these three different G proteins were nearly identical. However, the maximum GTPase activity observed was greatest with Gα_{i2}, whereas reconstitution with either Gα_{i1} or Gα_{i3} resulted in more modest 2MeSADP-stimulated GTPase activity (Fig. 7). To rule out the possibility that the differences in P2Y$_{12}$-R/Gα_i coupling efficiencies were caused by differences in G protein activity, similar reconstitution studies also were carried out with the human M$_2$ muscarinic receptor and each of the Gα_i-subunits. The M$_2$ muscarinic receptor coupled equally well to Gα_o and all three Gα_i isoforms of the Gi family in vesicles also containing G$\beta_1\gamma_2$ (Fig. 7, inset). Thus, the P2Y$_{12}$-R exhibits a selectivity of activation of Gα-subunits not observed under the same conditions with a similar Gi-coupled receptor.

Discussion

This study reports the successful purification of the P2Y$_{12}$-R to near homogeneity. The purified receptor, when stimulated by 2MeSADP, functionally activates reconstituted purified G proteins, promoting GTPase activity that is greatly augmented by RGS4. This reconstitution system provides the most unambiguous means available to date to assess the pharmacological selectivity of the P2Y$_{12}$-R and to directly determine its Gα-subunit selectivity.

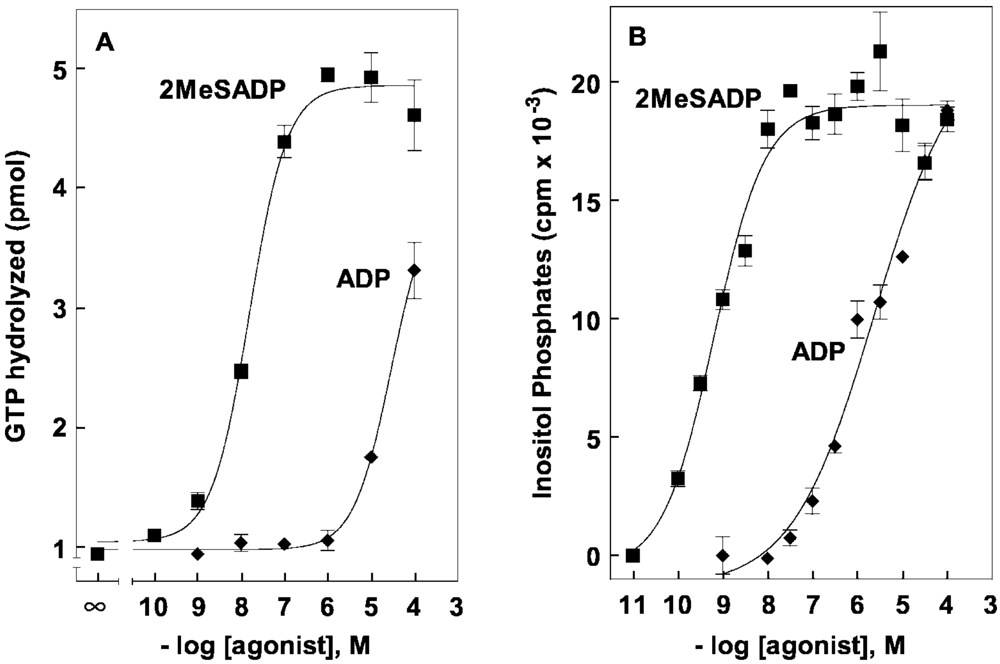

Fig. 4. Action of 2MeSADP and ADP at the purified or transiently expressed $P2Y_{12}$-R. A, purified $P2Y_{12}$-R, $G\alpha_{i2}$, and $G\beta_1\gamma_2$ were reconstituted into proteoliposomes as described under *Materials and Methods*. Steady-state GTP hydrolysis was measured in proteoliposomes incubated with 100 nM RGS4 and the indicated concentration of $P2Y_{12}$-R agonists. B, COS-7 cells were transfected with $P2Y_{12}$-R and $G\alpha_{q/i}$, which enables Gi-linked receptors to activate phospholipase C-β. Inositol phosphate accumulation was measured in transfected cells with the indicated concentrations of $P2Y_{12}$-R agonist. The results are presented as mean ± S.E.M. of an experiment representative of three independent experiments.

The $P2Y_{12}$-R was one of the first receptors illustrated to inhibit adenylyl cyclase and the first P2Y receptor studied biochemically (Cooper and Rodbell, 1979). Subsequent investigations revealed the $P2Y_{12}$-R to be a unique member of the P2Y receptor family in that it coupled to $G\alpha_i$ rather than $G\alpha_q$ (Hollopeter et al., 2001). The eventual cloning of the $P2Y_{12}$-R revealed a sequence with very low homology to the five previously cloned Gq/phospholipase C-coupled P2Y receptors (Foster et al., 2001; Hollopeter et al., 2001; Takasaki et al., 2001; Zhang et al., 2001). Although identification of $P2Y_1$-R antagonists led to the conclusion that two independent ADP receptors mediate platelet aggregation, this was confirmed with the molecular identification of these receptors (Hechler et al., 1998b; Savi et al., 1998; Takasaki et al., 2001). The existence of convergent G protein signaling is not unprecedented, yet the requirement of two independent signaling cascades mediated by two related yet independent receptors activated by a common extracellular signaling molecule is mechanistically provocative and a unique characteristic of the platelet ADP response. Although these two P2Y receptors and their signaling pathways may always function as coactivated signaling partners, inherent differences between the two indicate the possibility of independent functions. For example, previous studies have shown that ADP is more effective at inducing calcium mobilization via the $P2Y_1$-R relative to cAMP inhibition via the $P2Y_{12}$-R (Takasaki et al., 2001). Thus, signaling potentially could occur via the $P2Y_1$-R through the action of ADP without stimulating the $P2Y_{12}$-R. From the studies reported here, we conclude ATP to be a weak antagonist at the $P2Y_{12}$-R, which contrasts with its action at the $P2Y_1$-R, where it acts as a partial agonist. Thus, ATP could further prevent $P2Y_{12}$-R signaling and therefore promote independent activation of $P2Y_1$-R-mediated signaling responses to ADP.

The dual receptor and pathway involvement is well established in the action of ADP in platelets. Disruption of the genes for $P2Y_1$-R or $G\alpha_q$ results in loss of ADP-promoted Ca^{2+} mobilization, shape change, and platelet aggregation (Offermanns et al., 1997; Fabre et al., 1999; Leon et al., 1999). Similarly, ADP-induced inhibition of adenylyl cyclase and platelet aggregation

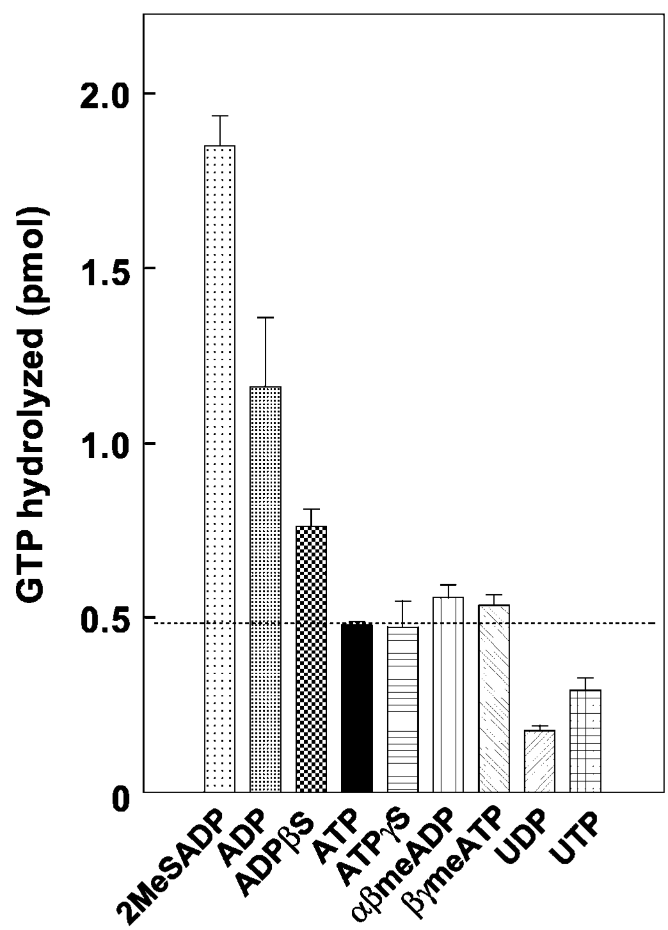

Fig. 5. Agonist selectivity of the purified $P2Y_{12}$-R. Steady-state GTPase assays were carried out with proteoliposomes containing purified $P2Y_{12}$-R, $G\alpha_{i2}$, and $G\beta_1\gamma_2$ incubated in the presence of 100 nM RGS4 and 100 μM of the indicated nucleotide. Dashed line represents activity measured in the presence of RGS4 alone. The results are the mean ± S.E.M. of an experiment representative of three independent experiments. ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); ATPγS, adenosine-5′-*O*-(3-thiotriphospate); αβmeADP, α,β-methylene ADP; βγmeATP, β,γ-methylene ATP.

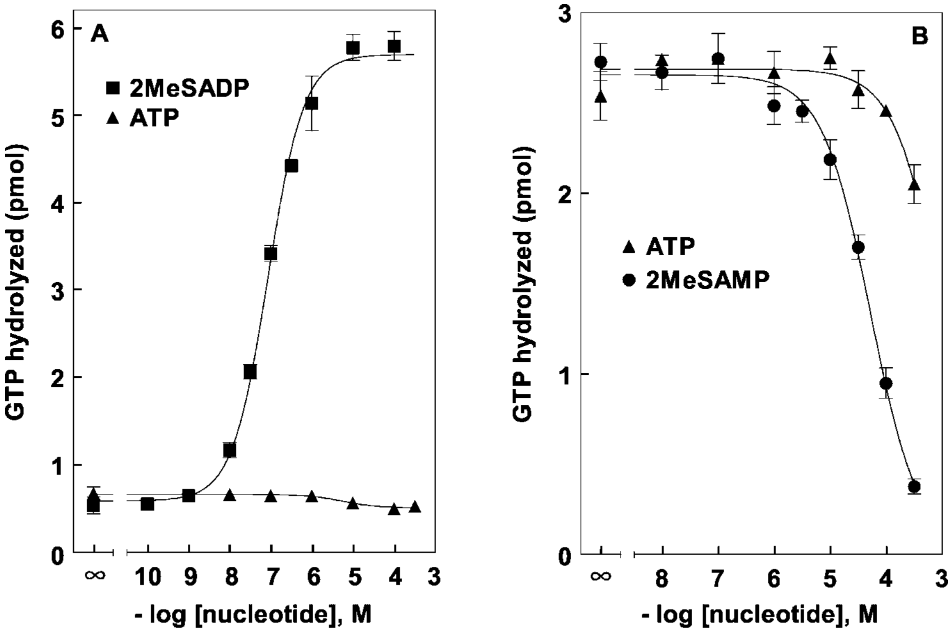

Fig. 6. Action of ATP at the purified P2Y$_{12}$-R. Purified P2Y$_{12}$-R, Gα_{i2}, and G$\beta_1\gamma_2$ were reconstituted into proteoliposomes. A, steady-state GTP hydrolysis was measured in proteoliposomes incubated with 100 nM RGS4 and the indicated concentration of drug. B, steady-state GTP hydrolysis was measured in proteoliposomes incubated with the indicated concentration of drug in the presence of 1 μM 2MeSADP. The results are the mean ± S.E.M. of an experiment representative of three independent experiments.

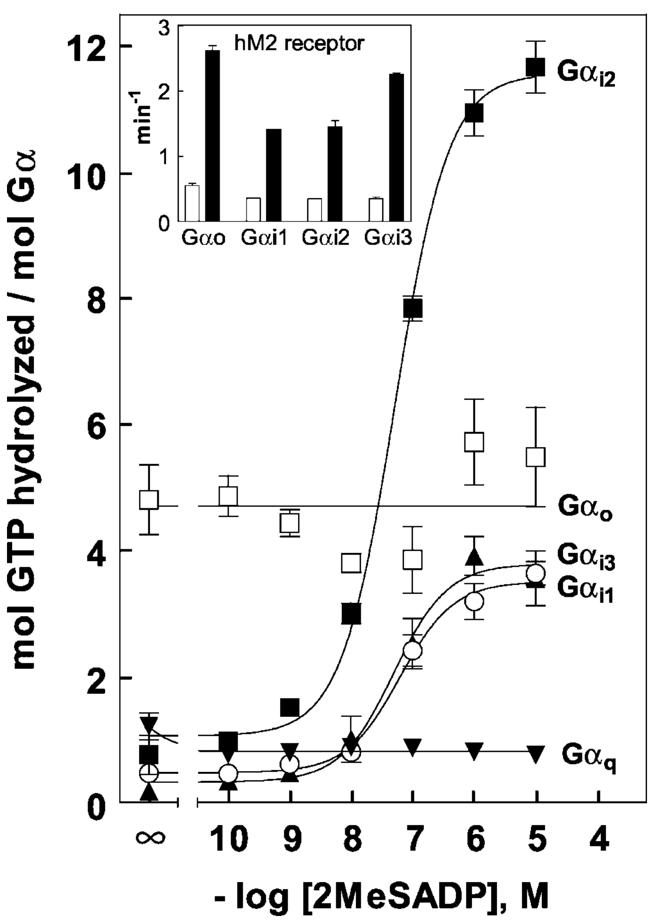

Fig. 7. Gα protein selectivity of purified P2Y$_{12}$-R. Purified P2Y$_{12}$-R and G$\beta_1\gamma_2$ were reconstituted into proteoliposomes with either Gα_q, Gα_o, Gα_{i1}, Gα_{i2}, or Gα_{i3}. Steady-state GTP hydrolysis was measured in proteoliposomes incubated with 100 nM RGS4 and the indicated concentration of 2MeSADP. Gα incorporation into proteoliposomes was measured by guanosine-5′-O-(3-[^{35}S]thiotriphosphate) binding. Inset, steady-state GTP hydrolysis measured in proteoliposomes reconstituted with purified human M$_2$ muscarinic receptor, G$\beta_1\gamma_2$, and the indicated Gα protein with RGS4 in the presence (■) and absence (□) of 100 μM carbachol. The results are the mean ± S.E.M. of an experiment representative of three independent experiments.

is lost with the knockout of the P2Y$_{12}$-R gene as well as with disruption of the gene for Gα_{i2} (Foster et al., 2001; Jantzen et al., 2001). The purified reconstitution system used here allows definitive assessment of the Gα-selectivity of the P2Y$_{12}$-R.

Platelets express four members of the Gi family of G proteins, Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_z. However, the last three are most abundant (Williams et al., 1990; Gagnon et al., 1991). Ohlmann et al. (1995) initially suggested selectivity of coupling of the “platelet receptor” to Gα_{i2} based on immunoprecipitation of photoaffinity-labeled Gα-subunits after stimulation of platelets with ADP. However, this work predated identification of two independent ADP-activated P2Y receptors in platelets; therefore, the analyses did not distinguish which receptor coupled to Gα_{i2}. A more recent study demonstrates a reduction in P2Y$_{12}$-R promoted signaling in Gα_{i2} knockout mice, supporting the hypothesis of preferential coupling of the P2Y$_{12}$-R to Gα_{i2} (Jantzen et al., 2001). Reconstitution of the purified P2Y$_{12}$-R with either Gα_q or Gα_o resulted in no appreciable coupling assessed by measuring GTPase activity in the presence of increasing amounts of 2MeSADP. Although the purified receptor coupled to both Gα_{i1} and Gα_{i3}, our findings with the purified receptor support previous observations that preferential coupling to Gα_{i2} occurs relative to other Gα subunits. Our results do not rule out the possibility that composition of the G$\beta\gamma$ dimer or the RGS protein may contribute to the coupling preference of the receptor. However, parallel experiments with purified M$_2$ muscarinic receptor failed to reveal selectivity of this receptor among Gα_o, Gα_{i1}, Gα_{i2}, or Gα_{i3}. (Fig. 7, inset; S. B. Hooks and T. K. Harden, unpublished data). In addition, independent studies with the purified human P2Y$_1$-R have revealed selective coupling of this receptor to Gα_q but not Gα_i or Gα_o under these conditions (G. L. Waldo and T. K. Harden, unpublished observations).

Although the role of the P2Y$_{12}$-R in ADP-promoted platelet aggregation is well established, the pharmacological selectivity of this receptor has yet to be unequivocally determined. Purification and functional reconstitution of the P2Y$_{12}$-R in model phospholipid vesicles provides an ideal system for studying drug selectivity under conditions free from the problems inherent in studying the $P2Y_{12}$-R in situ. For example, ATP is recovered completely unchanged under the incubation conditions of these assays. In addition, the purified receptor behaves as anticipated by studies of the native receptor, and $G\alpha_{i2}$ is activated in an agonist-dependent manner, which is amplified by the presence of an RGS protein through stimulation of the endogenous GTPase activity.

Whereas the $P2Y_{12}$-R clearly is defined as an "ADP receptor", the status of ATP as a regulator of $P2Y_{12}$-R activity is unclear. Recent studies with exogenously expressed $P2Y_{12}$-R receptor in C6-15 rat glioma cells (Takasaki et al., 2001), recombinantly expressed in 1321N1 cells and B10 cell native expression (Simon et al., 2002) show ATP to be a full agonist. Our findings are in direct contrast to the previously mentioned studies in that ATP exhibited no stimulatory activity at the $P2Y_{12}$-R; indeed, antagonist effects by ATP were observed in the presence of 2MeSADP. This result would indicate that ATP binds to the receptor but is unable to stimulate its activity. In contrast, studies by our lab and others show ATP to be at least a partial agonist at the $P2Y_1$-R (Webb et al., 1993; Filtz et al., 1994; Boyer et al., 1996; Schachter et al., 1996; Palmer et al., 1998). This conclusion is supported by recent studies with purified human $P2Y_1$-R reconstituted with $G\alpha_q\beta_1\gamma_2$ under conditions similar to those described here. That is, ATP is a classic partial agonist at the purified $P2Y_1$-R (G. L. Waldo and T. K. Harden, unpublished observations). The differential effect of ADP and ATP at the $P2Y_1$-R versus the $P2Y_{12}$-R may be a subtle mechanism through which precise regulation of platelet aggregation occurs.

Because of the importance of the $P2Y_{12}$-R in platelet physiology, understanding of the pharmacological properties of this receptor is of critical importance. Studies with P2Y receptors expressed in mammalian cells can be complicated by release of cellular nucleotides as well as by their metabolism and interconversion. The reconstitution system with the purified $P2Y_{12}$-R developed here allows for a definitive assessment of receptor binding selectivity. In addition, we are in a position to further analyze the details of G protein-coupled receptor/G protein coupling and the influence of additional regulatory proteins, including $G\beta\gamma$ dimers, RGS proteins, effectors, and other proteins involved in G protein signaling.

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