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Agonist Binding and Gq-Stimulating Activities of the Purified Human $P2Y_1$ Receptor

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ABSTRACT

The human $P2Y_1$ receptor ($P2Y_1$-R) was purified after high-level expression from a recombinant baculovirus in Sf9 insect cells. Quantification by protein staining and with a radioligand binding assay using the high-affinity $P2Y_1$-R antagonist [^{3}H]MRS2279 ([^{3}H]2-chloro-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine 3′,5′-*bis*-phosphate) indicated a nearly homogenous preparation of receptor protein. K_i values determined in [^{3}H]MRS2279 binding assays for antagonists with the purified $P2Y_1$-R were in good agreement with the K_i and K_B values determined for these molecules in membrane binding and activity assays, respectively. Availability of $P2Y_1$-R in purified form allowed direct determination of nucleotide agonist affinities under conditions not compromised by nucleotide metabolism/interconversion, and an order of affinities of 2-methylthio-ADP (2MeSADP) > ADP = 2-methylthio-ATP = adenosine-5′-*O*-(3-thio)triphosphate = adenosine-5′-*O*-(2-thiodiphosphate) ≫ ATP was obtained. The signaling activity of the purified $P2Y_1$-R was quantified after reconstitution in proteoliposomes with heterotrimeric G proteins. Steady-state GTP hydrolysis in vesicles reconstituted with $P2Y_1$-R and $G\alpha_q\beta_1\gamma_2$ was stimulated by the addition of either 2MeADP or RGS4 alone and was increased by up to 50-fold in their combined presence. EC_{50} values of agonists for activation of the purified $P2Y_1$-R were similar to their respective K_i values determined in radioligand binding experiments with the purified receptor. Moreover, ATP exhibited 20-fold higher EC_{50} and K_i values than did ADP and was a partial agonist relative to ADP and 2MeSADP under conditions in which no metabolism of the nucleotide occurred. Both RGS4 and PLC-β1 were potent and efficacious GTPase-activating proteins for $G\alpha_q$ and $G\alpha_{11}$ in $P2Y_1$-R–containing vesicles. These results illustrate that the binding and signaling properties of the human $P2Y_1$-R can be studied with purified proteins under conditions that circumvent the complications that occur in vivo.

Burnstock's hypothesis (Burnstock, 1972) of purinergic signaling largely has been confirmed over the past two decades by demonstration of regulated release of cellular nucleotides, by elucidation of the mechanisms of metabolism of extracellular nucleotides by a complex array of ecto-enzymes, and by delineation of myriad nucleotide-promoted physiological responses (Dubyak and El-Moatassim, 1993; Harden et al., 1995; Ralevic and Burnstock, 1998; Lazarowski et al., 2003). Moreover, the molecular cloning of two classes of ubiquitously distributed receptors for extracellular nucleotides, the ligand-gated P2X receptors (Khakh et al., 2000) and the G protein-coupled P2Y receptors (Harden et al., 1998), comprising at least 15 human genes, has amplified the importance of nucleotide-promoted signaling.

Although a burgeoning interest in the biology and therapeutic potential of extracellular nucleotide signaling now exists, the complex regulatory pathways that underlie the action of extracellular nucleotides remain difficult to study. Few truly selective agonists and antagonists are available for the P2 receptors, and studies with intact tissues are compromised both by nucleotide release and by metabolism and interconversion of P2 receptor agonists. In the face of these difficulties in the study of nucleotide signaling, little molecular insight into the mechanisms of action of P2 receptors has accrued. This holds particularly true with the P2Y receptors, which, with the exception of the $P2Y_1$-R of turkey erythrocyte membranes (Boyer et al., 1989, 1996b) and the $P2Y_{12}$-R on platelet membranes (Cooper and Rodbell, 1979; Kunapuli, 1998), have been investigated only in intact cell studies. As with other G protein-coupled receptors, the P2Y receptors function in a multiprotein cohort of receptor, heterotrimeric G protein, and effector enzyme or ion channel that transduces nucleotide-promoted signals across the

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ABBREVIATIONS: $P2Y_1$-R, $P2Y_1$ receptor; $P2Y_{12}$-R, $P2Y_{12}$ receptor; PLC, phospholipase C; GAP, GTPase activating protein; MRS2279, 2-chloro-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine 3′,5′-*bis*-phosphate; RGS, regulator of G-protein signaling; 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide *N*-glycosidase; MRS2179, N^6-methyl-2′-deoxyadenosine 3′,5′-bisphosphate.

plasma membrane. The recent discovery of a large class of regulators of G protein signaling (RGS) proteins that promote GTPase activities of heterotrimeric G proteins (Dohlman and Thorner, 1997; Ross and Wilkie, 2000; Hollinger and Hepler, 2002) adds a fourth component of complexity. Observation of activities of RGS proteins in addition to the promotion of GTP hydrolysis (Siderovski et al., 1999; Sierra et al., 2000) as well as the interaction of G protein-coupled receptors with other proteins (Brzostowski and Kimmel, 2001) suggests that traditional models of G protein-coupled receptor signaling are insufficient to describe fully hormone action. Indeed, G protein-coupled receptors may interact directly with proteins to produce signals that largely or entirely circumvent involvement of heterotrimeric G proteins.

We reasoned that problems inherent in the mechanistic study of the P2Y receptors can be circumvented in part by purifying these proteins to homogeneity and reconstituting them with their functional signaling cohorts in model vesicles. This task is made problematic because of the anticipated selectivity of coupling of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors to members of the Gq family of G protein α subunits, which are difficult to purify and study. However, the elegant studies of Ross and coworkers (Berstein et al., 1992; Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999) with purified M1 muscarinic receptors functionally reconstituted with G_q provide an encouraging model. As such, we generated an appropriately tagged construct of the $P2Y_1$-R and purified this protein to homogeneity after expression from a recombinant baculovirus in Sf9 insect cells. A radioligand binding assay was developed for the soluble, purified $P2Y_1$-R using a high-affinity radiolabeled antagonist, [^{3}H]MRS2279, and this assay was applied to determine directly the affinities of ligands, including nucleotide agonists, for the purified receptor. The purified $P2Y_1$-R also was reconstituted functionally in phospholipid vesicles with either $G\alpha_q$ or $G\alpha_{11}$ in heterotrimeric form with $G\beta_1\gamma_2$. Activation kinetics of the receptor were quantified with these model vesicles to establish unambiguously both agonist activity and agonist selectivity for the $P2Y_1$-R.

Materials and Methods

Materials. Phosphatidylserine and phosphatidylethanolamine were obtained from Avanti Polar Lipids (Birmingham, AL), and cholesterol hemisuccinate was from Sigma (St. Louis, MO). Superdex 200, chelating Sepharose FF, and Mono-Q and HiTrap SP FPLC columns were from Amersham Biosciences Inc. (Piscataway, NJ). Ni-NTA agarose and Penta-His antibody were from QIAGEN (Valencia, CA). Anti-FLAG M1 monoclonal antibody was from Sigma. High-purity digitonin was obtained from Calbiochem (San Diego, CA), and Centricon YM-30 concentrators were from Millipore Corporation (Bedford, MA). Other reagents were from sources cited previously (Waldo et al., 2002).

Preparation of Membranes from $P2Y_1$-R–Expressing Sf9 Insect Cells. A recombinant baculovirus was constructed for expression of the $P2Y_1$-R to high levels as was described previously (Waldo et al., 2002). The recombinant virus includes the coding sequence of the human $P2Y_1$-R with a FLAG tag incorporated at the amino terminus and a hexahistidine tag at the carboxyl terminus. Preliminary experiments optimized cell density, multiplicity of infection of virus particles/cell, and time of incubation for optimal $P2Y_1$-R expression. Eight 500-ml spinner cultures (4 liters total) typically were grown to a density of 1.4×10^6 Sf9 cells/ml before infection (multiplicity of infection = 2 virus particles/cell) with recombinant $P2Y_1$-R baculovirus. After incubation at 27°C for 48 h, the infected cells were collected by centrifugation for 10 min at 500*g* in a J6 centrifuge (Beckman Coulter, Inc., Fullerton, CA). All subsequent steps in the membrane preparation were performed at 4°C. The cell pellet was resuspended in cavitation buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 2 mM 2-mercaptoethanol, 100 μg/ml PMSF, 100 μM benzamidine, and 50 μg/ml TPCK), transferred to a Parr cavitation apparatus, and maintained under 600 psi nitrogen for 1 h with constant stirring. The cells were disrupted by rapid return to atmospheric pressure, and disrupted cells were collected after centrifugation for 30 min at 30,000*g* in a JA-14 rotor and J2-21 preparative centrifuge (Beckman Coulter). The pellet was collected, and the supernatant was centrifuged for 30 min at 41,600*g* in a JA-20 rotor (Beckman Coulter). Both pellets were combined and homogenized with a Dounce homogenizer in 200 ml of cavitation buffer. Nuclei and intact cells were removed by centrifugation in conical bottles for 10 min at 200*g* in a J6 centrifuge. The plasma membrane-containing supernatant was collected, and the nuclear pellet was washed twice by Dounce homogenization in 100 ml of cavitation buffer followed by centrifugation for 10 min at 200*g* in the J6 centrifuge. The supernatants from the two nuclear fraction washes were combined with the plasma membrane fraction and centrifuged for 30 min at 41,600*g* in a JA-20 rotor. The resultant pellet was resuspended in 100 ml of freezing buffer (20 mM Tris, pH 8.0, 250 mM sucrose, 2 mM 2-mercaptoethanol, 100 μg/ml PMSF, 100 μM benzamidine, and 50 μg/ml TPCK) by Dounce homogenization and stored at −80°C. Approximately 140 mg of plasma membrane protein was obtained per liter of infected cells.

Purification of $P2Y_1$-R. Plasma membranes (~1.1 g of protein) prepared from 8 liters of $P2Y_1$-R–expressing cells were thawed and combined. The combined membrane fraction was centrifuged for 30 min at 100,000*g* at 4°C in a type 35 rotor and L8-70 ultracentrifuge (Beckman Coulter). The resultant pellet was resuspended by Dounce homogenization in 370 ml (~3 mg membranes/ml) of extraction buffer (70 mM Na_2HPO_4, pH 8.0, 10% glycerol, 5 mM 2-mercaptoethanol, 1% digitonin, 20 mM imidazole, 500 mM NaCl, 100 μg/ml PMSF, 50 μg/ml TPCK, 100 μM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and stirred for 1 h at room temperature. The digitonin-extracted membranes were centrifuged for 1 h at 100,000*g* in a type 35 rotor (Beckman Coulter). The soluble $P2Y_1$-R fraction was incubated for 4 h at room temperature with 12 ml of chelating Sepharose FF charged previously with $NiSO_4$ according to the manufacturer's instructions. The $P2Y_1$-R–bound resin was transferred to a 2.5-cm diameter chromatography column and washed with 4 bed volumes of extraction buffer followed by 12 bed volumes of extraction buffer containing a lower concentration (0.1%) of digitonin. The recombinant $P2Y_1$-R was eluted with 36 ml of buffer containing 50 mM Na_2HPO_4, pH 7.4, 150 mM NaCl, 0.1% digitonin, 10% glycerol, 250 mM imidazole, 100 μg/ml PMSF, 50 μg/ml TPCK, 100 μM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The $P2Y_1$-R–containing eluant was desalted on a 100-ml column of Sephadex G-50 equilibrated with buffer A (50 mM Na_2HPO_4, pH 7.4, 10% glycerol, 0.1% digitonin, 100 μg/ml PMSF, 50 μg/ml TPCK, 100 μM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The desalted $P2Y_1$-R–containing fraction was loaded onto a 1-ml HiTrap SP FPLC column and eluted with a 30-ml gradient from 0 to 400 mM NaCl in buffer A. $P2Y_1$-R–containing fractions eluting from the HiTrap SP column were concentrated to 2 ml by passage through a Centricon YM-30 membrane (Millipore) and applied to a Superdex 200 (1.6 × 66 cm, 133 ml) gel-filtration column equilibrated with 50 mM Na_2HPO_4, pH 7.4, 10 mM glycerol, 100 mM NaCl, and 0.1% digitonin. The $P2Y_1$-R–containing fraction eluting from the Superdex 200 column was concentrated to 0.6 ml by passage through a Centricon YM-30 membrane. The final yield was approximately 2 nmol of $P2Y_1$-R.

Purification of $G\alpha_q$, $G\alpha_{11}$, $G\beta_1\gamma_2$, RGS4, and PLC-β1. $G\alpha_q$ and $G\alpha_{11}$ were purified according to the method described by Kozasa and Gilman (1996). Briefly, membranes expressing recombinant $G\beta_1$, hexahistidine-tagged $G\gamma_2$, and either $G\alpha_q$ or $G\alpha_{11}$ were prepared

from Sf9 insect cells 48 h after infection with the corresponding recombinant baculoviruses. Heterotrimeric G protein was extracted from the Sf9 membranes with a buffer containing 1% sodium cholate. The soluble extract was diluted 4-fold with buffer containing 0.5% $C_{12}E_{10}$ before binding the soluble G protein heterotrimer to nickel nitrilotriacetic acid affinity resin via the hexahistidine-tagged $G\gamma_2$ subunit. The $G\alpha$ subunit was eluted from the affinity matrix with a buffer containing 50 mM $MgCl_2$, 10 mM NaF, and 0.03 mM $AlCl_3$. The $G\alpha$ subunit was further purified by Mono-Q FPLC ion-exchange chromatography as described elsewhere.

$G\beta_1\gamma_2$ purification (Kozasa and Gilman, 1996) was performed with use of a strategy similar to that used for the purification of $G\alpha_q$ and $G\alpha_{11}$, except that $G\beta_1$ and $G\gamma_2$ were coexpressed in Sf9 cells with hexahistidine-tagged $G\alpha_{i1}$. RGS4 was purified after bacterial expression as described by Hepler et al. (1997). PLC-β1 was purified from the cytosol of Sf9 insect cells after infection with a recombinant baculovirus as described previously (Paterson and Harden, 1996).

***N*-Glycosidase F Treatment of Purified $P2Y_1$-R and SDS-PAGE Analysis.** Approximately 6 pmol of $P2Y_1$-R was incubated overnight at room temperature with or without 1000 U of peptide *N*-glycosidase (PNGase) F (New England BioLabs, Beverly, MA). After the overnight incubation SDS-PAGE sample buffer was added to each reaction and the proteins were separated on 12.5% SDS-PAGE gels. The proteins were either stained with Coomassie blue or transferred to nitrocellulose for immunoblot analysis with anti-FLAG M1 or anti-Penta-His monoclonal antibodies.

Radioligand Binding Assay of Soluble $P2Y_1$-R. Approximately 18 ng of purified $P2Y_1$-R was incubated with [^{3}H]MRS2279 (Waldo et al., 2002) and buffer or the indicated concentration of competing drug for 60 min in an ice-water bath in a total assay volume of 50 μl. All subsequent steps were performed at 4°C. Saturation-binding isotherms were generated by incubation of soluble receptor with [^{3}H]MRS2279 at concentrations of radioligand ranging from 0.5 to 60 nM. Specific binding was defined as total [^{3}H]MRS2279 binding minus binding occurring in the presence of 10 μM MRS2179. Agonist and antagonist competition curves were generated by incubation of approximately 20,000 cpm (~5 nM) of [^{3}H]MRS2279 with increasing concentrations of competing drug. After the 60-min incubation, bound radioligand was separated from free radioligand by Sephadex G-50 spin columns. Briefly, 4 ml of Sephadex G-50 in Poly-Prep chromatography columns (0.8 × 4 cm) (Bio-Rad, Hercules, CA) was washed with 20 ml of wash buffer (20 mM Tris, pH 7.5, 145 mM NaCl, 1 mM EDTA, and 5 mM $MgCl_2$) and then was equilibrated with 4 ml of wash buffer containing 0.1% digitonin. The columns were centrifuged for 10 to 15 s at 750*g* in a JS-3.0 rotor (Beckman Coulter) before application of samples. Bound radioligand was isolated sequentially from each set of triplicate assays by transferring 40 μl of each reaction to a prespun G-50 column and immediately centrifuging the three columns for 5 min at 1775*g* in a JS-4.2 rotor (Beckman Coulter). Bound radioligand was collected directly into miniscintillation vials during the centrifugation. Scintillant was added, and radioactivity was quantified by liquid scintillation spectrometry. K_i values were calculated for the agonists and antagonists from the EC_{50} values obtained in competition experiments using the average K_d value (3.8 nM) from three saturation-binding isotherms for [^{3}H]MRS2279.

Reconstitution of $P2Y_1$-R into Proteoliposomes. Purified $P2Y_1$-R was co-reconstituted with either $G\alpha_q$ or $G\alpha_{11}$ and $G\beta_1\gamma_2$ into proteoliposomes by a modification of the method used by Brandt et al. (1983) and as described in detail by Cunningham et al. (2001). Briefly, 15 pmol of purified $P2Y_1$-R, 50 pmol of $G\alpha$, and 150 pmol of $G\beta_1\gamma_2$ were reconstituted by Sephadex G-50 chromatography into phospholipid vesicles containing L-α-phosphatidylserine from brain, L-α-phosphatidylethanolamine from liver, and cholesteryl hemisuccinate.

Quantification of Steady-State GTP Hydrolysis. Steady-state GTPase activity of the reconstituted proteoliposomes was determined in the absence or presence of the indicated concentrations of RGS4 or PLC-β1 and with or without the indicated concentrations of $P2Y_1$-R agonist or antagonist. Unless otherwise indicated, the assays (final volume, 50 μl) were incubated for 30 min at 30°C and contained 20 mM HEPES, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 2 μM [γ-^{32}P]GTP (~4500 cpm/pmol). The assays were terminated by the addition of 950 μl of a 4°C solution of 5% activated charcoal in 20 mM H_3PO_4. After centrifugation, liberated [^{32}P]P_i in the supernatant was quantified by liquid scintillation spectrometry.

Results

With the goal of purifying the $P2Y_1$-R to homogeneity in a fully functional form, a recombinant baculovirus was constructed for high-density expression of the human $P2Y_1$-R in Sf9 insect cells. Conditions, including multiplicity of virus infection, time of incubation, and cell density, were optimized for the expression of immunoreactivity of the recombinant receptor (see *Materials and Methods*). Although both dodecylmaltoside and digitonin were effective in solubilizing a large percentage of the immunoreactivity from a membrane preparation of $P2Y_1$-R–expressing Sf9 cells, receptor signaling activity only was retained in the presence of digitonin. Therefore, the purification and all of the analyses of the human $P2Y_1$-R described below were carried out with receptor purified in the presence of digitonin.

We recently reported that [^{3}H]MRS2279 is a reliable radioligand for quantification of both recombinant and natively expressed $P2Y_1$-R (Waldo et al., 2002). Much of our initial analyses of this radioligand were accomplished with membranes prepared from Sf9 cells expressing the recombinant human $P2Y_1$-R to high levels. Given the relatively high hydrophilicity of [^{3}H]MRS2279 and the reliability of this radioligand for quantification of membrane $P2Y_1$-R, we examined whether [^{3}H]MRS2279 binding might also be a useful means to directly assess the receptor in a soluble form. As was reported previously (Waldo et al., 2002), [^{3}H]MRS2279 bound to the solubilized receptor with an affinity similar to that of the membrane receptor. The relative affinities of competing $P2Y_1$-R ligands determined with the soluble receptor (see below) also were similar to those obtained with the $P2Y_1$-R in its native membrane form. Thus, [^{3}H]MRS2279 was used as a means to quantify active $P2Y_1$-R during purification.

The $P2Y_1$-R was expressed to high levels in Sf9 cells, membranes were prepared, and the receptor was purified as described in detail under *Materials and Methods*. The [^{3}H]MRS2279 binding assay was applied to quantify soluble $P2Y_1$-R (Table 1), and approximately 45% of receptor binding activity solubilized from membranes was recovered in the final purification step. Approximately 2 nmol of [^{3}H]MRS2279 binding sites was purified in a typical preparation from 8 liters of infected Sf9 cells, and approximately 1000-fold purification of [^{3}H]MRS2279 binding activity was observed. Saturation-binding studies of the purified $P2Y_1$-R are considered below. Analyses of the purified receptor fraction by SDS-PAGE revealed a diffusely migrating protein species of approximately 44 kDa identified either by protein staining or by immunoblots with anti-FLAG or anti-His antiserum (Fig. 1). A much less abundant, slower migrating species also was observed at approximately 95 kDa with most receptor preparations. The amount of this 95-kDa species markedly increased after incubation of the purified receptor at room temperature or 37°C; incubation also increased the occurrence of both an immunoreactive species of approxi-

mately 130 kDa and an aggregated protein that did not enter the gel (Fig. 1). Thus, multimeric forms of the $P2Y_1$-R were barely detectable with the freshly purified receptor but could be induced to occur by maintaining the receptor preparation at elevated temperature.

Treatment of the purified $P2Y_1$-R preparation with *N*-glycosidase F resulted in a faster migrating receptor species presumably because of the removal of N-linked carbohydrate moieties (Fig. 1). Although the faster migrating species was somewhat less diffuse than the native $P2Y_1$-R, the appearance of heterogeneity remained. Variation in the temperature and time of incubation with glycosidase treatment did not produce a sharper $P2Y_1$-R species. Similarly, denaturation of the preparation with SDS and treatment with β-mercaptoethanol before incubation with *N*-glycosidase F also failed to decrease the apparent heterogeneity. We are uncertain whether these observations follow from incomplete deglycosylation or the occurrence of additional covalent modifications (e.g., phosphorylation, palmitoylation) of the $P2Y_1$-R.

The purified $P2Y_1$-R bound [^{3}H]MRS2279 with high affinity (K_d = 3.8 ± 0.3 nM) and by law of mass action-binding kinetics for ligand interaction with a single site (Fig. 2). The density of binding sites (approximately 3 nmol/mg protein) represents a stoichiometry of binding that is only 10 to 15% of theoretical binding to a single site of a protein with a molecular size of approximately 44,000. This observation was surprising given the apparent purity of the protein determined in protein-staining analyses (Fig. 1). Several factors probably contribute to the somewhat low binding stoichiometry, including the difficulty of accurately quantifying protein in relatively small samples and the somewhat cumbersome G-50 Sephadex centrifugation technique used to separate bound from free [^{3}H]MRS2279, which allows the dissociation of significant amounts of specific radioligand binding (k_2 = 0.721 min^{-1} in membrane filtration assay) (Waldo et al., 2002). Thus, the values obtained in the soluble binding assay underestimate the true B_{max} values. Extrapolation of a first-order rate plot (data not shown) of the dissociation of radioligand– $P2Y_1$-R complexes that occurs after the addition of reactants to the G-50 column predicts that the specific activity of [^{3}H]MRS2279 binding to the purified $P2Y_1$-R is at least 5.5 nmol/mg (approximately 25% of theoretical). Moreover, because the purification scheme used here resulted in the recovery of up to 50% of the $P2Y_1$-R present in the detergent extract (Table 1), we believe that a significant amount of the $P2Y_1$-R present in the starting membrane preparation represents $P2Y_1$-R protein that lacks functional binding activity. That is, significant loss of binding activity does not occur during purification, and protein-stained gels indicate >90% purity of the $P2Y_1$-R protein.

The pharmacological properties of the $P2Y_1$-R have been widely studied. However, consensus conclusions on the relative potencies and efficacies of agonists at the $P2Y_1$-R have not been attained despite rigorous attempts to do so by many laboratories. This deficit has followed from the propensity of most of these nucleotide agonists to undergo breakdown and/or interconversion. The recent elucidation of a direct binding assay using a high-affinity non-nucleotide antagonist of the $P2Y_1$-R has for the first time provided a means to directly determine ligand affinities for the $P2Y_1$-R (Waldo et al., 2002). However, assessment with [^{3}H]MRS2279 of binding affinities of nucleotide agonists using membranes expressing recombinant $P2Y_1$-R to high levels is still compromised by nucleotide metabolism, as we reported previously (Waldo et al., 2002). In contrast, application of the [^{3}H]MRS2279 binding assay to $P2Y_1$-R purified to homogeneity by definition circumvents any problems contributed by enzymatic metabolism of nucleotides. Therefore, we carried out detailed radioligand binding analyses with the purified human $P2Y_1$-R. Competition binding isotherms were generated for nucleotides and nucleotide analogs known to interact with the $P2Y_1$-R (Fig. 3), and K_i values of these analogs for the $P2Y_1$-R were determined. It should be pointed out that, because these binding affinities were determined with purified receptor in the absence of heterotrimeric G_q, they represent affinities for the uncoupled low-affinity agonist binding state of the $P2Y_1$-R. The Hill slopes of all competition curves were near unity (data not shown). The nucleotide analog 2MeADP exhibited the lowest K_i, and ADP, the cognate agonist of the receptor, exhibited approximately 10-fold lower affinity than did 2MeSADP. ATP, which has been concluded to be an agonist (Webb et al., 1993; Filtz et al., 1994; Henderson et al., 1995; Schachter et al., 1996), partial agonist (Palmer et al., 1998), or antagonist (Leon et al., 1997; Hechler et al., 1998) of the $P2Y_1$-R, exhibited a K_i value (18 μM) approximately 20-fold higher than that of ADP. K_i values of 1 to 2 μM were observed for ADPβS, 2MeSATP, and ATPγS (Table 2). The molecule adenosine 3′,5′-bisphosphate, originally identified (Boyer et al., 1996a) as a selective bisphosphate antagonist of the $P2Y_1$-R, exhibited a K_i value of approximately 1 μM (Table 1), whereas the K_i values of

TABLE 1

Recovery of specific binding sites for [^{3}H]MRS2279 during $P2Y_1$-R purification

[^{3}H]MRS2279 binding assays were carried out on membranes prepared from $P2Y_1$-R–expressing Sf9 cells and in a digitonin extract from these membranes that was subsequently purified by multiple column steps as described under *Materials and Methods*. Binding activity is indicated in the pooled receptor-containing sample obtained after Ni-Sepharose, Sephadex G-50, HiTrap SP, and Superdex 200 column chromatography. The data are presented as specific binding of [^{3}H]MRS2279 in the total pool from each step in the purification, total protein in that pool, calculated specific activity of binding sites/mg protein, and fold purification of [^{3}H]MRS2279 binding sites. The binding data were determined from a single concentration of radioligand (8.9 nM; ~2.2 × K_d) and were corrected to reflect B_{max} values. In addition, saturation-binding isotherms were performed on the membrane preparation, on the digitonin extract, and on the purified $P2Y_1$-R, and the B_{max} values obtained were identical with those obtained in assays using a single radioligand concentration. The K_d values of [^{3}H]MRS2279 in the soluble binding assay with the digitonin extract and the purified $P2Y_1$-R were both approximately 4 nM. The results are representative of four different purifications.

Sample	[^{3}H]MRS2279 Bound	Total Protein	Specific Activity	-Fold Purification
	nmol/pool	*mg/pool*	*nmol/mg*	
Sf9 membranes	4.60 ± 0	1100	0.004	
Digitonin extract	4.99 ± 0.91	635	0.008	2
Ni Sepharose	3.80 ± 0.04	30.4	0.125	31
Sephadex G-50	3.59 ± 0.09	23.3	0.154	39
HiTrap SP	2.52 ± 0.41	2.19	1.15	287
Superdex 200	2.25 ± 0.25	0.737	3.05	762

MRS2179 and MRS2279, which are bisphosphate analogs synthesized (Boyer et al., 1998, 2002; Camaioni et al., 1998; Nandanan et al., 2000) to enhance affinity for the $P2Y_1$-R, were 15 and 11 nM, respectively. These K_i values determined with the purified $P2Y_1$-R are in good agreement with the respective K_i values determined in binding assays with membranes and with respective K_B values determined in studies of antagonism of 2MeSADP-stimulated responses in intact cells.

These direct binding studies with the purified human $P2Y_1$-R provide the first unequivocal determination of binding affinities of ligands for this receptor. They do not, however, indicate whether the purified $P2Y_1$-R retains signaling activity, and they also do not provide assessment of the relative agonist activities of nucleotides and nucleotide analogs at the purified receptor. As such, a model phospholipid-vesicle system was developed in which the purified $P2Y_1$-R was reconstituted with various purified $G\alpha$ subunits and purified $G\beta_1\gamma_2$, as described under *Materials and Methods*. GTPase activity of the reconstituted $G\alpha$ subunits was quantified by measuring the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]GTP$, and potential agonist activity of $P2Y_1$-R ligands was assessed by measuring the concentration-dependent capacity of these molecules to increase GTPase activity of the reconstituted $G\alpha$ subunits. Addition of the $P2Y_1$-R agonist 2MeSADP to proteoliposomes reconstituted with purified $P2Y_1$-R, $G\alpha_{11}$, and $G\beta_1\gamma_2$ resulted in a small but reproducible increase in GTPase activity (Fig. 4). Addition of the RGS protein, RGS4, to these vesicles in the absence of a $P2Y_1$-R agonist also elicited a small increase in GTPase activity. Guanine nucleotide exchange is rate-limiting in the catalytic cycle of heterotrimeric G proteins. Therefore, coaddition of 2MeSADP to promote guanine nucleotide exchange and RGS4 to activate the GTPase of $G\alpha_{11}$ resulted in GTP hydrolysis at rates that were up to 50-fold greater than those observed with either agonist or RGS protein alone (Fig. 4). GTP hydrolysis was linear for up to 45 min, and therefore, steady-state GTPase activity was measured under the conditions of these assays. The rate of GTP hydrolysis also was linearly dependent on the amount of proteoliposomes in the assay (data not shown). The purified $P2Y_1$-R also functionally coupled to $G\alpha_q\beta_1\gamma_2$, as is illustrated below. In contrast, relatively small effects of 2MeSADP and RGS4 were observed in vesicles reconstituted with purified $P2Y_1$-R and $G\alpha_0$, $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ in heterotrimeric form with $G\beta_1\gamma_2$ (data not shown). Under these same conditions, marked agonist and RGS4-stimulated GTP hydrolysis was observed in proteoliposomes reconstituted with M2 muscarinic receptors and $G\alpha_0\beta_1\gamma_2$, $G\alpha_{i1}\beta_1\gamma_2$, $G\alpha_{i2}\beta_1\gamma_2$, and $G\alpha_{i3}\beta_1\gamma_2$ (data not shown). Thus, as is suggested from studies with intact cell systems, the $P2Y_1$-R preferentially couples to $G\alpha_q$ rather than to $G\alpha$ subunits of the G_i family.

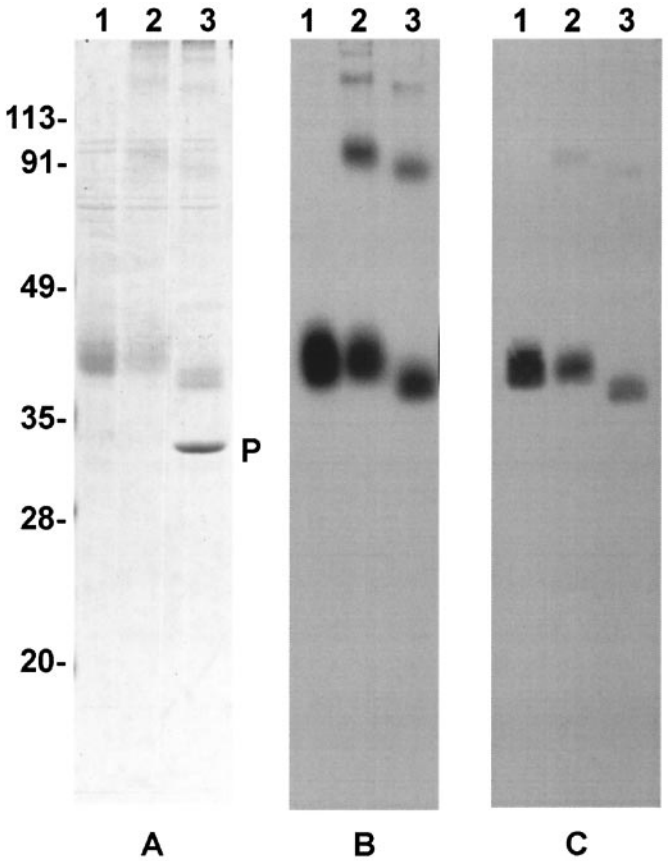

Fig. 1. SDS-PAGE and immunoblot analysis of purified recombinant human $P2Y_1$-R. Recombinant $P2Y_1$-R tagged with His-6 and FLAG epitopes was purified from a digitonin extract of Sf9 insect cell plasma membranes, and *N*-glycan moieties were removed from the glycoprotein as described under *Materials and Methods*. Purified $P2Y_1$-R incubated with PNGase F (lane 3), without PNGase F (lane 2), or untreated $P2Y_1$-R (lane 1) were subjected to SDS-PAGE analysis. The resulting gels were stained with Coomassie blue (A) or transferred to nitrocellulose and immunoblotted with anti-FLAG antibody (B) or anti-Penta-His antibody (C). The PNGase enzyme is identified (P) in the Coomassie-stained gel.

The responses illustrated in Fig. 4 suggested that this reconstitution system can be used to determine pharmacological properties of the $P2Y_1$-R by varying agonist and antagonist concentrations in the presence of a fixed concentration of RGS protein. Pharmacological studies are demonstrated with $P2Y_1$-R/$G\alpha_{11}$/$G\beta_1\gamma_2$ vesicles in Fig. 5. In the absence of added GTPase-activating protein, 2MeSADP elicited a small but significant concentration-dependent increase in steady-state GTPase activity (Fig. 5A). However, in the presence of 100 nM RGS4, a marked concentration of 2MeSADP-dependent stimulation of GTP hydrolysis was observed. As illustrated in Fig. 5B, the $P2Y_1$-R antagonist MRS2279 inhibited the capacity of 1 μM 2MeSADP to stimulate GTPase with an IC_{50} value consistent with the affinity of MRS2279 for binding to the human $P2Y_1$-R.

This reconstitution system using purified proteins provides a well-controlled system that circumvents many of the difficulties inherent in studying activities of P2Y receptors under conditions in which significant hydrolysis and intercoversion of added nucleotides and/or release of cellular nucleotides occurs. Given that the purified receptor exhibited signature responses to an agonist and antagonist of the $P2Y_1$-R (Fig. 5, A and B), we generated a series of concentration-effect curves for activation of the purified receptor (Fig. 6). These studies were carried out with vesicles reconstituted with purified recombinant $G\alpha_q$ rather than with the very similar $G\alpha$ subunit $G\alpha_{11}$, which was used in the experiments presented in Figs. 4 and 5. The maximal effects of 2MeSADP and 2MeSATP on GTPase activity were equivalent, and the potency of 2MeSADP (EC_{50} = 150 nM) was approximately 20-fold greater than that of 2MeSATP (EC_{50} = 3 μM). The presence of 2MeSADP in the preparation of 2MeSATP apparently did not contribute to its activity because HPLC purification of

commercial 2MeSATP just before its use did not result in a significant change in its activity (Fig. 6A). The maximal effect observed with ADP was similar to that observed with 2MeSADP, and ADP exhibited an EC_{50} value of approximately 1 μM (Fig. 6B). Thus, 2MeSADP, 2MeSATP, and ADP are all full agonists at the purified P2Y$_1$-R. ADPβS and ATPγS were less effective agonists than 2MeSADP and ADP for activation of the purified P2Y$_1$-R, and both ADPβS and ATPγS seemed to be partial agonists. UTP, UDP, $\alpha\beta$MeATP, and $\beta\gamma$MeATP all were inactive at the purified P2Y$_1$-R (Fig. 6C).

Whether ATP is an agonist or an antagonist at the P2Y$_1$-R is an important question in understanding the biological regulation of this receptor. This issue has not been entirely resolved, despite rigorous studies from a number of laboratories (Hechler et al., 1998, Palmer et al., 1998). Availability of purified P2Y$_1$-R reconstituted with purified G_q allowed us to assess the action of ATP under conditions in which no conversion of ATP to ADP occurs. ATP exhibited an efficacy for stimulation of GTPase activity that was approximately 30% of that of the full P2Y$_1$-R agonists 2MeSADP, 2MeSATP, and ADP (Figs. 6B and 7A). The partial agonist activity of ATP was further confirmed by illustrating that over the range of ATP concentrations that stimulated GTPase activity, inhibition of the stimulatory activity of nearly maximally effective concentrations of 2MeSADP also occurred (Fig. 7B).

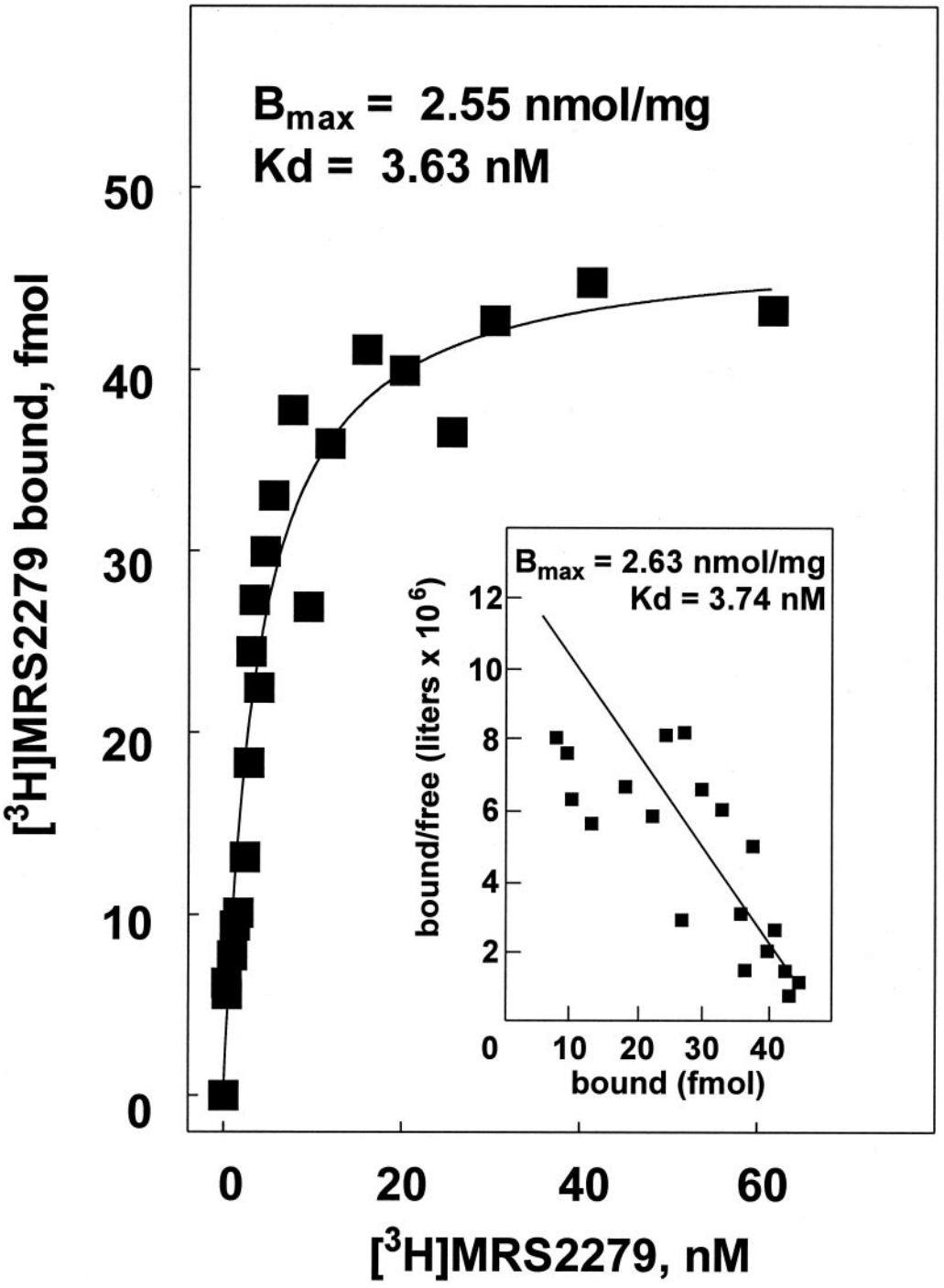

Fig. 2. Saturation-binding isotherm for [^{3}H]MRS2279 binding to purified P2Y$_1$-R. P2Y$_1$-Rs were purified from a digitonin extract of P2Y$_1$ Sf9 membranes as described under *Materials and Methods*. Approximately 18 ng of purified P2Y$_1$-R was incubated with the indicated concentrations (0.5–60 nM) of [^{3}H]MRS2279 for 60 min at 4°C. Bound radioligand was separated from free by Sephadex G-50 chromatography. Data are plotted as specific binding and are representative of three similar experiments. A Scatchard transformation of the data is presented in the inset.

Metabolism of [γ-^{32}P]ATP was simultaneously measured under the conditions of the assays presented in Fig. 7, A and B, to ascertain whether the formation of ADP from ATP accounted for any of the activity of ATP. Only 0.07% of the ATP was hydrolyzed in a 30-min incubation under these conditions (Table 3). This level of metabolism would not result in a $P2Y_1$-R–activating concentration of ADP, even assuming that all of the conversion was to ADP alone and that this ADP was present during an entire 30-min test of agonist activity. Thus, we conclude that ATP is a relatively weak partial agonist at the $P2Y_1$-R.

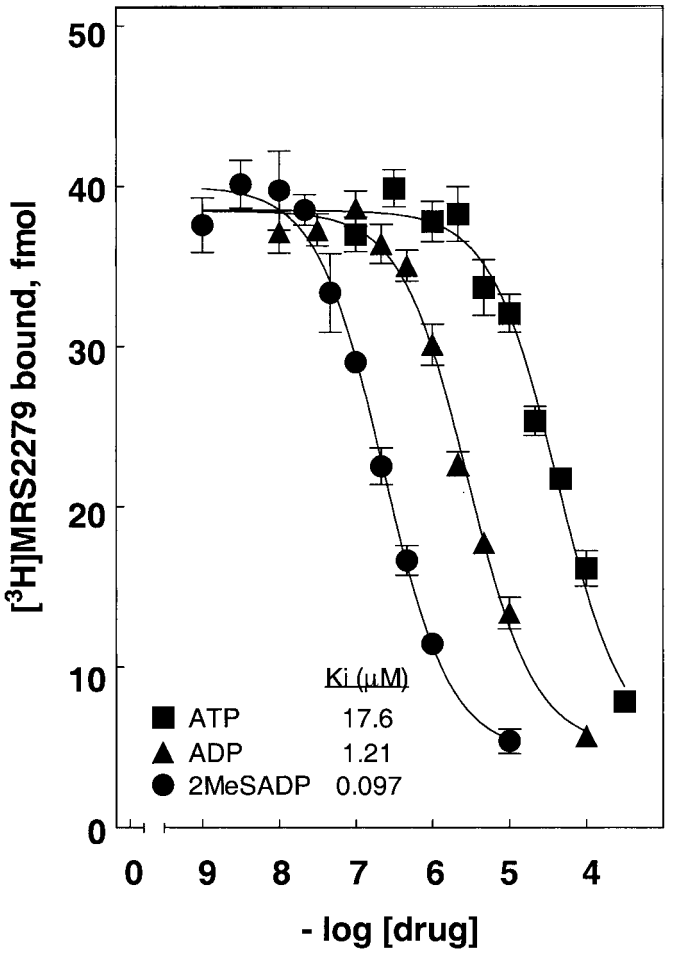

Fig. 3. Pharmacological selectivity of 2MeSADP, ADP, and ATP for the inhibition of [^{3}H]MRS2279 binding to purified $P2Y_1$-R. Approximately 18 ng of purified $P2Y_1$-R was incubated for 60 min at 4°C with 5.4 nM [^{3}H]MRS2279 and the indicated concentrations of the $P2Y_1$-R nucleotide ligands 2MeSADP (●), ADP (▲), and ATP (■). Bound radioligand was separated from free by Sephadex G-50 chromatography. Data are representative of five similar experiments. K_i values are indicated.

TABLE 2

Pharmacological selectivity of $P2Y_1$-R agonists and antagonists for inhibition of [^{3}H]MRS2279 binding to purified $P2Y_1$-R

K_i values were calculated as described under *Materials and Methods*. Data presented are mean ± S.E. determined from three to five different experiments each carried out in triplicate assays.

Substance	*n*	K_i
		μM
Agonist		
2MeSADP	5	0.099 ± 0 0.015
ADP	5	0.92 ± 0.23
ATPγS	5	1.33 ± 0.42
2MeSATP	5	1.87 ± 0.48
ADPβS	5	2.42 ± 0.44
ATP	5	17.7 ± 2.39
Antagonist		*nM*
MRS2279	3	11.5 ± 3.0
MRS2179	3	15.4 ± 2.5
A3′P5′P	3	754 ± 107

In the presence of a $P2Y_1$-R agonist, guanine nucleotide exchange is no longer rate-limiting, and the capacity of various GTPase activating proteins to stimulate GTP hydrolysis by $G\alpha_q$ can be assessed. Thus, we also applied vesicles reconstituted with purified $P2Y_1$-R and heterotrimeric G_q or G_{11} to assess the action of proteins known to be GAPs of G_q family $G\alpha$ subunits. As illustrated in Fig. 8, both RGS4 and PLC-β1 caused a concentration-dependent stimulation of the GTPase activity of $G\alpha_q$ and $G\alpha_{11}$. The maximal activity observed with RGS4 was slightly greater than that of PLC-β1, whereas PLC-β1 was consistently slightly more potent than RGS4 under the conditions of these assays. $G\alpha_q$ and $G\alpha_{11}$ exhibit approximately 90% sequence identity, and these ubiquitously expressed $G\alpha$ subunits exhibit completely interchangeable activities in a variety of biological test conditions. As illustrated in Fig. 8, the concentration-dependence for stimulation of GTPase activities by RGS4 and of PLC-β1 also were essentially identical whether $G\alpha_q$- or $G\alpha_{11}$-containing vesicles were studied.

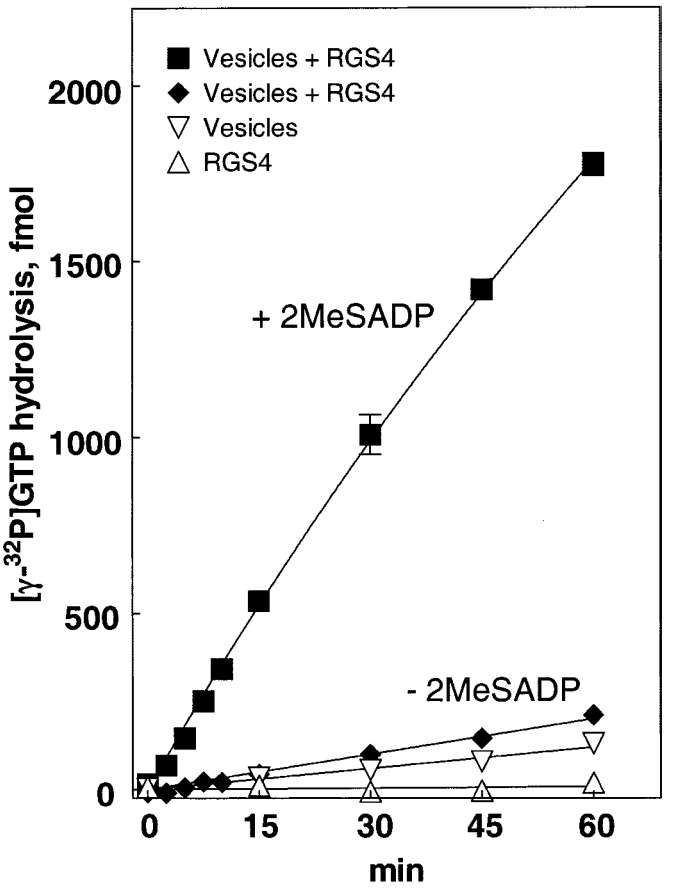

Fig. 4. 2MeSADP-promoted steady state GTP hydrolysis by $P2Y_1$-R/$G\alpha_{11}$ proteoliposomes. Purified human $P2Y_1$-R, $G\alpha_{11}$, and $G\beta_1\gamma_2$ were reconstituted in phospholipid vesicles. GTP hydrolysis was measured at 30°C for the indicated times in the presence of 100 nM RGS4 without proteoliposomes (△), in the presence of proteoliposomes without RGS4 (▽), or in the presence of 100 nM RGS4 and proteoliposomes in the absence (◆) or presence (■) of 1 μM 2MeSADP.

Discussion

High-level expression of a hexahistidine-tagged P2Y$_1$-R in Sf9 insect cells provided technological advantages for purifying the human P2Y$_1$-R. When reconstituted with its protein-signaling cohorts in model phospholipid vesicles, the purified receptor exhibited signaling and pharmacological characteristics reminiscent of those predicted from studies of the native P2Y$_1$-R observed in a variety of target tissues. Thus, recombinant P2Y$_1$-R can be obtained in a nearly homogeneous form that retains activity in its agonist-binding site and in the domain(s) that functionally interacts with G$_q$. Approximately 25% of the purified P2Y$_1$-R is active as assessed by radioligand binding, which is similar to that reported for epitope-tagged β_2 adrenergic receptor (30% of theoretical) after purification to homogeneity and before a final ligand affinity-purification step (Kobilka, 1995). This model system allowed the unambiguous illustration of the overall agonist selectivity of the P2Y$_1$-R and the partial agonist activity of ATP at the P2Y$_1$-R. P2Y$_1$-R–containing proteoliposomes also were used to illustrate that the interaction of the purified receptor with downstream regulatory proteins RGS4 and PLC-β1 could be studied and to establish that neither RGS4 nor PLC-β1 exhibits selectivity as GTPase-activating proteins for Gα_q versus Gα_{11}.

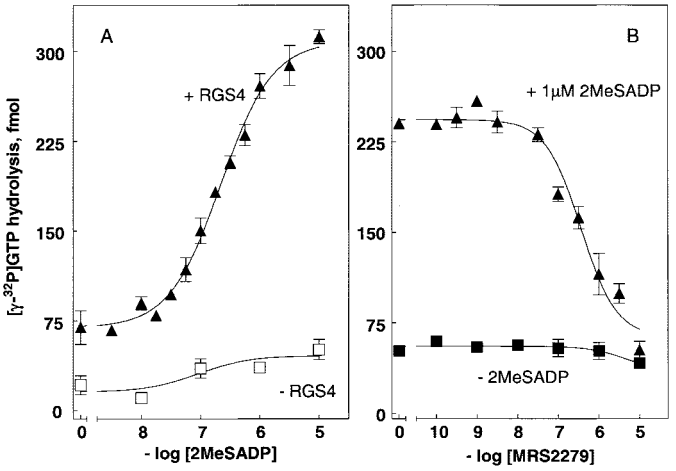

Fig. 5. Concentration-dependence of agonist and antagonist activities measured with purified P2Y$_1$-R reconstituted in proteoliposomes. Purified P2Y$_1$-R, Gα_{11}, and G$\beta_1\gamma_2$ were reconstituted in proteoliposomes. A, steady-state GTP hydrolysis was measured in proteoliposomes incubated in the absence (□) or presence (▲) of 100 nM RGS4 and the indicated concentration of the P2Y$_1$-R agonist 2MeSADP. B, steady-state GTP hydrolysis was measured in proteoliposomes incubated with 100 nM RGS4 and the indicated concentrations of the P2Y$_1$-R antagonist MRS2279 with (▲) or without (■) 1 μM 2MeSADP.

The affinities of agonists for the P2Y$_1$-R have not been known because reliable means for their determination have not been available. Tissues contain high levels of nucleotide-metabolizing enzymes, and we found that expression of recombinant P2Y$_1$-R to very high levels still does not allow sufficiently low membrane concentrations in biochemical assays to overcome nucleotide metabolism (Waldo et al., 2002). Drugs of sufficient selectivity and affinity also are not available to effect pharmacological blockade of endogenous nucleotide metabolism. The development of [^{3}H]MRS2279 as an antagonist radioligand for the P2Y$_1$-R provided the first reliable means to quantify directly the interaction of ligands with membrane P2Y$_1$-R. As we described recently in detail, this methodology alone did not circumvent the aforementioned problems encountered in determining agonist affinity at membrane P2Y$_1$-R (Waldo et al., 2002). However, the [^{3}H]MRS2279 binding assay now applied to studies of the human P2Y$_1$-R purified to homogeneity have circumvented these concerns. Thus, we directly measured the binding of

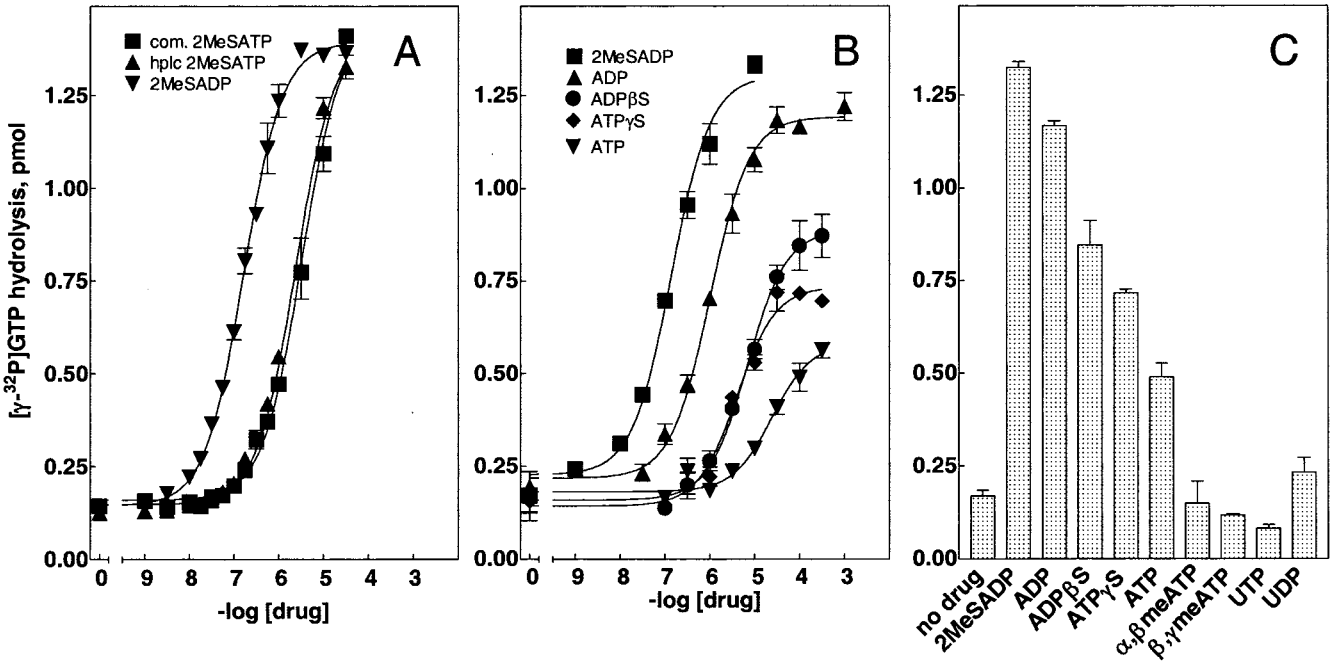

Fig. 6. Agonist selectivity of the purified P2Y$_1$-R. Purified P2Y$_1$-R, Gα_q, and G$\beta_1\gamma_2$ were reconstituted in proteoliposomes. A and B, Steady-state GTP hydrolysis was measured in proteoliposomes incubated with 100 nM RGS4 and the indicated concentration of P2Y$_1$-R agonist: A, commercial 2MeSATP (■), HPLC-purified 2MeSATP (▲), and 2MeSADP (▼); B, 2MeSADP (■), ADP (▲), ADPβS (●), ATPγS (◆), and ATP (▼). C, Steady-state GTP hydrolysis was measured in proteoliposomes incubated with no drug, with 10 μM 2MeSADP, or with 100 μM of the indicated drugs.

molecules to the $P2Y_1$-R under conditions in which molecules are not metabolized or interconverted to other nucleotides. Although the general consensus has been that the $P2Y_1$-R is preferentially activated by ADP, both the affinity and agonist activity of ATP relative to ADP have been questions of considerable disagreement. We show here by direct and unambiguous means that the affinity of ADP for the human $P2Y_1$-R is approximately 20-fold greater than that of ATP.

Cell signaling through heterotrimeric G proteins is ultimately governed by the rate of exchange of guanine nucleotides and the rate of hydrolysis of GTP by the involved G protein α subunit (Gilman, 1987). Thus, in conditions in which multiple rounds of nucleotide hydrolysis occur, GTPase activity is regulated through the capacity of receptors to act as guanine nucleotide exchange factors and through the activity of various classes of proteins to increase the $k_{catalysis}$ of the GTPase activity of the involved G protein α subunit. We took advantage of the rate-limiting nature of both of these steps in the catalytic cycle of G proteins to analyze the concentration-dependence of agonists at the $P2Y_1$-R for enhancing GTPase activity in the presence of a saturating concentration of a GAP, and the concentration-dependence of GAPs, e.g., PLC-β1 or RGS4, for enhancing GTPase activity in the presence of a maximally effective concentration of a $P2Y_1$-R agonist. Our results indicate that under the conditions of these assays, the level of agonist-stimulated GTPase activity observed is proportional to the amount of reconstituted $P2Y_1$-R. Thus, the relative potencies and maximal effects of agonists observed under these conditions are related to their relative affinities and intrinsic efficacies for the $P2Y_1$-R. Similarly, in the presence of a maximally effective concentration of agonist, relative affinities and maximal activities for GAPs can be compared for $P2Y_1$-R–activated G protein α subunits.

Studies designed to minimize the confounding contributions of nucleotide release, metabolism, and interconversion have led to the view that ADP is the preferred natural agonist for the $P2Y_1$-R, with ATP acting either as a partial agonist or antagonist, possibly depending on the level of expression of $P2Y_1$-R in the test system (Hechler et al., 1998; Palmer et al., 1998; Simon et al., 2001). This view is strongly supported by the results of the current study with a functional system reconstituted from purified components. Negligible hydrolysis of ATP to ADP occurred under conditions in

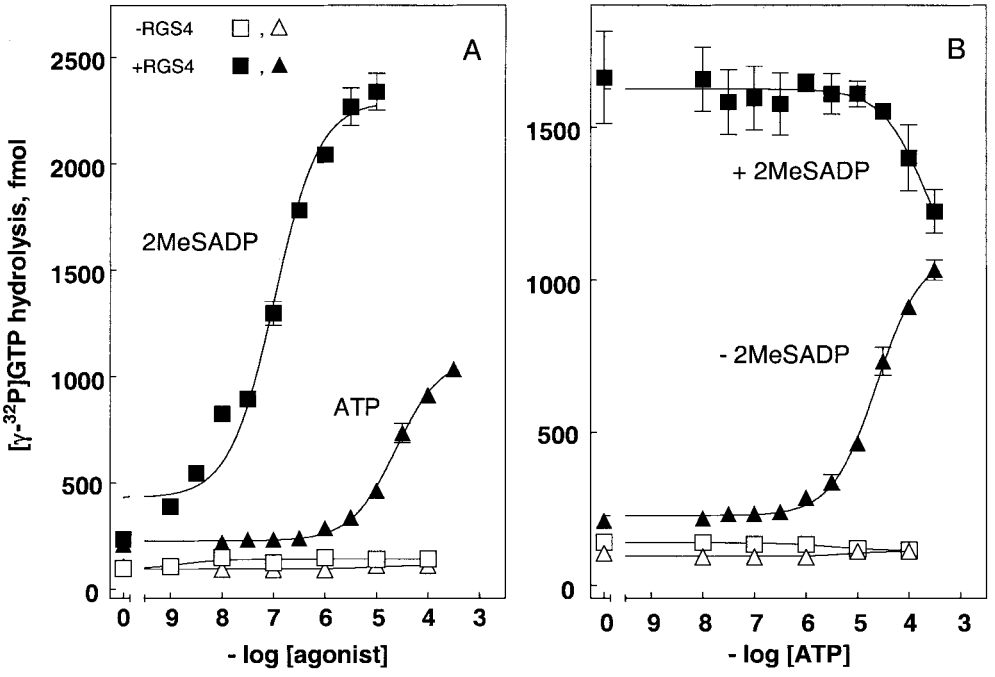

Fig. 7. Concentration-dependence of ATP as a partial agonist at the $P2Y_1$-R. Purified $P2Y_1$-R, $G\alpha_q$, and $G\beta_1\gamma_2$ were reconstituted in proteoliposomes. A, steady-state GTP hydrolysis was measured in proteoliposomes incubated with (■, ▲) or without (□, △) 100 nM RGS4 and with the indicated concentrations of 2MeSADP (■, □) or ATP (▲,△). B, Steady-state GTP hydrolysis was measured in proteoliposomes incubated with the indicated concentrations of ATP in the presence (■, ▲) or absence (□, △) of 100 nM RGS4 and with (■, □) or without (▲, △) 1 μM 2MeSADP.

TABLE 3

Metabolism of [γ^{32}P]ATP under GTPase assay conditions

ATP hydrolysis was determined by measuring the release of ^{32}P from [γ-^{32}P]ATP after incubation at 30° C for the indicated times in the presence or absence of $P2Y_1/G\alpha_q/\beta_1\gamma_2$ proteoliposomes, 100 nM RGS4, and 10 μM 2MeSADP as indicated. The assay conditions were identical with those used in Fig. 7 except that [γ-^{32}P]ATP was substituted for [γ-^{32}P]GTP.

Assay Composition	Incubation Time		
	0 min	15 min	30 min
	pmol of ATP hydrolyzed/50-μl assay		
10 μM (500 pmol) [γ-^{32}P]ATP	0	0.138 ± 0.022	0.283 ± 0.038
10 μM (500 pmol) [γ-^{32}P]ATP + 100 nM RGS4	0.005 ± 0.008	0.213 ± 0.014	0.392 ± 0.068
10 μM (500 pmol) [γ-^{32}P]ATP + $P2Y_1/G\alpha_q/\beta_1\gamma_2$ proteoliposomes + 100 nM RGS4	0.020 ± 0.009	0.320 ± 0.020	0.602 ± 0.008
10 μM (500 pmol) [γ-^{32}P]ATP + $P2Y_1/G\alpha_q/\beta_1\gamma_2$ proteoliposomes + 100 nM RGS4 + 10 μM 2MeSADP	0.033 ± 0.013	0.261 ± 0.002	0.609 ± 0.003
1 mM (50,000 pmol) [γ-^{32}P]ATP + $P2Y_1/G\alpha_q/\beta_1\gamma_2$ proteoliposomes + 100 nM RGS4 + 10 μM 2MeSADP	−1.49 ± 0.50	2.51 ± 0.55	10.7 ± 1.59

which ATP was illustrated to be a partial agonist exhibiting an EC_{50} approximately 20-fold greater than that of ADP. In contrast, 2MeSATP was a full agonist exhibiting a potency approximately 20-fold less than 2MeSADP and similar to that of ADP. Thus, we conclude that ADP is the cognate agonist for the P2Y$_1$-R and that the much lower efficacy and apparent affinity of ATP are both consistent with the idea that this nucleotide is not of major importance as a physiological regulator of the P2Y$_1$-R.

A number of studies using transient and stable heterologous expression of the recombinant human P2Y$_1$-R in mammalian cell lines have concluded that the P2Y$_1$-R is predominantly G_q-coupled (Filtz et al., 1994; Schachter et al., 1996, 1997). However, for many years, the unrealized presence of two different ADP-activated receptors in platelets resulted in uncertainty over the nature of the involved receptor(s) and signaling responses that accounted for the aggregation response to ADP. Furthermore, the identification of the P2Y$_1$-R in platelets before the unambiguous identification of the G_i/adenylyl cyclase-linked P2Y$_{12}$-R led to uncertainties over whether the P2Y$_1$-R coupled to both G_q/phospholipase C and G_i/adenylyl cyclase. Indeed, the eventual identification of the P2Y$_{12}$-R receptor gene (Zhang et al., 2000; Hollopeter et al., 2001) and the G_i-activating/adenylyl cyclase-inhibiting activity of this receptor does not formally prove that the P2Y$_1$-R couples only to G_q rather than to both G_q and G_i in the platelet or other tissues. However, the purified P2Y$_{12}$-R apparently couples to three different $G\alpha_i$ subunits but not to $G\alpha_q$ (Bodor et al., 2003), and the purified P2Y$_1$-R studied here primarily couples to G_q, supporting the view that the two ADP-activated receptors of platelets signal in a relatively linear manner down a G_q-mediated pathway, i.e., the P2Y$_1$-R, or G_i-mediated pathway, i.e., the P2Y$_{12}$-R.

$G\alpha_q$ and $G\alpha_{11}$ exhibit approximately 90% amino acid identity and, with the exception of the mouse platelet (Offermanns et al., 1997), seem to be redundantly expressed in all tissues studied. These two ubiquitously expressed signaling proteins also have exhibited essentially identical signaling functions in all processes studied to date. Thus, a generally consensus view has been that these $G\alpha$ subunits are interchangeable in most if not all of their actions. Ross and coworkers first observed GAP activity of an effector protein for heterotrimeric G proteins in their studies with phospholipase C-β1 (Berstein et al., 1992). Thus, the capacity of a purified fraction containing both $G\alpha_q$ and $G\alpha_{11}$ to hydrolyze GTP was markedly increased by PLC-β1 in a concentration-of-enzyme–dependent manner. Subsequent work elegantly elucidated the kinetics of agonist-promoted guanine nucleotide exchange and enhancement of GTPase activity by PLC-β1 in proteoliposomes reconstituted with M1 muscarinic receptors and heterotrimeric G_q (Biddlecome et al., 1996, Mukhopadhyay and Ross, 1999). As was illustrated here, the interaction of the purified P2Y$_1$-R with downstream signaling components can be studied in proteoliposomes with purified proteins, and both RGS4 and PLC-β1 stimulated GTPase activities of $G\alpha_q$ versus $G\alpha_{11}$ with equal effectiveness.

In summary, the data presented in this study illustrate that the human P2Y$_1$-R can be purified to near homogeneity in active form and that downstream signaling components can be reconstituted in a functional proteoliposome system with the purified P2Y$_1$-R. This model system should be useful in obtaining further molecular insight into this important signaling protein.

Acknowledgments

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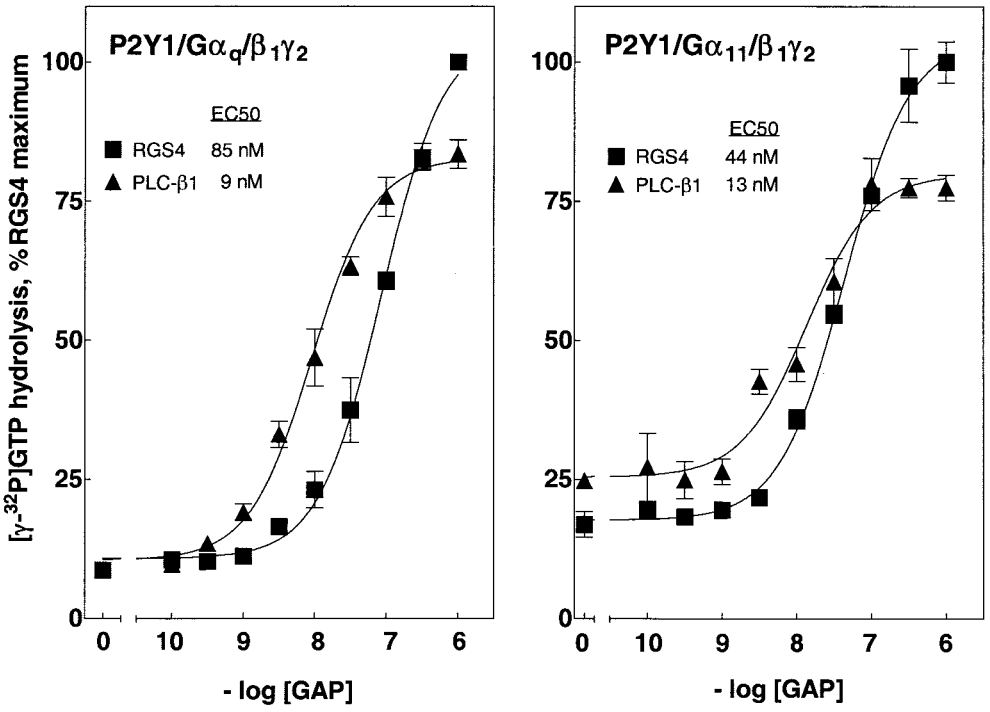

Fig. 8. Concentration-dependence of RGS4 and phospholipase C-β1 for promoting 2MeSADP-stimulated GTP hydrolysis in $G\alpha_q$ and $G\alpha_{11}$ proteoliposomes. P2Y$_1$-Rs were reconstituted with $G\beta_1\gamma_2$ and $G\alpha_q$ (left) or $G\alpha_{11}$ (right). Steady-state GTP hydrolysis was measured in the presence of 10 μM 2MeSADP and the indicated concentrations of PLC-β1 (▲) or RGS4 (■).

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Correction to "Agonist Binding and Gq-Stimulating Activities of the Purified Human $P2Y_1$ Receptor"

In the print version of the above article [Waldo GL and Harden TK (2004) *Mol Pharmacol* **65:**426–436], the type in the inset to Fig. 2 was too small. The online version contains a different rendering of the figure with larger type. The data in the print and online versions are identical.