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A Chimeric Rat Brain $P2Y_1$ Receptor Tagged with Green-fluorescent Protein: High-affinity Ligand Recognition of Adenosine Diphosphates and Triphosphates and Selectivity Identical to That of the Wild-type Receptor

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ABSTRACT. We tested how the green fluorescent protein (GFP) tag affects signaling of the nucleotide-activated $P2Y_1$ receptor. Therefore, we generated stably transfected human embryonic kidney 293 cells expressing the rat $P2Y_1$ wild-type receptor ($rP2Y_1$-wt) or the receptor tagged at the C-terminus with the enhanced GFP ($rP2Y_1$-eGFP). The chimeric $rP2Y_1$-eGFP receptor is localized mainly to the plasma membrane as revealed by Western blotting of subcellular fractions. Both receptors were analyzed by measuring Ca^{2+} responses to short pulses of the agonists in single cells by continuous superfusion with medium. The $rP2Y_1$-eGFP receptor was coupled to Ca^{2+} release as was the $rP2Y_1$-wt receptor. 2-Methylthio adenosine 5′-diphosphate and -triphosphate (2-MeSATP and 2-MeSADP) were the most potent agonists at the heterologously expressed receptors, with EC_{50} values of 50 to 70 nM for $rP2Y_1$-eGFP and 0.06 to 0.4 nM for $rP2Y_1$-wt. These potencies of the two P2Y-selective agonists at $rP2Y_1$-wt receptor-expressing cells are the highest values reported so far. This increase is probably due to a receptor reserve. In both $rP2Y_1$-wt- and in $rP2Y_1$-eGFP-expressing cells, the effect of 2-MeSATP was inhibited equally by the antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid. We established that ATP as well as adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) are full agonists at the $rP2Y_1$ receptor at both transfected cell lines. The $rP2Y_1$-eGFP receptor has the same ligand selectivity as the $rP2Y_1$-wt receptor (2-MeSADP = 2-MeSATP > ADP > ATPαS, ATP ≫ UTP). Thus, the GFP-tagged $P2Y_1$ receptor is fully active and shows regular signal transduction coupling. It provides the means for biochemical characterization, since it can be solubilized and is a tool for further physiological analysis. BIOCHEM PHARMACOL **59**;7:791–800, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. purinergic receptor; receptor expression; GFP tag; human embryonic kidney cells (HEK 293)

The extracellular purine nucleotide ATP acts as intercellular signaling molecule in the central and peripheral nervous system through activation of specific membrane receptors, the P2 receptors [1]. Stimulation of P2 receptors by ATP leads either to fast excitatory responses by activation of ionotropic P2X receptors, which are ligand-gated, non-selective cation channels, or to metabotropic responses mediated by the G protein-coupled receptor family of P2Y receptors [1, 2]. The members of the P2Y receptor family show varied sensitivity to purine and pyrimidine nucleotides. The $P2Y_2$ (formerly P2U) subtype is activated by uridine and adenine nucleotides with equal potency. The $P2Y_1$ and p2y3 receptor subtypes prefer purine nucleotide, whereas $P2Y_4$ and $P2Y_6$ receptors are selective for pyrimidine nucleotide [1, 3, 4]. Multiple P2Y receptor subtypes, classified pharmacologically and molecularly cloned, are linked to activation of phospholipase C, leading to increased levels of inositol 1,4,5-triphosphate, diacylglycerol, and intracellular free Ca^{2+} [3, 4]. Moreover, the activation of the mitogen-activated protein kinase signaling pathway by P2Y receptors has been discovered recently [5, 6]. Activation of phospholipase A_2 and phospholipase D, inhibition of adenylate cyclase, and modulation of K^+ channels by P2Y receptors have also been described (reviewed in [4]). A receptor modification using the eGFP† is a valuable biochemical tool for functional studies investigating receptor desensitization and internalization in living

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† *Abbreviations:* ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); eGFP, enhanced green fluorescent protein; 2-MeSADP, 2 methylthio adenosine 5′-diphosphate; 2-MeSATP, 2 methylthio adenosine 5′-triphosphate; $rP2Y_1R$, rat $P2Y_1$ receptor; $rP2Y_1$-wt, wild type; TMR, transmembrane region; HEK, human embryonic kidney; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; DMEM, Dulbecco's Modified Eagle's Medium; RT-PCR, reverse transcription-polymerase chain reaction; HBS, HEPES buffered saline; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonium) propane; and $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

cells and to elucidate the involvement of receptor internalization in mitogenic receptor signaling. Thus, the recently developed technique of tagging with eGFP is most helpful for combining studies of functions and local distribution of proteins as diverse as receptors, structural proteins, or ion channels [7]. Moreover, the eGFP tag is useful for establishing conditions for the isolation of native receptors.

However, the correct membrane insertion and the functional coupling of the chimeric receptor protein have yet to be established. Here, we demonstrate the first successful stable expression of the eGFP-tagged $P2Y_1$ receptor from rat in HEK 293 cells. The chimeric $rP2Y_1$-eGFP protein is a high affinity P2Y receptor functionally coupled to Ca^{2+} release. The functional characteristics of the $rP2Y_1$-eGFP receptor were confirmed by comparison with the $rP2Y_1$-wt receptor, also stably expressed in HEK 293 cells. The recombinant receptor was used to demonstrate that ATP and ATPαS are high-affinity $P2Y_1$ receptor agonists. Moreover, we could solubilize the $P2Y_1$-eGFP protein.

MATERIALS AND METHODS

Materials

ATPαS was from Calbiochem, 2-MeSADP and 2-MeSATP from Biotrend, PPADS and all other nucleotides or nucleotide analogues from Sigma, fura-2/acetoxymethyl ester from Biomol, the primer pairs from MWG Biotec, and the plasmid expression vector pEGFPN and polyclonal "Living colors" GFP antibody were from Clontech. The transfection reagent DOTAP was from Boehringer, goat anti-mouse horseradish peroxidase-conjugated antibody from Dianova, Renaissance chemiluminescence Western blot reagent from NEN, and Immobilon-PSQ membranes from Millipore. All other chemicals were of the highest purity available and either from Merck or Serva. The purity of ligands was checked by HPLC analysis.

Construction of $rP2Y_1$-wt and $rP2Y_1$-eGFP Expression Vectors

The full open reading frame of rat $P2Y_1$ receptor (nucleotides 620–1741; [8]) was amplified using PCR with appropriate forward and reverse primers (5′-tggaattcatgacggaggttccgtggtcg-3′ and 5′-acgaattctcacaaggtggtgtcgccatt-3′; primers 1 and 2) containing *Eco*RI restriction sites (underlined) and the original stop codon (tca). We used a $P2Y_1$ receptor clone obtained from a rat brain library (gift from Dr. G. Schmalzing, Frankfurt, Biozentrum) as template. To generate a product suitable for $rP2Y_1$-eGFP expression, the stop codon of the rat wild-type receptor ($rP2Y_1$-wt) was mutated to alanine using a modified reverse primer (5′-taggatcctgccaaggtggtgtcgccatt-3′; primer 3) containing a *Bam*HI restriction site (underlined). PCR products were purified by agarose gel electrophoresis, digested with *Eco*RI and/or *Bam*HI, respectively, and subcloned into pEGFPN vector at the *Eco*RI and/or *Bam*HI sites. Orientation of the ligation product was determined via restriction analysis. Clones identified to contain $rP2Y_1$-wt or the modified $rP2Y_1$-alanine PCR product in sense orientation were subjected to DNA sequencing to verify the correctness of the DNA sequence.

Generation of Stably Transfected Cell Lines

Highly purified plasmid DNA of the wild-type $rP2Y_1$-wt or $rP2Y_1$-eGFP expression vector constructs were stably transfected into HEK 293 cells by lipofection. Linearized plasmid DNA (5–10 μg) of pEGFPN containing PCR products corresponding either to $rP2Y_1$-wt or $rP2Y_1$-eGFP were mixed with 6 μg of DOTAP reagent in a final volume of 600 μL 20 mM HEPES buffer (pH 7.4). HEK 293 cells ($1–5 \times 10^5$) in logarithmic growth phase (ca. 70% confluency) were washed once with DMEM/Ham's F12 without serum and exposed to 5 mL of the same culture medium supplemented with DOTAP/DNA mixture. After incubation for 6 to 8 hr at 37°, 5% CO_2 atmosphere, 5 mL DMEM/Ham's F12 with 20% fetal bovine serum was added to each culture dish. Another 24 hr later, cell clones expressing the receptors were selected by G-418 resistance (Calbiochem; 500 μg of G-418 per mL). Single cell colonies were isolated and checked for receptor expression by Ca^{2+} measurements of fura-2-loaded cells and/or GFP fluorescence microscopy at 488 nm (argon ion laser) with 507-nm long-pass emission filter. Identified stably expressing single cell colonies were further maintained in DMEM/Ham's F12, 10% fetal bovine serum supplemented with 500 μg G-418 per mL.

Cell Culture

HEK 293 cells were plated at a density of $1–2 \times 10^5$ cells/cm^2 and grown to 60–80% confluency on poly-D-lysine-coated, round glass coverslips (∅ 22 mm) for Ca^{2+} measurement or in Petri dishes in DMEM/Ham's F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 500 μg/mL geneticin (G-418) at 37° in a humidified atmosphere of 5% CO_2 and 95% air.

PCR Analysis of Total RNA Isolated from the Generated Cell Lines

The specific expression of the transfected $rP2Y_1$-wt or $rP2Y_1$-eGFP receptors was verified by RT–PCR experiments with primers specific for rat and human $P2Y_1$ receptors, respectively. Total RNA was isolated from control HEK 293 cells and from cells engineered to express the $rP2Y_1$-wt or $rP2Y_1$-eGFP by the phenol/guanidine isothiocyanate method. Total RNA from each cell line (1–3 μg) was used to perform reverse transcription by reverse transcriptase (MuMLV-RT; Promega). PCR amplification was done with: i) the primers 1 and 2 described above used to generate the PCR product of the $rP2Y_1$-wt expression vector construct, specifically amplifying the full-length

coding region of the rat $P2Y_1$ receptor; or ii) 5′-gaattc(ct)t(gc)ttcctcacctgcat(ac)ag-3′ and 5′-gaattca(ct)(ag)gggtcaacgca(ag)ctgtt-3′ (primers 4 and 5), suitable to amplify either a 560-bp spanning region (encompassing TMR-3 to TMR-7) of $P2Y_1$ or a 540-bp region of $P2Y_2$ of various vertebrate species.

Ca^{2+} Measurements

Changes in $[Ca^{2+}]_i$ were detected by fluorescence imaging analysis (TILL Photonics) of single cells loaded with fura-2/acetoxymethyl ester (2 μM) as described previously [9]. Cells grown on round coverslips were assayed in Na-HBS buffer (pH 7.4) containing 1.8 mM Ca^{2+} and 1 mM Mg^{2+} in the presence of varying concentrations of different nucleotides as indicated in the figure legends. Concentration–response data were analyzed with the Sigmaplot program (Jandel Scientific).

Preparation of Membranes

Plasma membranes were prepared from untransfected (control) or stably transfected HEK 293 cells by sucrose density gradient centrifugation. Briefly, confluent cells were harvested by trypsinization and pelleted at 400xg for 5 min, the pellet was washed with 10 mL PBS, lysed with 10 mL lysis buffer (5 mM Tris–HCl, pH 8.1; 50 μM $CaCl_2$, 3 mM $MgCl_2$, including Complete™ protease inhibitor cocktail from Boehringer Mannheim) per gram wet weight, homogenized with an Ultra Turrax (3 × 10 sec; setting 13,500 rpm), and the homogenate centrifuged at 500xg for 5 min. An aliquot (1 mL) of the supernatant was layered on top of a sucrose step gradient (0.8 to 2.0 M sucrose) and centrifuged for 40 min at 76,000xg. Plasma membranes were collected from the 1.0 M/1.2 M sucrose interface, pelleted at 120,000xg for 20 min, and stored at a protein concentration of 2 mg/mL at −80°. Protein was determined by the method of Bradford with bicinchinonic acid reagent using BSA as standard.

Western Blotting

For detection of the $rP2Y_1$-eGFP receptor, membrane proteins were solubilized with the nonionic detergent Igepal (0.5%; wt/wt) at a protein concentration of 0.6 mg/mL. The different fractions of $rP2Y_1$-eGFP-transfected HEK 293 cells were prepared by differential centrifugation, as stated above for preparation of membranes. Aliquots (15–40 μg of protein) were dissolved in SDS sample buffer, separated by SDS–PAGE on 10% polyacrylamide gels, transferred to Immobilon-PSQ membranes, and processed for immunodetection as described previously [10]. The GFP antibody was used at 1:400 dilution and the horseradish peroxidase-coupled secondary antibody at a dilution of 1:5000. Reactive bands were visualized by chemiluminescence with Renaissance reagent (NEN) by exposure of the blots to Kodak X-Omat blue film.

RESULTS

Expression of $P2Y_1$ Receptors in HEK 293 Cells

For the following study, clones of HEK 293 cells stably transfected with the rat $P2Y_1$ receptor were used. The heterologous expression of the $rP2Y_1$-eGFP fusion protein and the $rP2Y_1$-wt receptor in HEK 293 cells was verified by RT–PCR using primers specific for the rat $P2Y_1$ receptor with total RNA as template. A 1.1-kbp PCR product, which corresponds to the full-length clone, was detected by PCR amplification with the $rP2Y_1$ receptor-specific primers, with HEK 293 cells stably transfected with the $rP2Y_1$-wt or the $rP2Y_1$-eGFP receptor (Fig. 1A, lanes 2 and 3), but not in control HEK 293 cells (Fig. 1A, lane 1). This clearly demonstrates the expression of $rP2Y_1$-wt and $rP2Y_1$-eGFP in the transfected HEK 293 cell lines.

To corroborate the specificity of the primer pair for rat $P2Y_1$ receptor and, further, to check the expression of endogenous human $P2Y_1$ and $P2Y_2$ receptors in HEK 293 cells [11], we designed primers specific for two TMR of the P2Y receptors, i.e. TMR-3 and TMR-7. These regions are highly conserved between rat and human $P2Y_1$ and $P2Y_2$ receptors. RT–PCR amplification yielded two DNA bands of similar size in the two transfected HEK cell lines (Fig. 1B, lanes 2 and 3). Using plasmid DNA from clones which contain the coding region for either the rat $P2Y_1$ or the $P2Y_2$ receptor for template in further PCR experiments, the larger band (560 bp) was identified to correspond to the $P2Y_1$ receptor (Fig. 1B, lane 5) and the smaller band (540 bp) to the $P2Y_2$ receptor (Fig. 1B, lane 4). The upper band appeared with strong intensity with the transcribed cDNA sample obtained from $rP2Y_1$-wt-expressing cells (Fig. 1B, lane 2) as well as in $P2Y_1$-eGFP-expressing cells, albeit with reduced intensity (Fig. 1B, lane 3). In untransfected HEK 293 cells, this band was at the detection limit (Fig. 1B, lane 1), indicating a very weak expression of the endogenous $hP2Y_1$. The lower band (540 bp), which was seen with similar intensity in all HEK cell lines, demonstrates the expression of the endogenous $hP2Y_2$ receptor.

Localization of the Fusion Protein

Studies of HEK 293 cells transfected with $rP2Y_1$-eGFP by confocal fluorescence microscopy showed bright fluorescence at the plasma membrane (data not shown). To ascertain that the $rP2Y_1$-eGFP receptor protein was mainly inserted into the plasma membrane, we used a polyclonal antibody to GFP to examine the distribution of the expressed protein in subcellular fractions. A protein band migrating with a molecular mass of 82 kDa was the only one specifically recognized in $rP2Y_1$-eGFP-expressing cells. This band was identified in the plasma membrane fraction (Fig. 2, lane 3) and in the detergent-soluble fraction (Fig. 2, lane 4). The band also appeared, albeit with much weaker intensity, in a fraction enriched in endoplasmic reticulum and Golgi and in a microsomal fraction, but not in nuclear or mitochondrial fractions (data not shown). The GFP

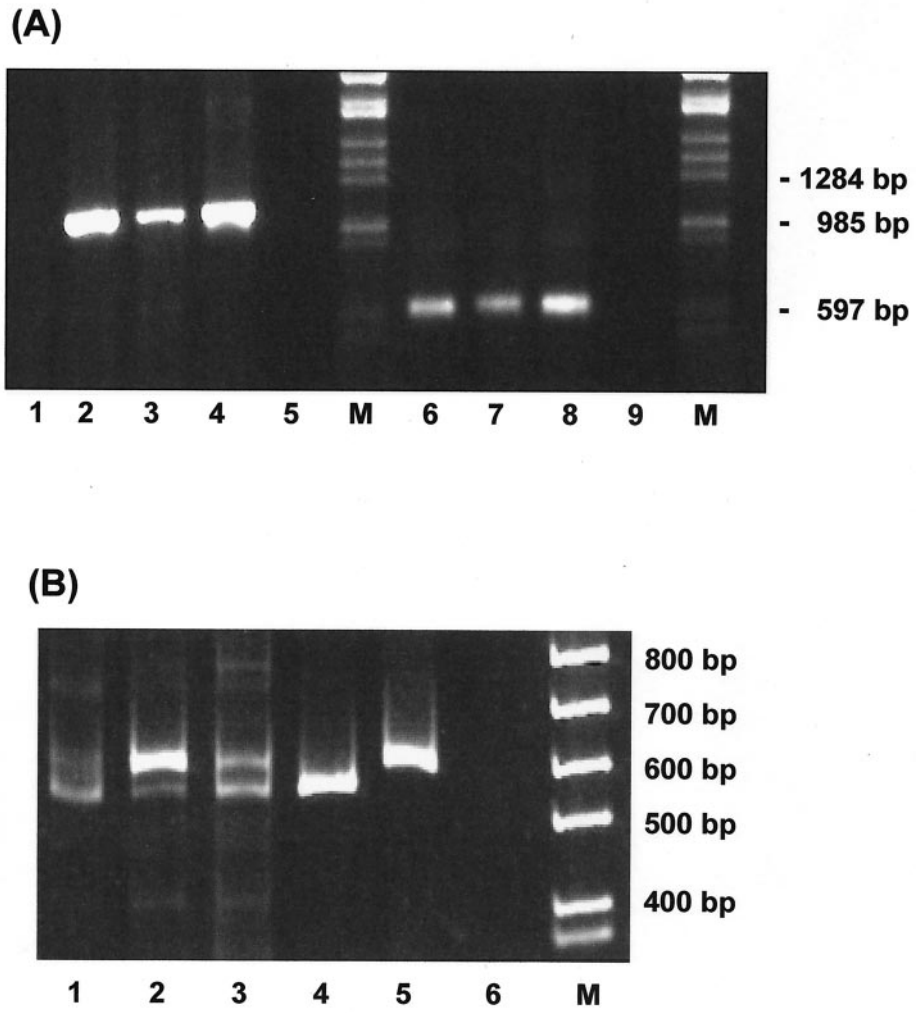

FIG. 1. Determination of the expression of rat $P2Y_1$ receptors in HEK 293 cells. (A) RT–PCR products using RNA from untransfected HEK 293 cells (control, lane 1), HEK cells transfected with the $rP2Y_1$-wt vector (lane 2) or the $rP2Y_1$-eGFP vector (lane 3) with primers specific for the full-length $rP2Y_1$ receptor, with PCR product using the $rP2Y_1$-wt plasmid as template for comparison (lane 4). Lanes 6–9 contain RT–PCR products using primers specific for the housekeeping enzyme glycerinaldehyde phosphate dehydrogenase (GAPDH) with RNA from untransfected cells (lane 6), HEK 293 cells expressing the $rP2Y_1$-wt (lane 7) or the $rP2Y_1$-eGFP receptor (lane 8). The DNA size standard is λDNA digested with *Eco*RI and *Hind* III. (B) RT–PCR products with the primers for the transmembrane regions (TMR-3 and TMR-7) of P2Y receptors with RNA from untransfected cells (lane 1), and cells expressing the $rP2Y_1$-wt receptor (lane 2) or the $rP2Y_1$-eGFP receptor (lane 3). Plasmid DNA containing the $P2Y_1$ receptor (lane 5) or the $P2Y_2$ receptor (lane 4) are used as templates to identify the corresponding PCR products. In (B), the position of the markers (M) is distorted. Total RNA was isolated, transcribed, and amplified by PCR as described under Methods. Negative controls without template DNA are shown in lanes 5 and 9 in A and lane 6 in B. M: DNA size standard as indicated. Comparable results were seen in three experiments.

antibody reacted unspecifically and with a significant intensity with one band at 64 kDa in the cytosolic and membrane fractions from both transfected cell lines (Fig. 2, lanes 1, 2, and 6) and with some protein bands above a molecular mass of 100 kDa. The intense immunostaining at the top of the gel possibly represents aggregated $rP2Y_1$-eGFP receptor protein. The 82 kDa protein band is very likely the fully processed (probably glycosylated) chimeric $P2Y_1$ receptor, as labeling of a protein with a molecular mass of 70 kDa is expected for the chimeric $rP2Y_1$-eGFP protein from the sequence data. Such a protein band, however, was not found with the polyclonal GFP antibody. Therefore, the detection of the expressed eGFP fusion protein in the plasma membrane fractions confirms the correct insertion of the fully processed $rP2Y_1$-eGFP receptor.

Ca^{2+} Measurements

In fura-2-loaded cells, changes in $[Ca^{2+}]_i$ elicited by short stimulation (60 sec) with $P2Y_1$-selective adenine nucleotides were examined to determine the cellular response of the eGFP-tagged $rP2Y_1$ and $rP2Y_1$-wt receptors. Figure 3 demonstrates $[Ca^{2+}]_i$ responses induced by short stimuli of the agonist 2-MeSADP at 1 pM, 1 nM, and 1 μM concentrations. In HEK 293 control cells, 1 μM agonist was required to cause a response, whereas in the $rP2Y_1$-eGFP-

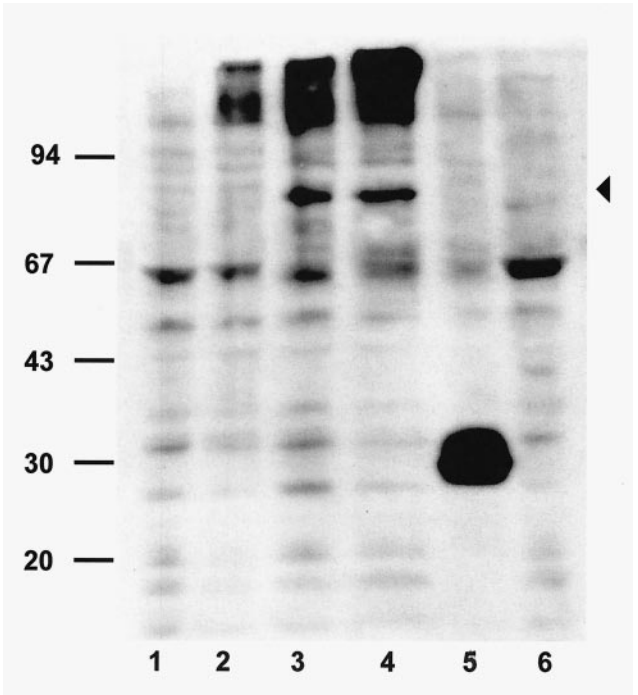

FIG. 2. Immunodetection of the rP2Y$_1$-eGFP receptor in subcellular fractions. Western blotting of subcellular fractions from HEK cells stably transfected with the rP2Y$_1$-wt receptor (lane 1) or with the rP2Y$_1$-eGFP receptor (lanes 2–4 and 6) were carried out with the polyclonal GFP antibody as described in Methods. The arrow at 82 kDa marks the band corresponding to the fusion protein rP2Y$_1$-eGFP. Lanes were loaded with homogenate (40 μg of protein; lanes 1 and 2), purified plasma membranes (30 μg of protein; lane 3), Igepal-soluble plasma membranes (30 μg of protein; lane 4), or cytosolic fraction (30 μg of protein; lane 6). Lane 5 contains the cytosolic fraction (15 μg of protein) of HEK 293 cells expressing the GFP protein only. Positions of molecular weight standards are shown on the left. Comparable results were seen in two experiments.

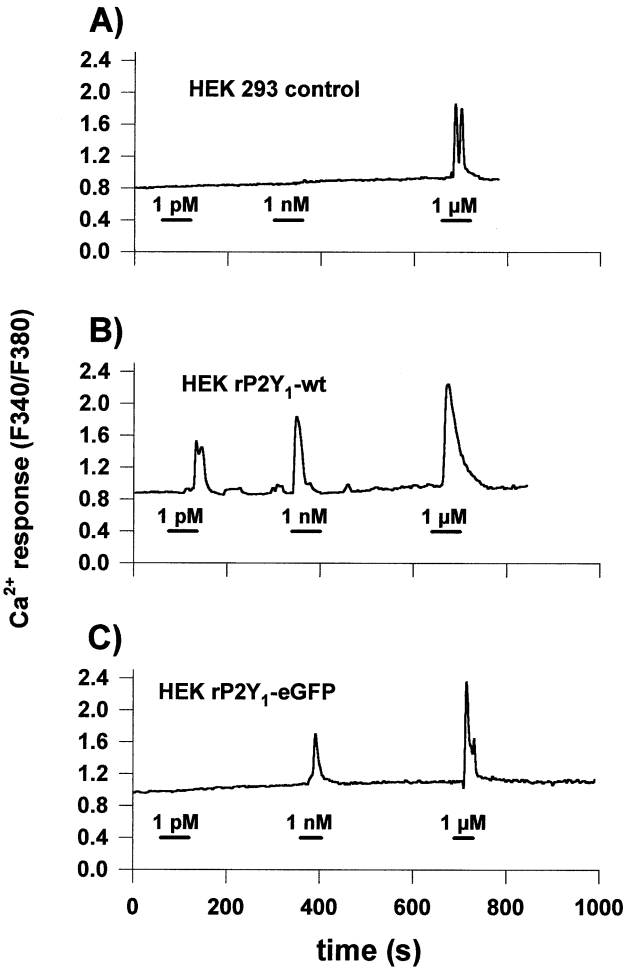

FIG. 3. Ca^{2+} responses induced by 2-MeSADP in HEK 293 cell lines. HEK 293 cell lines were loaded with fura-2/acetoxymethyl ester to measure changes in intracellular $[Ca^{2+}]_i$. Cells were stimulated with three different concentrations of 2-MeSADP at the times indicated by the bars. The traces shown are the responses from single cells as indicated in the respective graphs.

expressing cells, a clear response was already apparent at 1 nM. In the rP2Y$_1$-wt-expressing cells, even 1 pM agonist was enough to cause a comparable $[Ca^{2+}]_i$ rise. A statistical comparison of the Ca^{2+} responses induced by physiological concentrations (10 nM to 10 μM) of 2-MeSADP and other P2Y$_1$ receptor-selective agonists demonstrates that their potency for inducing increases in $[Ca^{2+}]_i$ is much higher in the cell lines expressing the rat P2Y$_1$ receptors than in untransfected HEK 293 control cells (Table 1). At these concentrations, the agonists elicit increases in inositol 1,4,5-trisphosphate concentrations in HEK 293 cells and other cell lines [12, 13].

The different sensitivity of the three cell lines can be assessed at low concentrations of the agonists, as shown in Table 1. At a concentration as low as 10 nM of the P2Y$_1$-selective agonists 2-MeSADP and 2-MeSATP, the rP2Y$_1$-wt-expressing HEK cells responded at a frequency of 100% with an almost maximal Ca^{2+} increase. At this agonist concentration, about 70–80% of the HEK 293 cells expressing the rP2Y$_1$-eGFP receptor responded. In the untransfected cells, the same low concentration of 2-MeSATP or 2-MeSADP evoked a small increase in $[Ca^{2+}]_i$ (less than 20% of the maximal response) in only 31% and 5% of the cells, respectively. The high sensitivity of the heterologously expressed rP2Y$_1$ receptors to the P2Y$_1$-selective agonists 2-MeSADP and 2-MeSATP is underlined by the fact that low concentrations in the range of 10 to 100 nM induced nearly maximal $[Ca^{2+}]_i$ increases in 100% of the cells, whereas concentrations of 1 μM and higher were needed to activate all of the control cells (Table 1). The 10-nM concentration of ATP induced smaller Ca^{2+} responses in rP2Y$_1$-eGFP-expressing cells (15% responding cells) and in rP2Y$_1$-wt-expressing cells (70% responding cells), whereas no increase in $[Ca^{2+}]_i$ could be induced with concentrations of ATP up to 100 nM in HEK control cells (Table 1). ADP was more effective in increasing $[Ca^{2+}]_i$ in the stably transfected cells than ATP, demonstrating the typical pharmacological potency order of agonists at P2Y$_1$ receptors. At a concentration of 10 nM, ADP evoked Ca^{2+} responses in 100% of rP2Y$_1$-wt-expressing cells, with a $[Ca^{2+}]_i$ increase similar to that observed for the two

TABLE 1. Sensitivity of rP2Y$_1$-wt- and rP2Y$_1$-eGFP-transfected HEK 293 cells and of untransfected HEK 293 control cells

Nucleotide / Cell type	Concentration											
	10 nM			0.1 μM			1 μM			10 μM		
	$\Delta F_{340/380}$	% resp.	N	$\Delta F_{340/380}$	% resp.	N	$\Delta F_{340/380}$	% resp.	N	$\Delta F_{340/380}$	% resp.	N
2-MeSATP												
HEK rP2Y$_1$-wt	1.49	98	95	1.58	100	125	1.85	100	93	1.29	100	20
HEK rP2Y$_1$-eGFP	0.47	78	119	0.81	96	289	0.86	100	233	1.27	100	152
HEK control	0.18	31	133	0.55	71	266	0.91	98	341	1.03	100	114
2-MeSADP												
HEK rP2Y$_1$-wt	1.43	100	64	1.69	100	143	1.49	100	106	1.48	100	66
HEK rP2Y$_1$-eGFP	0.47	71	109	0.83	98	156	1.02	100	171	1.26	100	110
HEK control	0.03	5	72	0.42	63	146	0.90	100	145	1.18	100	116
ATP												
HEK rP2Y$_1$-wt	0.47	70	117	1.82	100	174	1.03	84	133	1.70	100	112
HEK rP2Y$_1$-eGFP	0.05	15	67	0.39	74	75	0.74	99	105	1.08	100	118
HEK control	0	0	46	0.06	34	95	0.12	23	125	1.02	97	101
ADP												
HEK rP2Y$_1$-wt	1.16	100	203	1.82	100	56	1.94	100	128	1.96	100	38
HEK rP2Y$_1$-eGFP	0.11	36	88	0.39	74	101	0.95	96	199	1.17	100	101
HEK control	0	0	86	0.06	34	102	0.66	77	188	1.46	100	102
ATPαS												
HEK rP2Y$_1$-wt	0.66	66	71	1.04	100	81	1.18	100	68	1.51	100	28
HEK rP2Y$_1$-eGFP	0.08	29	37	0.61	90	43	0.94	100	41	1.38	100	32
HEK control	0	0	46	0	0	64	0.31	74	35	0.84	97	34

The $[Ca^{2+}]_i$ rise ($\Delta F_{340/380}$) and the frequency of cells responding (% resp.) from N cells studied at different concentrations of nucleotide agonists are given.

P2Y$_1$-selective agonists 2-MeSADP and 2-MeSATP. However, at rP2Y$_1$-eGFP-expressing cells, ADP was less potent than 2-MeSADP or 2-MeATP (36% cells responding at 10 nM) (Table 1).

Concentration–effect curves (Fig. 4) were used to compare the sensitivity of the three cell lines to 2-MeSATP (A), 2-MeSADP (B), ADP (C), and ATP (D). From the concentration–effect curves for the P2Y$_1$-selective agonists seen with the two cell lines expressing the rP2Y$_1$-wt and rP2Y$_1$-eGFP receptors, EC_{50} values were derived which are presented in Table 2 together with the values obtained for HEK 293 control cells. Table 2 demonstrates that 2-MeSADP and 2-MeSATP activated the receptors in the two transfected cell lines with almost identical affinity. The EC_{50} values for the rP2Y$_1$-wt receptor in the high picomolar range (60 to 350 pM) are about three orders of magnitude lower than those found with the HEK 293 control cells (Table 2). The rP2Y$_1$-eGFP receptor was half maximally activated at concentrations in the nanomolar range (50 to 70 nM). These agonist concentrations were 200- to 1100-fold higher than those needed for half-maximal activation of the rP2Y$_1$-wt receptor-expressing cells. The untransfected HEK 293 cells showed substantially lower affinity, since nearly micromolar concentrations of the agonists were needed for half-maximal stimulation. The chimeric rP2Y$_1$-eGFP receptor as well as the rP2Y$_1$-wt receptor revealed the same agonist selectivity, since the rank order in eliciting a half-maximal $[Ca^{2+}]_i$ increase was 2-MeSADP $\cong$ 2-MeSATP > ADP > ATPαS > ATP $\gg$ UTP, which is typical for the ligand selectivity of P2Y$_1$ receptors. Taking into account the statistical error implied in the concentration–response curve analysis, both 2-methylthio-substituted nucleotides appear to be equipotent. ADP and ATP achieved their highest affinities in the rP2Y$_1$-wt receptor-expressing cell line. For ADP, the EC_{50} value of 0.2 μM in the rP2Y$_1$-eGFP receptor (Table 2) was about 1000-fold higher than the half-maximal concentration needed to activate Ca^{2+} responses in rP2Y$_1$-wt cells. Apparently, ATP has a lower affinity for P2Y$_1$ receptors than ADP (Table 2), which is consistent with the data in Table 1, which showed that only 70% of the rP2Y$_1$-wt cells and only 15% of the rP2Y$_1$-eGFP-expressing cells displayed Ca^{2+} responses to 10 nM ATP. In both stably transfected HEK 293 cell lines as well as in the untransfected cells, the P2Y$_2$-selective agonist UTP evoked identical maximal Ca^{2+} responses with identical concentration–effect curves (Fig. 4F) and nearly identical potencies (Table 2). These results demonstrate that the heterologous expression of both rat P2Y$_1$ receptors does not interfere with the signal transduction of the endogenous hP2Y$_2$ receptor. This conclusion is also supported by the similar expression level derived from the intensity of the PCR product, which can be attributed to P2Y$_2$ receptors in all three HEK 293 cell lines (Fig. 1B, lanes 1–3).

The potency of ATPαS to stimulate P2Y$_1$ receptors was investigated, since the radioactively labeled compound was previously used in attempts to identify P2Y$_1$-like receptors in brain membranes [14–16]. When the two stably transfected HEK 293 cell lines expressing the rP2Y$_1$ receptors were challenged with 100 nM ATPαS, higher Ca^{2+} responses than those caused by the same concentration of ATP could be detected (Table 1). rP2Y$_1$-eGFP-transfected

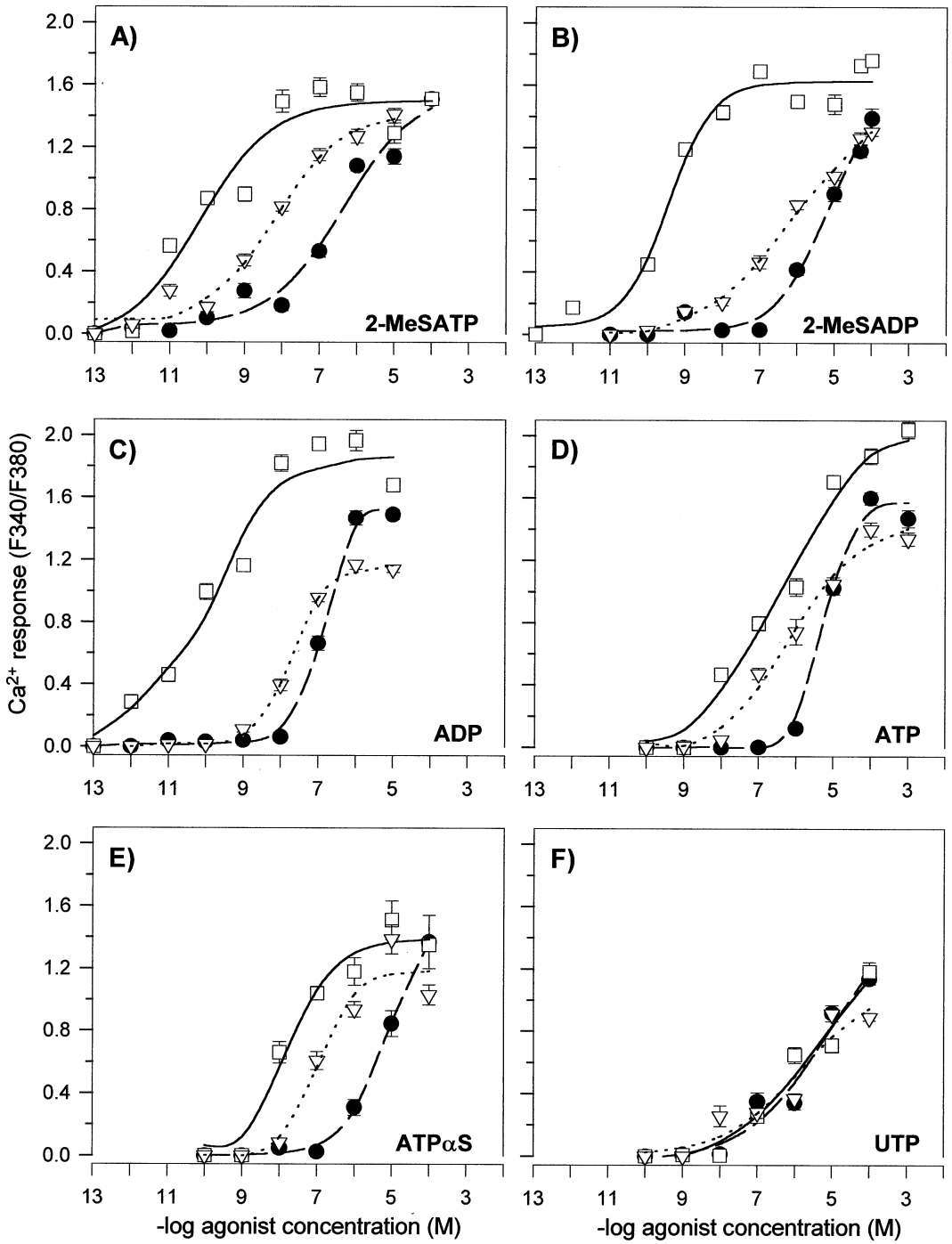

FIG. 4. Concentration–effect curves for nucleotide-stimulated Ca^{2+} increases in HEK 293 cells. Increases in $[Ca^{2+}]_i$ were measured in fura-2-loaded cells as described in Methods. Untransfected HEK 293 control cells (●) or cells transfected with the $rP2Y_1$-wt (□) or the $rP2Y_1$-eGFP (▽) receptor were stimulated with different concentrations of the $P2Y_1$-selective agonists 2-MeSADP (A), 2-MeSATP (B), ADP (C), ATP (D), ATPαS (E), or the $P2Y_2$-selective agonist UTP (F). Individual curves represent the means ± SEM of 3 to 5 experiments each measuring at least 20 single cells. Data are derived from 20–200 cells studied in two to five different experiments.

cells needed concentrations of 100 nM to 1 μM for 100% of the cells to respond (Table 1), while untransfected HEK 293 cells did not respond to ATPαS at concentrations up to 100 nM. The $rP2Y_1$-wt receptor and the $rP2Y_1$-eGFP receptor-expressing cells exhibited a sensitivity to ATPαS 3- to 6-fold higher than that for ATP (Table 2). The maximal Ca^{2+} responses of the stably transfected cells to saturating concentrations (10 μM) of ATPαS (Fig. 4E) were not significantly different from those elicited by 2-MeSADP and 2-MeSATP, but were lower than those

TABLE 2. Affinity of different P2Y receptor agonists at the rP2Y$_1$-eGFP and rP2Y$_1$-wt receptor in HEK 293 cells compared with untransfected HEK 293 control cells

	Nucleotide					
Cell line	2-MeSATP	2-MeSADP	ADP	ATPαS	ATP	UTP
	EC_{50} (nM)					
HEK 293 rP2Y$_1$-eGFP	69	51	200	110	640	1500
HEK 293 rP2Y$_1$-wt	0.06	0.35	0.13	13	43	6600
HEK 293 control	450	450	1200	8400	6200	8700

EC_{50} values were calculated from concentration–effect curves (Fig. 4) fitted with the Sigmaplot program.

elicited by ATP (Fig. 4D). These results support the notion that ATPαS is a full agonist preferentially activating the P2Y$_1$ receptor.

In further experiments, inhibition of Ca^{2+} responses induced by 2-MeSADP in both rP2Y$_1$-wt and rP2Y$_1$-eGFP-transfected cells was assayed by PPADS, an antagonist active at most recombinant P2Y$_1$ receptors [13]. Figure 5, A and B shows concentration–effect curves obtained with cells stimulated at approximately the half-maximal agonist concentration which was found for the respective cell clone (see Table 1). In both cases, a comparable IC_{50} value was obtained (250 μM for P2Y$_1$-wt and 430 μM for P2Y$_1$-eGFP). In HEK 293 control cells, the response to 1 μM 2-MeSATP was inhibited by PPADS (Fig. 5C) with an IC_{50} value which was considerably lower (3.8 μM). This difference may be due to a difference in sensitivities to PPADS between the human and rat P2Y$_1$ receptors.

Thus, the Ca^{2+} responses evoked by the P2Y agonists clearly demonstrate activation of the rat P2Y$_1$ receptors in the stably transfected HEK 293 cells, proving their functional expression. Moreover, the ATP analogue ATPαS exhibited properties of a full agonist at the expressed rP2Y$_1$ receptors with a potency slightly higher than that of ATP.

DISCUSSION

The stable expression of the rat P2Y$_1$-eGFP fusion protein or the rat P2Y$_1$-wt receptor confers on HEK 293 cells a high sensitivity to different P2Y receptor agonists with a rank order of potency typical for P2Y$_1$ receptors expressed in different cells (reviewed in [4]). The responsiveness of the cells was determined by a functional Ca^{2+} response. In the cell line stably expressing the rat P2Y$_1$ receptor tagged with eGFP at its C-terminus, we ascertained that the rP2Y$_1$-eGFP receptor was mainly inserted into the plasma membrane. The examination of the correct localization is important. Overexpression of the G protein-coupled CCK$_A$ receptor, tagged with GFP at its C-terminus, led to saturation of all intracellular membranes (endosomes, mitochondria, nuclei, etc.), whereas the N-terminal GFP tag prohibited the insertion of the receptor into the plasma membrane [17]. Like several other receptors, the β$_2$-adrenergic receptor was successfully tagged with the green fluorescent protein, which enabled the authors to visualize agonist-induced sequestration of the receptor [18]. The rP2Y$_1$-eGFP receptor displays functional responses to nanomolar concentrations of 2-MeSADP and 2-MeSATP that selectively activate P2Y$_1$ receptors. These low concentrations of the two agonists do not lead to the activation of P2Y$_2$ receptors, as 2-MeSATP acts as low potency agonist at the hP2Y$_2$ receptor at concentrations higher than 10 μM [12]. Thus, the rP2Y$_1$-eGFP chimeric protein has no restrained receptor coupling to G proteins due to steric hindrance of the eGFP tag, and the regulatory properties of the rP2Y$_1$-eGFP receptor can be studied in HEK 293 cells without interference with the endogenous hP2Y$_2$ receptor. However, we cannot completely rule out the possibility that the GFP tag somehow affects the sensitivity of the receptor by changing the protein conformation. The rP2Y$_1$-eGFP receptor was activated by the P2Y$_1$-selective agonists 2-MeSADP and 2-MeSATP with a potency similar to that reported for the stably expressed human and turkey P2Y$_1$ receptors [13]. However, the rP2Y$_1$-wt receptor expressed in HEK 293 cells was activated at subnanomolar concentrations. These apparent potencies are the highest reported so far in the literature. They are about 100-fold higher than those found for the human and turkey P2Y$_1$ receptors stably expressed in human astrocytoma 1321N1 cells [13]. This can be explained by the existence of a receptor reserve of the rP2Y$_1$-wt receptor.

In the classical receptor theory [19], increased levels of receptor expression generate a receptor reserve which leads to increases in the potencies of agonists without causing changes in the maximal responsiveness. Elegant experimental studies by Hermans *et al.* [20] inducing different expression levels of mGlu1α receptor provided a convincing demonstration of this relationship. Therefore, the high potency of the P2Y$_1$-selective agonists at the transfected rP2Y$_1$-wt receptor can be convincingly explained by a high expression level of the receptors. This interpretation is supported by the much stronger intensity of the rP2Y$_1$-wt DNA band in the RT–PCR experiments (Fig. 1). A receptor reserve for the stably expressed human P2Y$_2$ [12] and the turkey P2Y$_1$ receptor [13] was already used to explain the high agonist potencies found for activation by the P2Y subtype-selective agonists UTP and 2-MeSADP/2-MeSATP, respectively. The availability of a receptor-selective irreversible antagonist would allow the unequivocal delineation of the reasons for the high agonist potencies. Nevertheless, we were also able to demonstrate that

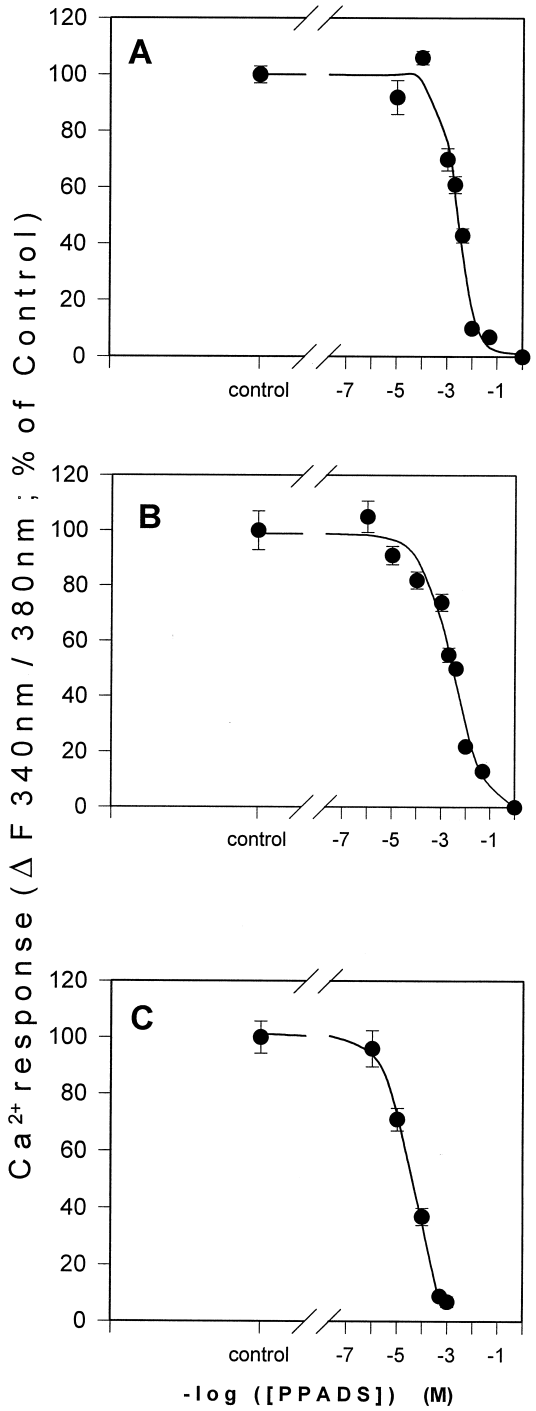

FIG. 5. PPADS inhibits Ca^{2+} responses due to $P2Y_1$ receptor stimulation in HEK 293 cells. Increases in $[Ca^{2+}]_i$ were measured in fura-2-loaded cells as described in Methods. (A) HEK 293 $rP2Y_1$-wt cells were stimulated with 2-MeSADP (30 pM) without addition (control) or together with increasing concentrations of the antagonist PPADS. (B) Values from HEK 293 $rP2Y_1$-eGFP cells stimulated with 30 nM 2-MeSADP. (C) Response amplitudes from HEK 293 control cells stimulated with 1 μM 2-MeSATP. All values represent means ± SEM from at least 20 cells.

UTP was no agonist at either heterologously expressed $P2Y_1$ receptor, especially under conditions with a more than 10-fold ($rP2Y_1$-eGFP) or even 1000-fold ($rP2Y_1$-wt) increased sensitivity of these receptors for the genuine $P2Y_1$-selective agonists.

ATP has been described to act as agonist or partial agonist/antagonist at recombinant $P2Y_1$ receptors and endogenous $P2Y_1$-like receptors [4], e.g. in rat mesenteric arteries not responding to ATP [21]. This has been found especially in tissues or cells where co-localization of $P2Y_1$ and $P2Y_2$ receptors has been suggested from physiological experiments. We did not find evidence for an antagonistic or partial agonistic activity of ATP at the recombinant $rP2Y_1$-wt or $rP2Y_1$-eGFP receptors. In our case, ATP elicited maximal Ca^{2+} responses in the transfected HEK cell lines, almost identical to those induced by 2-MeSADP, 2-MeSATP, or ATPαS, albeit at higher concentrations. These results demonstrate that ATP is a full agonist at recombinantly expressed $P2Y_1$ receptors from rat. The full agonistic potency of ATP was also found for the stably expressed human $P2Y_1$ receptor [13]. This conclusion was confirmed recently [22] for the adenosine triphosphates ATP and 2-MeSATP by analyzing Ca^{2+} responses from single cells as done here. In our experimental setup, the continuous and rapid superfusion of a small number of cells by a relatively large volume of medium excludes the possibility that metabolic products generated by ectonucleotidases on the HEK cells might accumulate to induce the cellular responses observed. However, it is to be noted that at the platelet purinoceptor, which has not yet been identified, ATP is a competitive antagonist [23].

Our findings that the maximal Ca^{2+} responses of the three cell lines induced by 10 μM UTP were of comparable size and that the half-maximal UTP concentrations were identical demonstrate that the heterologous expression of either rat $P2Y_1$ receptor did not interfere with the signal transduction of the endogenous $P2Y_2$ receptors. In addition, these results confirm that UTP is inactive at both expressed $rP2Y_1$ receptors and that UTP-mediated $[Ca^{2+}]_i$ increases are solely due to activation of endogenous $P2Y_2$ receptors. Moreover, comparison of the maximal Ca^{2+} responses to 2-MeSADP and UTP seen with control HEK cells indicates that endogenous $P2Y_1$ and $P2Y_2$ receptors are present in HEK 293 cells at a similar functional ratio, as the two agonists are considered to selectively activate $P2Y_1$ and $P2Y_2$ receptors, respectively. However, this functional ratio is different from the mRNA levels estimated from RT–PCR using the transmembrane region-specific primers (Fig. 1B). The intensity of the band encoding the $P2Y_2$ receptor was much higher than the intensity of the band representing the $P2Y_1$ receptor. Schachter *et al.* [11] reported for HEK 293 control cells a functional ratio for $P2Y_1$ and $P2Y_2$ receptors of 2 to 1, based on the maximal production of inositol phosphates by 2-MeSADP and UTP, respectively. Thus, conclusions about the numerical relationship of $P2Y_1$ and $P2Y_2$ receptors from functional studies or from estimations of the mRNA level do not necessarily

correlate. Therefore, the problem of calculating the actual number of functional $P2Y_1$ or $P2Y_2$ receptors can only be solved when subtype-specific antibodies or radiolabels are available.

In conclusion, this is the first report describing a functional eGFP-tagged $rP2Y_1$ receptor, yielding a cell line with $P2Y_1$ receptors functionally coupled to Ca^{2+} release. The pharmacological properties are comparable to those of the $rP2Y_1$-wt receptor. The expressed receptors are activated at nanomolar concentrations of $P2Y_1$-selective agonists and are both inhibited by the antagonist PPADS. Thus, the $rP2Y_1$-eGFP-expressing cells are suitable to further elucidate the involvement of receptor translocation in physiological $P2Y_1$ receptor signaling.

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References

1. Burnstock G, The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* **36:** 1127–1139, 1997.
2. Abbracchio MP and Burnstock G, Purinoceptors: Are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* **64:** 445–475, 1994.
3. King BF, Townsend-Nicholson A and Burnstock G, Metabotropic receptors for ATP and UTP: Exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol Sci* **19:** 506–514, 1998.
4. Ralevic V and Burnstock G, Receptors for purines and pyrimidines. *Rev Pharmacol* **50:** 413–492, 1998.
5. Huwiler A, van Rossum G, Wartmann M and Pfeilschifter J, Stimulation by extracellular ATP and UTP of the stress-activated protein kinase cascade in rat renal mesangial cells. *Br J Pharmacol* **120:** 807–812, 1997.
6. Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP and Burnstock G, Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci* **19:** 13–18, 1996.
7. Lopatin AN, Makhina EN and Nichols CG, Novel tools for localizing ion channels in living cells. *Trends Pharmacol Sci* **19:** 395–398, 1998.
8. Tokuyama Y, Hara M, Jones EM, Fan Z and Bell GI, Cloning of rat and mouse P_{2Y} purinoceptors. *Biophys Biochem Res Commun* **211:** 211–218, 1995.
9. Ubl JJ, Vöhringer C and Reiser G, Co-existence of two types of $[Ca^{2+}]_i$-inducing protease-activated receptors (PAR-1 and PAR-2) in rat astrocytes and C6 glioma cells. *Neuroscience* **86:** 597–609, 1998.
10. Stricker R, Kalbacher H, Lottspeich F and Reiser G, Characterization of a high-affinity Ins-P_4 (inositol 1,3,4,5 tetrakisphosphate) receptor from brain by an anti-peptide antiserum. *FEBS Lett* **370:** 236–240, 1995.
11. Schachter JB, Sromek SM, Nicholas RA and Harden TK, HEK293 human embryonic kidney cells endogenously express the $P2Y_1$ and $P2Y_2$ receptors. *Neuropharmacology* **36:** 1181–1187, 1997.
12. Lazarowski ER, Watt WC, Stutts MJ, Boucher RC and Harden TK, Pharmacological selectivity of the cloned human P_{2U}-purinoreceptor: potent activation by diadenosine tetraphosphate. *Br J Pharmacol* **116:** 1619–1627, 1995.
13. Schachter JB, Li Q, Boyer JL, Nicholas RA and Harden TK, Second messenger cascade specificity and pharmacological selectivity of the human P_{2Y1}-purinoceptor. *Br J Pharmacol* **118:** 167–173, 1996.
14. Reiser G, Laubinger W and Schäfer R, Nucleotide radiolabels as tools for studying P2Y receptors in membranes from brain and lung tissue. *Prog Brain Res* **120:** 45–58, 1999.
15. Schäfer R and Reiser G, Characterization of [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP binding in rat brain cortical synaptosomes: Regulation of ligand binding by divalent cations. *Br J Pharmacol* **121:** 913–922, 1997.
16. Schäfer R and Reiser G, ATPαS is a ligand for P2Y receptors in synaptosomal membranes: Solubilization of [^{35}S]ATPαS binding proteins associated with G-proteins. *Neurochem Int* **34:** 303–317, 1999.
17. Tarasova NI, Stauber RH, Choi JK, Hudson EA, Czerwinski G, Miller JL, Pavlakis GN, Michejda CJ and Wank SA, Visualization of G protein-coupled receptor trafficking with the aid of the green fluorescent protein: Endocytosis and recycling of cholecystokinin receptor type A. *J Biol Chem* **272:** 14817–14824, 1997.
18. Kallal L, Gagnon AW, Penn RB and Benovic JL, Visualization of agonist-induced sequestration and down-regulation of a green fluorescent protein-tagged β_2-adrenergic receptor. *J Biol Chem* **273:** 322–328, 1998.
19. Kenakin T, Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:** 456–464, 1997.
20. Hermans E, Challiss RA and Nahorski SR, Effects of varying the expression level of recombinant human mGlu1α receptors on the pharmacological properties of agonists and antagonists. *Br J Pharmacol* **126:** 873–882, 1999.
21. Ralevic V and Burnstock G, Discrimination by PPADS between endothelial P_{2Y}- and P_{2U}-purinoceptors in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **118:** 428–434, 1996.
22. Palmer RK, Boyer JL, Schachter JB, Nicholas RA and Harden TK, Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol Pharmacol* **54:** 1118–1123, 1998.
23. Léon C, Hechler B, Vial C, Leray C, Cazenave JP and Gachet C, The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett* **403:** 26–30, 1997.