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Novel modified adenosine 5′-triphosphate analogues pharmacologically characterized in human embryonic kidney 293 cells highly expressing rat brain $P2Y_1$ receptor: biotinylated analogue potentially suitable for specific $P2Y_1$ receptor isolation

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Abstract

Rat brain $P2Y_1$ ($rP2Y_1$) receptor-transfected human embryonic kidney cells (HEK 293) were recently shown to have enhanced reactivity to both ATP and ADP (Vöhringer C, Schäfer R, Reiser G. Biochem Pharmacol 2000;59:791–800). Here, we demonstrated the usefulness of this cell line as a system for further studying novel adenine nucleotide analogues (Halbfinger *et al*. J Med Chem 1999;42:5325–37) and for the biochemical characterization of the $P2Y_1$ receptor. By measurement of intracellular Ca^{2+} release, for 2-butylthio-, 2-butylamino-, and 2-butyloxy-ATP (2-BuS-, 2-BuNH-, 2-BuO-ATP), EC_{50} values of 1.3, 5, and 60 nM were determined, markedly lower than the value for ATP (130 nM). The EC_{50} for 2-BuSADP was 1.1 nM. The corresponding 8-substituted ATP analogues showed a substantially lower potency than ATP (ATP > 8-BuSATP > 8-BuNHATP ≈ 8-BuOATP). AMP induced intracellular Ca^{2+} release with a very low potency; 2- and 8-substitutions on AMP caused no significant potency shift, except for 2-BuSAMP (EC_{50} = 180 nM). Another new P2Y receptor probe, 2-[(6-biotinylamido)-hexylthio]ATP, was 22-fold more potent than ATP (EC_{50} = 6 nM), revealing that even more bulky substituents linked to the C-2 position bind with high affinity at the $P2Y_1$ receptor. This biotinylated probe was successfully used for the enrichment of the $P2Y_1$ receptor tagged with green fluorescent protein from a crude membrane fraction. This one-step enrichment provides a substantial advance for $P2Y_1$ receptor purification. Thus, human embryonic kidney 293 cells stably transfected with the $rP2Y_1$ receptor represent a powerful model system for pharmacological characterization of the $P2Y_1$ receptor, circumventing problems associated with natural systems. They provide a means for the development of $P2Y_1$ ligands of high potency and a good source for obtaining purified $P2Y_1$ receptor.

Keywords: Purinergic receptor; Receptor expression; $P2Y_1$ receptor ligands; Adenine nucleotides; Biotin

1. Introduction

On various peripheral tissues as well as in the central nervous system, P2 receptors activated by extracellular adenine nucleotides produce a broad range of physiological responses [1,2]. Stimulation of G-protein-coupled P2Y receptors mediates activation of phospholipase C [3,4], stimulation of adenylate cyclase [5], or inhibition of adenylate cyclase [6]. Recent studies have implicated extracellular nucleotides as trophic agents in the nervous system. Stimulation of P2 receptors resulted in an increase in DNA synthesis in astrocytes [7], and enhanced glial fibrillary acidic protein content as well as increased stellation and process elongation [8,9].

To date, we have gained profound insight into the mo-

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Abbreviations: 2-MeSATP, 2-methylthio adenosine 5′-triphosphate; HEK cells, human embryonic kidney cells; $rP2Y_1$ receptor, rat brain $P2Y_1$ receptor; $[Ca^{2+}]_i$, intracellular free calcium concentration; GFP, green fluorescent protein; DEAE-, diethylaminoethyl-; and fura 2-AM, fura 2-acetoxymethyl ester.

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lecular amino acid sequence structure of P2Y receptors, since more than eleven types of P2Y receptors have been reported, five of which ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) are currently accepted as molecularly distinct entities [10,11]. However, the enrichment and purification of P2Y receptors by biochemical methods, which is quite important for their understanding, has not yet been successful. This would be useful specifically for the widely distributed $P2Y_1$ subtype, which is intimately involved with a number of important physiological mechanisms, particularly considering the striking neuronal localization of the $P2Y_1$ receptor demonstrated for human brain [12]. It is thus highly desirable to identify a suitable expression system of $P2Y_1$ receptors with great sensitivity for the development of potent ligands for P2Y receptors. C-2-substituted alkylthio nucleotides (e.g. 2-MeSATP, 2-methylthio-ADP, 2-hexylthio-ATP, 2-hexenylthio-ATP, 2-cyanohexylthio-ATP) have already been found to be potent and selective agonists at the $P2Y_1$ receptors [2,13]. To investigate whether steric effects or the electron-donating character of ligands which affects the electronic distribution of the adenine ring system play a major role in ligand binding to the $P2Y_1$ receptor, we recently investigated ATP analogues bearing butylthio-, butylamino-, and butyloxy substitution at either the C-2 or C-8 positions of ATP and AMP by inducing activation of phospholipase C in turkey erythrocytes and Ca^{2+} response in rat astrocytes [14]. Based on a molecular model, it could be shown that steric effects realized by substitution at C-2 and C-8 and hydrophobic interactions of C-2 substituent, rather than electronic effects, play a major role in ligand binding to the $P2Y_1$ receptor [15].

To get a clear potency series that was not possible to obtain with rat astrocytes due to the low expression level [14], in the present study we evaluated the above agonists in HEK 293 cells stably transfected with the $rP2Y_1$ receptor at a high density [16]. Most remarkably, the transfected cells are a suitable test system, since they are fully activated at those concentrations of adenine nucleotides, at which the endogenous $P2Y_1$ receptors from untransfected cells [17] did not respond [16]. Furthermore, ATP turned out to be a full agonist unequivocally at the heterologously expressed $rP2Y_1$ receptor, whereas at the human $P2Y_1$ receptor ATP is a partial agonist when expressed at high levels in 1321N1 cells [18].

The results presented here provide tools to evaluate the potency of new nucleotide derivatives at the $P2Y_1$ receptor and to solve, by a refined system, the question as to how different substituents on the nucleotide scaffold influence the potency of these agonists at the $P2Y_1$ receptor. Moreover, pharmacological analysis combined with protein chemistry was used to develop a new method for the enrichment of $P2Y_1$ receptors from solubilized HEK cell membrane proteins. A potent biotinylated P2Y receptor agonist allowed selective enrichment of $P2Y_1$ receptors from solubilized membrane proteins in a one-step method.

2. Materials and methods

2.1. Materials

2-MeSATP was obtained from RBI and ATP and AMP from Sigma Chemical Co. Fura 2-AM was purchased from Biomol, and Dulbecco's modified Eagle's medium and Ham's F12 medium were from Seromed. Monoclonal anti-GFP antibody was obtained from Eurogentec, and electrophoresis gels and buffer were from Invitrogen.

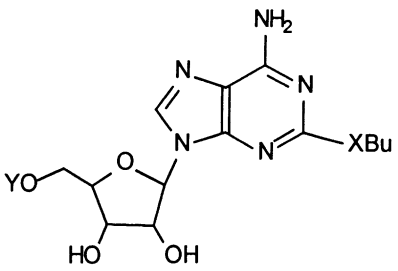
2.2. Synthesis of nucleotide analogues

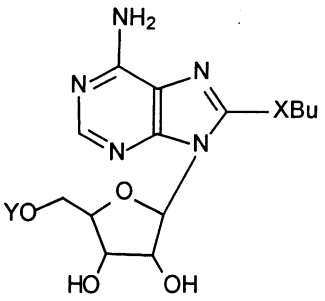
All commercial reagents were used without further purification. 2-Thioadenosine was synthesized as described in [14]. Tri-*n*-butylammonium pyrophosphate solution was prepared as described previously [13]. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AC-200 spectrometer. The chemical shifts are reported in ppm relative to tetramethylsilane (TMS) as an internal standard. Nucleotides were also characterized by ^{31}P-NMR in D_2O using 85% H_3PO_4 as an external reference on a Bruker AC-200 spectrometer. FAB and high resolution FAB spectra were obtained using a glycerol matrix on an AutoSpec-E-FISION VG high resolution mass spectrometer. Separation of the newly synthesized nucleotides was achieved on LC (Isco UA-6) using DEAE A-25 Sephadex (HCO_3^- form) anion exchanger as described below. A series of 2- and 8-substituted ATP and AMP analogues, 1a,b–6a,b, and 2-BuSADP, 1c (Fig. 1A), were synthesized, purified, and chemically characterized as described previously [13,14].

2.3. Synthesis of 2-[(6-biotinylamido)-hexylthio]ATP (10; Fig. 1B)

2-[(6-Bromohexyl)thio]adenosine (7): A suspension of 2-thiol-adenosine (0.28 g, 0.94 mmol in 12 mL MeOH) was dissolved in 0.25 M NaOH (4.2 mL). The clear, yellow solution was stirred at room temperature for 1 hr. After freeze drying, the thiolate sodium salt, obtained as a yellowish solid, was dissolved in dry dimethyl formamide (30 mL), and 1,6-dibromohexane (0.43 mL, 3 eq) was added. The solution was stirred under nitrogen at room temperature for 4 hr. The solvent was evaporated *in vacuo* and the residue was co-evaporated repeatedly with MeOH until it turned into a yellow solid. The solid was triturated with petroleum ether/ether 1:1, and then adsorbed on silica and separated on a silica column (eluted with chloroform/methanol 4:1). After evaporation and drying for two days *in vacuo*, the product was obtained in 86% yield (300 mg). ^{1}H-NMR (DMSO): δ 8.22 (s, 1H, H-8), 7.34 (br.s, 2H, NH_2), 5.81 (d, J = 6 Hz, 1H, H-1′), 5.42 (d, J = 6 Hz, 1H, OH-2′), 5.17 (d, J = 4.5 Hz, 1H, OH-3′), 5.07 (t, J = 5.5 Hz, 1H, OH-5′), 4.61 ("q", J = 5.5 Hz, 1H, H-2′), 4.19–4.08 (m, 1H, H-3′), 3.91 ("q", J = 3.5 Hz, 1H, H-4′), 3.69–3.48 (m, 2H, H-5′), 3.53 (t, J = 6.5 Hz, 2H, CH_2Br),

A





1a X = S Y = $P_3O_9^{4-}$
1b X = S Y = PO_3^{2-}
1c X = S Y = $P_2O_6^{3-}$
2a X = NH Y = $P_3O_9^{4-}$
2b X = NH Y = PO_3^{2-}
3a X = O Y = $P_3O_9^{4-}$
3b X = O Y = PO_3^{2-}

4a X = S Y = $P_3O_9^{4-}$
4b X = S Y = PO_3^{2-}
5a X = NH Y = $P_3O_9^{4-}$
5b X = NH Y = PO_3^{2-}
6a X = O Y = $P_3O_9^{4-}$
6b X = O Y = PO_3^{2-}

B

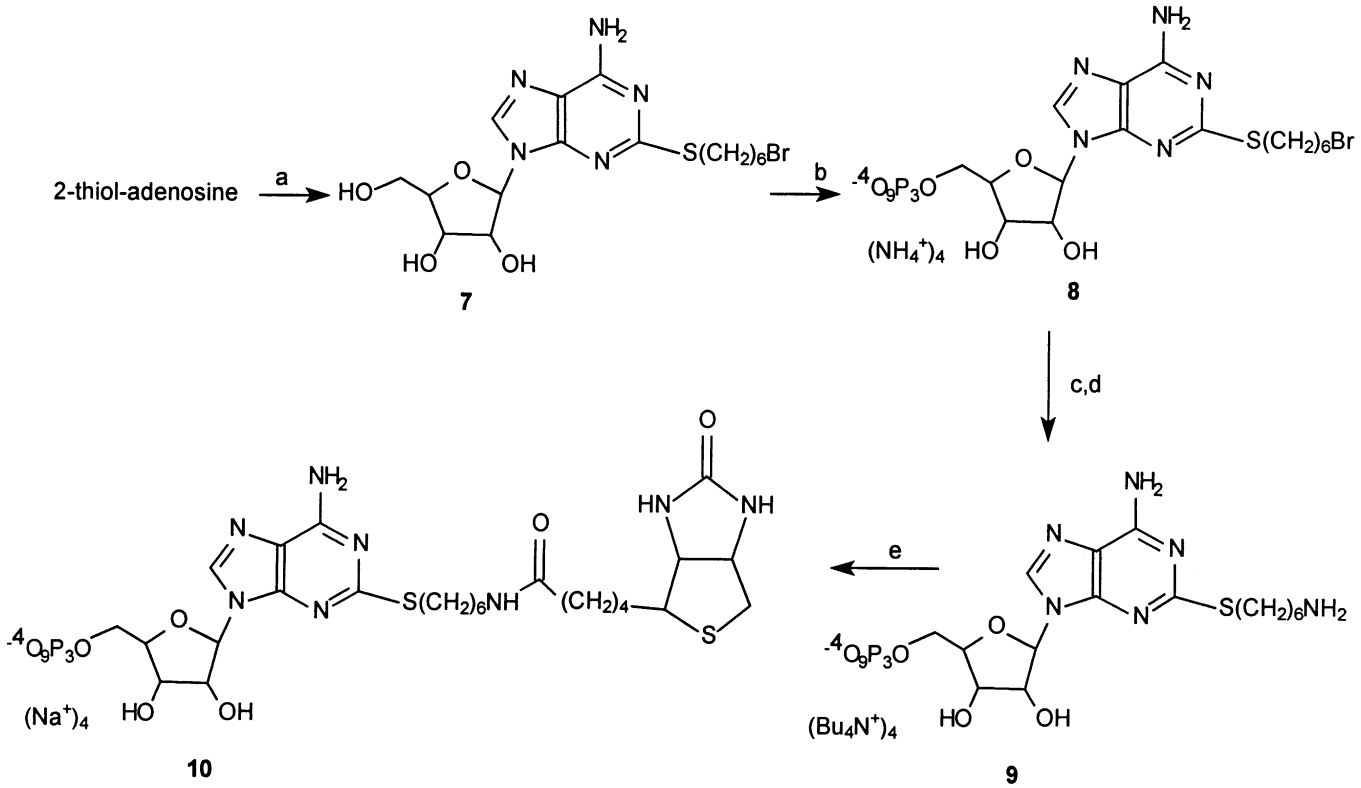


Fig. 1. (A) Structures of ATP and AMP analogues used. (B) Synthesis of 2-[(6-biotinylamido)-hexylthio]ATP. Reaction conditions: a. 1. NaOH/MeOH, then freeze drying 2. $Br(CH_2)_6Br$/DMF. b. 1. $POCl_3$/TMP/proton sponge 2. $P_2O_7H_2^{2-}(Bu_3NH^+)_2$/DMF 3. 0.2 M TEAB c. NH_4OH conc./RT d. Chelex-100 (Bu_4N^+)-form e. biotin-NHS/DMF.

3.14–3.00 (m, 2H, SCH_2), 1.86–1.76 (m, 1H), 1.75–1.59 (m, 3H), 1.48–1.35 (m, 4H); ^{13}C-NMR (DMSO): δ 163.64 (s, C-2), 155.04 (s, C-6), 150.04 (s, C-4), 139.26 (d, C-8), 116.60 (s, C-5), 87.75 (d, C-1′), 85.64 (d, C-4′), 73.48 (d, C-2′), 70.56 (d, C-3′), 61.67 (t, C-5′), 35.33 (t), 32.33 (t, SCH_2), 29.08 (t), 27.51 (t), 27.30 (t); FAB (positive ions): 462, 464 MH^+. High-resolution FAB: calculated for $C_{16}H_{25}N_5O_4SBr$ 462.0811; found 462.0822.

2-[(6-Bromohexyl)thio]ATP tetraammonium salt (8): A solution of 7 (150 g, 0.32 mmol) in dry trimethyl phosphate (2 mL) was added to a flame-dried flask under argon. The solution was cooled to 0°, then proton sponge (109 mg, 2 eq) was added. After 20 min, distilled phosphorous oxychloride (69 μL, 3 eq) was added dropwise and a purple clear solution was formed. Stirring was continued for 2 hr at 0°. TLC on a silica gel plate (1-propanol/28% NH_4OH/H_2O, 11:7:2) indicated the disappearance of starting material and the formation of a polar product (R_f = 0.38). The spot was typically intensely purple under UV light and dark brown in an I_2 chamber. A mixture of Bu_3N (0.32 mL) and 1 M $(Bu_3NH^+)_2P_2O_7H_2$ in *N*,*N*-dimethylformamide (2.1 mL) was added at once. After 2 min, 0.2 M tetraethylammonium bromide solution (44 mL) was added, and the clear solution was stirred at room temperature for 45 min. The latter was freeze-dried overnight. The semisolid obtained after freeze drying was chromatographed on an activated Sephadex DEAE-A25 column. The resin was washed with deionized water for 30 min and loaded with the crude reaction residue dissolved in a minimal volume of water. The separation was monitored by UV detection (ISCO, UA-6) at 280 nm. A buffer gradient of 1 L of water to 1 L of 0.5 M NH_4HCO_3 was applied. The relevant fractions were freeze-dried repeatedly to yield the product as a white solid in 52% yield (129 mg). ^{1}H-NMR (D_2O): δ 8.44 (s, 1H, H-8), 6.12 (d, J = 5.5 Hz, 1H, H-1′), 4.91–4.81 (m, 1H, H-2′), 4.64–4.54 (m, 1H, H-3′), 4.43–4.33 (m, 1H, H-4′), 4.30–4.15 (m, 2H, H-5′), 3.49 (t, J = 6.5 Hz, 2H, CH_2Br), 3.24–3.05 (m, 2H, SCH_2), 1.92–1.63 (m, 4H, $(CH_2)_2$), 1.57–1.36 (m, 4H, $(CH_2)_2$); ^{31}P-NMR (D_2O): δ −7.97 (d), −10.64 (d), −22.02 (t).

2-[(6-Aminohexyl)thio]ATP tetrabutylammonium salt (9): Compound 8 (129 mg, 0.168 mmol) was dissolved in 28% NH_4OH (8 mL) and stirred at room temperature for 3 hr. The crude reaction mixture was concentrated by freeze drying and chromatographed on an activated Sephadex DEAE-A25 column (as described above). The product was obtained as tetraammonium salt in 30% yield (35 mg) after repeated freeze drying. ^{1}H-NMR (D_2O): δ 8.31 (s, 1H, H-8), 6.08 (d, J = 5.5 Hz, 1H, H-1′), 4.91 (t, J = 5.5 Hz, 1H, H-2′), 4.59 (br. t, J = 5 Hz, 1H, H-3′), 4.43–4.32 (m, 1H, H-4′), 4.28–4.13 (m, 2H, H-5′), 3.08 (t, J = 7 Hz, 2H, SCH_2), 2.92 (t, J = 7 Hz, 2H, CH_2NH_2), 1.72–1.50 (m, 4H, $(CH_2)_2$), 1.49–1.24 (m, 4H, $(CH_2)_2$); ^{31}P-NMR (D_2O) δ: −5.92 (d), −10.44 (d), −21.36 (t); High resolution FAB: calculated for $C_{16}H_{28}N_6O_{13}P_3S$ 637.0648; found 637.0668. An aqueous solution of the product was passed through a Chelex-100 (Bu_4N^+)-form column and eluted with deionized water to obtain the corresponding tetrabutylammonium salt which is also soluble in organic solvents. The Chelex-100 (Bu_4N^+)-form column was prepared as follows: Chelex-100-Na^+ 100–200 mesh resin (4.5 mL) was set in a column, washed with an aqueous solution of tetrabutylammonium bromide (at least 3 eq, 2.25 g). The column was washed with deionized water (90 mL).

2-[(6-Biotinylamido)-hexylthio]ATP tetrasodium salt (10): Biotin *N*-hydroxysuccinimide ester (20 mg, 0.06 mmol) and 9 (0.05 mmol) were dissolved in dry CH_3CN/DMSO 3:1 solution (0.8 mL). The reaction mixture was stirred under nitrogen at room temperature for 3 hr. The crude reaction mixture was diluted with water and freeze-dried. The product was separated on a Sephadex DEAE-A25 column applying 0–0.5 M NH_4HCO_3 gradient (1 L of each). Finally, the product (dissolved in water) was passed through a Chelex-100-Na^+ 100–200 mesh resin column (eluted with deionized water) to obtain the product as sodium salt after freeze drying. Compound 10 was obtained in 62% yield (29.5 mg). ^{1}H-NMR (D_2O) δ: 8.38 (s, 1H, H-8), 6.10 (d, J = 5.5 Hz, 1H, H-1′), (H-2′ is hidden by the water peak), 4.64–4.54 (m, 1H, H-3′), 4.48–4.31 (m, 1H, H-4′), 4.29–4.12 (m, 2H, H-5′), 3.33–2.98 (m, 5H, SCH), 2.80 ('dd', J = 13, 5 Hz, 1H, biotin ring), 2.61 (d, J = 13 Hz, 1H, biotin ring), 2.22 (t, J = 6.5 Hz, 2H, CH_2CONH), 1.85–1.11 (m, 16H, aliphatic protons); ^{31}P-NMR (D_2O) δ: −6.39 (d), −10.49 (d), −20.88 (t); High resolution FAB: calculated for $C_{26}H_{42}N_8O_{15}P_3S_2$ 863.1424; found 863.1449.

2.4. Generation of stably transfected cell line and cell cultures

Transfection of HEK 293 cells with the $rP2Y_1$ receptor [19] was carried out as described [16]. HEK 293 cells were plated at a density of 1–2 × 10^5 cells/cm^2 and grown for 2 days to approximately 50% confluency on poly-D-lysine-coated, round glass coverslips (∅ 22 mm) for Ca^{2+} measurements or in Petri dishes in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% fetal bovine serum, penicilline (100 U/mL), streptomycin (100 μg/mL) at 37° in a humidified atmosphere of 5% CO_2/95% air.

2.5. $[Ca^{2+}]_i$ measurements

For Ca^{2+} measurements, cells grown on coverslips were loaded for 30 min with 2 μM fura 2-AM in HEPES-buffered saline (HBS) containing 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM HEPES, pH 7.4. Cells were assayed under continuous superfusion of 35° prewarmed HBS (1 mL/min) in the presence of varying concentrations of different nucleotides as indicated.

A critical issue in testing nucleotide analogues is enzymatic stability under the experimental conditions and the

possible occurrence of degradation products. Therefore, it is important to note that it was previously shown that analogues bearing a substitution at C-2 of the purine ring are resistant to enzymatic degradation [20]. NTPDase hydrolyzed 2-BuOATP to 75%, 2-BuSATP to 53%, 2-BuNHATP to 65%, and 2-BuSADP to 50%, compared to ATP. Rates of hydrolysis of C-8-substituted analogues were only 23% (8-BuNHATP) and 11% (8-BuOATP) compared to the rate of hydrolysis of ATP; 8-BuSATP was shown to be completely resistant to hydrolysis and even to inhibit nucleotidase activity [20]. The relative enzymatic stability of the analogues tested, in addition to the rapid superfusion system used to apply the different ATP analogues, precludes any appreciable enzymatic conversion. Thus, problems related to responses that may result from degraded or released nucleotides are circumvented. Constant superfusion of prewarmed buffer excluded unspecific Ca^{2+} responses caused by mechanical stress, temperature variation, or different components of the buffer.

Fluorescence changes in single cells were detected with an imaging system (TILL Photonics GmbH) attached to a Zeiss Axioscope using alternate excitation at 340/380 nm and emission at 500 nm. The fluorescence emission ratio was converted to $[Ca^{2+}]_i$ using the equation of Grynkiewicz *et al*. [21]. Concentration–response data were analyzed with the EXCEL program applying $\Delta F_{340nm}/F_{380nm}$ before and after addition of agonist. Curve fitting was carried out by using a four-parameter sigmoidal equation from the regression equation library of the SIGMAPLOT program. EC_{50} values represent the agonist concentration at which 50% of the maximal effect is achieved.

2.6. Preparation of solubilized rP2Y$_1$–GFP and Western blotting

The plasma membranes were prepared from HEK 293 cells stably transfected with the rP2Y$_1$–GFP receptor as described [16]. After solubilization of the plasma membrane with 1% (wt/wt) Igepal and centrifugation at $50{,}000 \times g$ for 30 min to pellet the insoluble material, the supernatant was incubated with 2-[(6-biotinylamido)-hexylthio]ATP coupled to 5 mL neutravidin matrix at 4° overnight with gentle agitation. The neutravidin matrix was poured into a column, washed with two column volumes of buffer A (25 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 5 mM KCl; 0.1% Igepal), and eluted with 5 mM ATP in buffer A at a flow rate of 0.5 mL/min.

Detergent-soluble proteins were precipitated in the cold with (final concentrations): 5% (w/w) trichloric acetic acid and 0.5% (w/w) sodium deoxycholate. After incubation for 15 min on ice, samples were centrifuged for 10 min at $20{,}000 \times g$ in an Eppendorf centrifuge. The pellets were washed twice with 500 μL ice-cold acetone, centrifuged as above, air-dried, and dissolved in SDS sample buffer (125 mM Tris/HCl, pH 6.8; 2% (w/v) SDS; 4 M urea; 1 mM 1,4-dithioerythritol; 0.005% (w/v) bromophenol blue) by heating at 95° for 3 min. Proteins were separated by SDS–PAGE on 10% polyacrylamide gels using the NOVEX MOPS system (NOVEX). The gel was transferred to nitrocellulose membranes (BA 83; Scheicher & Schüll) and processed for Western blotting as described [16]. The anti-GFP antibody was used at 1:6000 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.

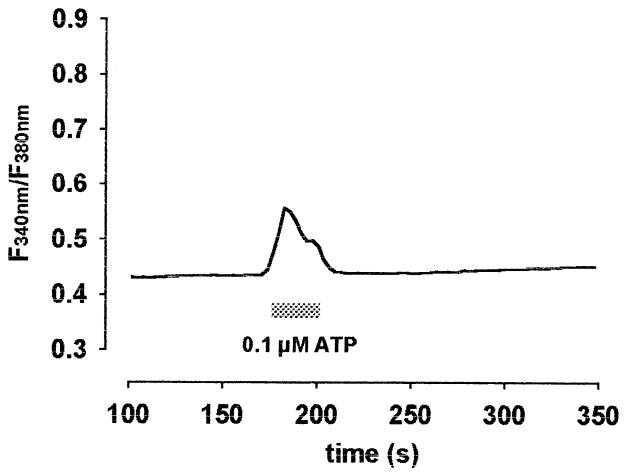
3. Results

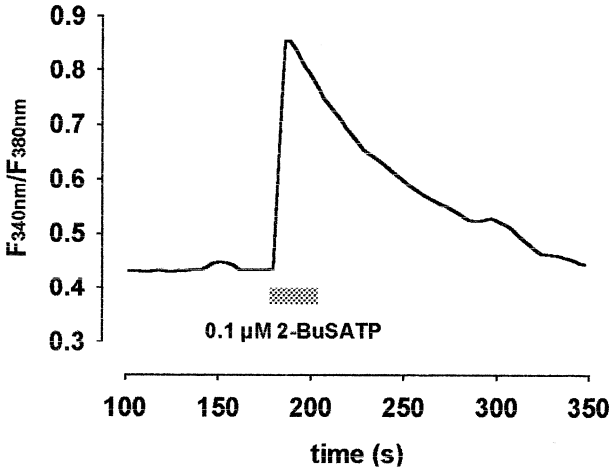
We used here a system expressing high levels of the rP2Y$_1$ receptor to test the agonistic potential of a series of ATP and AMP analogues where the C-2 or C-8 positions were substituted with electron-donating groups [14]. Changes in $[Ca^{2+}]_i$ were monitored in HEK 293 cells loaded with fura 2-AM to determine the effectiveness on P2Y$_1$ receptors of the nucleotide analogues bearing butylthio-, butylamino-, or butyloxy substitutions or a 2-[(6-biotinylamido)-hexylthio] group, compounds 1–6 and 10, respectively (Fig. 1). Compounds 1–6 were prepared as previously described [14], and the ATP probe, 10, was synthesized as described in Fig. 1B. The latter compound was designed based on the most potent ligand identified from the series described in Fig. 1A, namely compound 1a.

Stimulation of cells for 30 sec induced rapid Ca^{2+} responses, as shown in Fig. 2 after application of ATP, 2-BuSATP, and 2-BuOATP, each at 0.1-μM concentration. Their sensitivity to either 2- or 8-substituted adenine nucleoside 5′-mono- and 5′-triphosphate derivatives and to 2-BuSADP was analyzed by concentration–effect curves covering the range from 10 pM to 10 μM (Figs. 3–6). Agonist concentrations that induce the half-maximal response are defined as EC_{50} values. EC_{50} values were derived from the relative peak amplitude for those adenine nucleoside 5′-phosphate analogues where a maximal response was detectable at concentrations <10 μM (Table 1). In all these cases, maximal peak responses of similar amplitude were observed.

As shown in Fig. 3A, with all C-2-substituted ATP analogues complete concentration–response curves could be established, all of which displayed a higher potency to increase $[Ca^{2+}]_i$ than ATP. Based on their EC_{50} values, 2-BuOATP (60 nM) was approximately 2-fold and 2-BuNHATP (5 nM) 26-fold more potent than ATP (130 nM). 2-BuSATP (1.3 nM) had even a 100-fold higher potency than ATP. The potency of 2-BuSATP was as high as that of 2-MeSATP (1 nM, not shown), which represents a commonly used high potency P2Y$_1$-selective agonist.

The corresponding ATP analogues, bearing electron-donating groups at C-8 of the adenine ring, were also tested (Fig. 3B). The highest potency, as with the 2-substituted analogues, 1a–3a, was found with the thioether-substituted





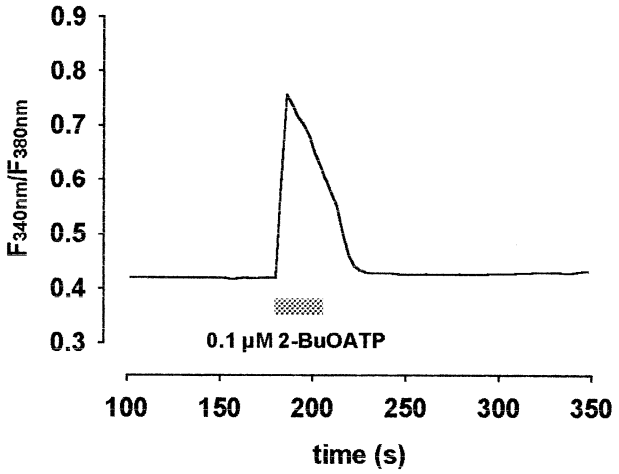


Fig. 2. Calcium responses of rat $P2Y_1$-transfected HEK 293 cells induced by adenine nucleotides. Bars below the traces show the times of addition of indicated nucleotides in the superfusing buffer as described in Methods. The traces depicted are those of the means of 30 single cells from a typical experiment.

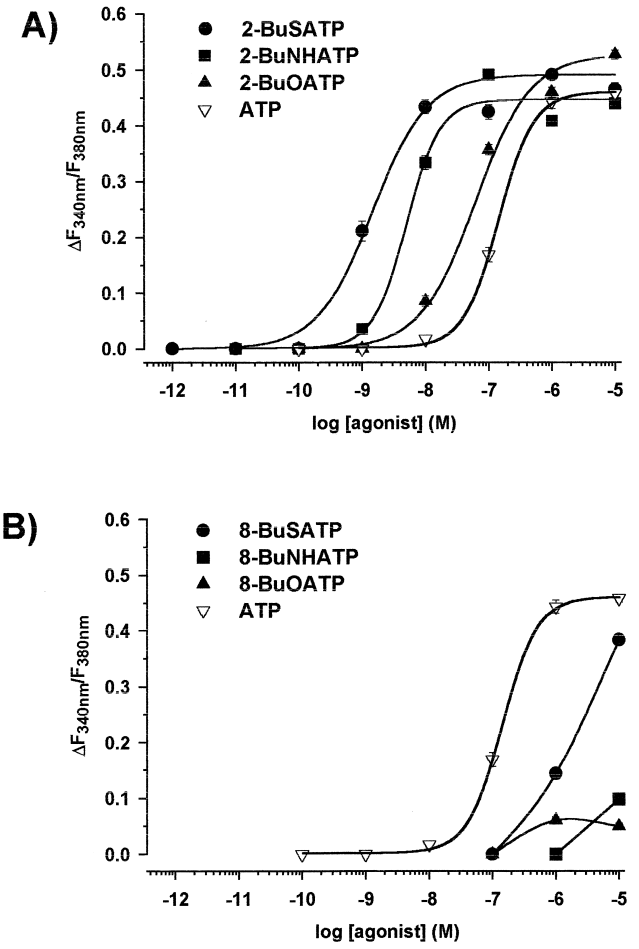


Fig. 3. Effect of different ATP analogues on Ca^{2+} release in HEK 293 cells expressing the $rP2Y_1$ receptor. The capacity of the indicated concentrations of (A) 2-BuSATP (1a; ●), 2-BuNHATP (2a; ■), and 2-BuOATP (3a; ▲) and (B) 8-BuSATP (4a; ●), 8-BuNHATP (5a; ■), and 8-BuOATP (6a; ▲) to raise the $\Delta F_{340}/F_{380}$ ratio was determined as described in Methods. Open triangles represent the dose–response curve for ATP. Data shown are the means ± SEM of 60–100 single cells and the results are representative of those obtained in at least three separate experiments.

analogue, but even at 10-μM concentration the plateau of response amplitude was not yet reached. Thus, the C-8 modification (compounds 4a–6a) resulted in a drastic diminution of the potency to induce intracellular Ca^{2+} release in comparison to that induced by ATP, since the EC_{50} values, if they could be estimated at all, were above 1 μM.

To elucidate the role of the triphosphate chain of the adenine nucleotides, 2-BuSADP, (1c), AMP, and the corresponding C-2- or C-8-substituted AMP analogues (1b–6b) were also investigated (Figs. 4 and 5). Table 1 shows that 2-BuSADP (EC_{50} = 1.1 nM) had nearly the same potency as 2-BuSATP. AMP had a very low potency ($EC_{50} > 1$ μM), and there was no significant difference after substitution in positions 2 or 8, respectively (Fig. 4). The only exception was 2-BuSAMP, which showed a potency similar to that of ATP (EC_{50} = 180 nM; Fig. 4A). However, there was no detectable effect of AMP and AMP analogues in non-transfected HEK 293 control cells (not shown). This difference confirms our previous conclusion

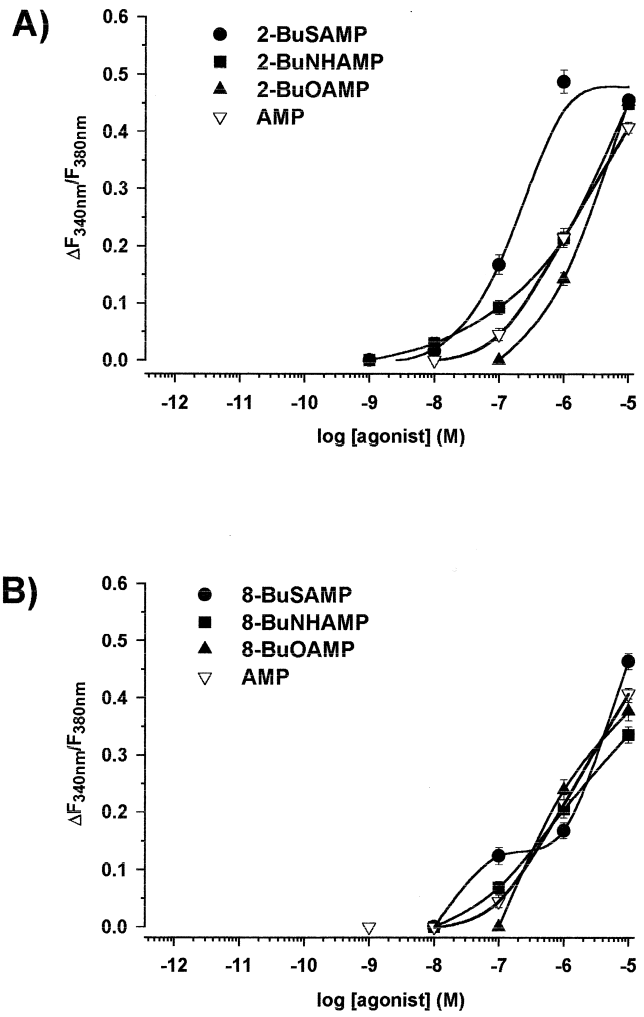


Fig. 4. Effect of different AMP analogues on Ca^{2+} release in HEK 293 cells expressing the rat $P2Y_1$ receptor. The capacity of the indicated concentrations of (A) 2-BuSAMP (1b; ●), 2-BuNHAMP (2b; ■), and 2-BuOAMP (3b; ▲) and (B) 8-BuSAMP (4b; ●), 8-BuNHAMP (5b; ■), and 8-BuOAMP (6b; ▲) to raise the $\Delta F_{340}/F_{380}$ ratio was determined as described in Methods. Open triangles represent the dose–response curve for AMP. Data shown are the means ± SEM of 60–100 single cells and the results are representative of those obtained in at least three separate experiments.

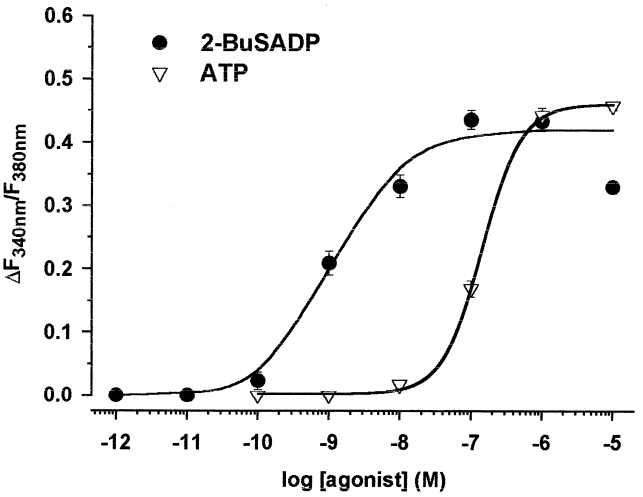


Fig. 5. Effect of different 2-BuSADP concentrations on Ca^{2+} release in HEK 293 cells expressing the $rP2Y_1$ receptor. The capacity of the indicated concentrations of 2-BuSADP (1c; ●) to raise the $\Delta F_{340}/F_{380}$ ratio was determined as described in Methods. Open triangles represent the dose–response curve for ATP. Data shown are the means ± SEM of 60–100 single cells and the results are representative of those obtained in at least three separate experiments.

[16] that the high level expression of the $P2Y_1$ receptor makes the cells considerably more sensitive.

To develop a practically useful derivative, we synthesized 2-[(6-biotinylamido)-hexylthio]ATP (10; Fig. 1B). This analogue with a biotin label can be used for various applications based on its conjugation with avidin. We found that 2-[(6-biotinylamido)-hexylthio]ATP had a high potency towards the $rP2Y_1$ receptor with an EC_{50} value of 6.0 nM (Fig. 6). This fact that the rat $P2Y_1$ receptor was 22-fold more potently activated by the biotinylated ATP derivative as compared to ATP prompted us to use it for affinity chromatography of the $rP2Y_1$ receptor tagged with GFP expressed in the HEK 293 cells [16]. The biotinylated nucleotide, coupled to a neutravidin matrix, selectively enriched the $rP2Y_1$–GFP receptor solubilized from a plasma membrane fraction from HEK 293 cells (Fig. 7). Western blotting with a monoclonal anti-GFP antibody demonstrated a strong reaction with a protein band with an apparent molecular mass of 85 kDa in the fraction eluted with ATP, which obviously represents the fully processed $rP2Y_1$–GFP receptor. The specific labeling of this band is in agreement with results we had obtained with a polyclonal anti-GFP antibody that labeled a protein with an apparent molecular mass of 82 kDa in the plasma membrane fraction in a different electrophoresis system [16]. The origin of the additional protein band (molecular mass 44 kDa) that was also recognized by the antibody is not known.

4. Discussion

For any of the P2Y receptor subtypes, as for the $P2Y_1$ receptor, the lack of agents suitable for reliable binding

Table 1
Potencies of substituted ATP analogues at the $rP2Y_1$ receptor-transfected HEK 293 cells

Agonist	EC_{50} (nM)
2-MeSATP	1.0
2-BuSATP	1.3
2-BuSADP	1.1
2-BuNHATP	5.0
2-[6-biotinylamido)-hexylthio]ATP	6.0
2-BuOATP	60
ATP	130
2-BuSAMP	180
2-Bu(NH,O)AMP	>1000

EC_{50} values were calculated from concentration–effect curves as represented in Figs. 3–5 and fitted with the Sigmaplot program.

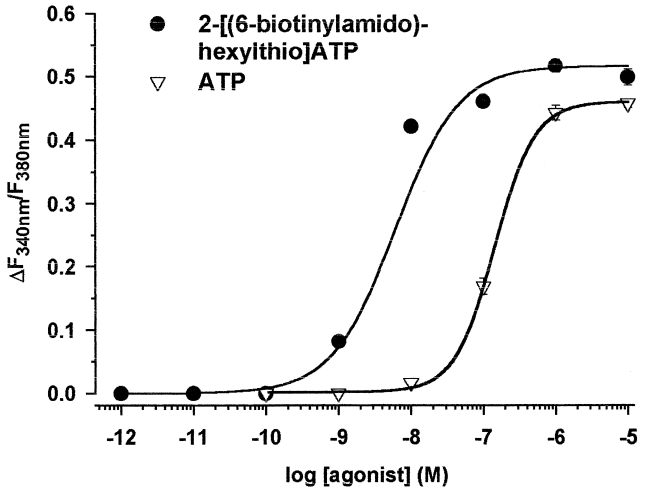


Fig. 6. Dose-dependent rise in the $\Delta F_{340}/F_{380}$ ratio of $rP2Y_1$ receptor-transfected HEK 293 cells after stimulation with indicated concentrations of 2-[(6-biotinylamido)-hexylthio]ATP (compound 10; ●) or ATP (▽). Data shown are the means ± SEM of 60–100 single cells and the results are representative of those obtained in five separate experiments.

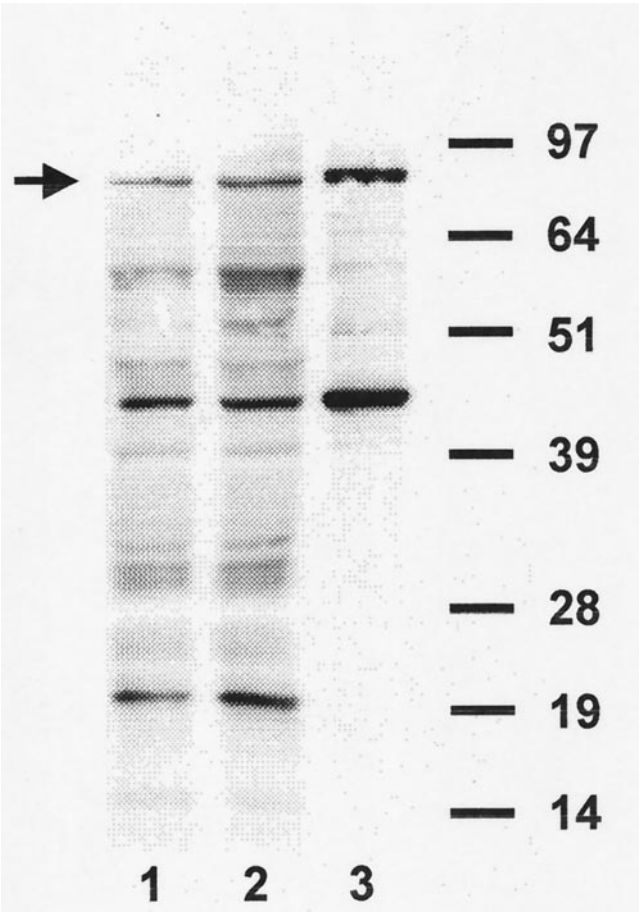


Fig. 7. Affinity chromatography of the $rP2Y_1$–GFP receptor using 2-[(6-biotinylamido)-hexylthio]ATP. Western blotting of protein fractions from HEK 293 cells stably transfected with the $rP2Y_1$–GFP receptor were carried out with the monoclonal anti-GFP antibody as described in Methods. Lanes were loaded with purified plasma membranes (20 μg of protein; lane 1), Igepal-soluble plasma membranes (20 μg of protein; lane 2), or the fraction eluted with 5 mM ATP (6 μg of protein; lane 3). The arrow indicates the position of the $rP2Y_1$–GFP receptor. Positions of molecular mass standards are shown on the right.

assays impedes progress in receptor pharmacology. Tests relying on downstream signaling responses have evolved as the primary means to assess ligand–receptor interactions. However, pharmacological characterization can be confounded by several factors that are specific for the model used [22], such as the presence of ectonucleotidase activity [23], release of nucleotides from cells after mechanical stimulation, the coexpression of additional P2Y receptors or P2X receptors, or the expression level of receptors [24,25]. The ability to insert receptors into foreign surrogate cells led to an explosion of information regarding the activity of drugs and the behavior of receptors. Hence, heterologously expressed receptors represent an important technology for drug discovery, allowing a better understanding of receptor–effector interaction [26].

In the present study, we used a heterologous receptor-expressing model system for the investigation of newly synthesized adenine nucleotides, which were previously tested at rat astrocytes and turkey erythrocyte membranes homologously expressing $P2Y_1$ receptor [14]. Due to their low sensitivity related to low expression levels of endogenous $P2Y_1$ receptors, rat astrocytes were not useful for establishing concentration–effect curves. Moreover, turkey erythrocytes express a P2Y receptor preferentially activated by ADP which is coupled to inhibition of adenylyl cyclase. An additional turkey P2Y receptor is activated by all trinucleotides [27,28]. Thus, using these systems the pharmacological potency of new nucleotide compounds for P2Y receptors does not necessarily reflect the activity of solely one receptor. The model system used here is a cell line that we recently generated by stably transfecting HEK 293 cells with $rP2Y_1$ wild-type or $rP2Y_1$–GFP receptor [16]. We found at the heterologously expressed $rP2Y_1$ receptor that ATP and ADP are full agonists at HEK 293 cells either expressing the wild-type or the GFP-tagged receptor and that transfection renders the cells up to 1000-fold more sensitive to different nucleotides than control cells [16].

The most potent and selective $P2Y_1$ receptor specific agonists currently known bear a thioether chain at C-2 of the adenine ring [6,13,29,30]. We recently investigated the influence of different electron-donating groups (2-BuS-, 2-BuNH-, 2-BuO-) on ATP on the potency of these derivatives at $P2Y_1$ receptors. Furthermore, we evaluated the importance of steric and conformational effects on the nucleotide by using the 8-BuS-, 8-BuNH-, and 8-BuOATP analogues. Long-chain 2-alkylthio substituents were shown to provide both potency and stability for the ligands. The butyl chain was chosen for practical synthetic advantages [20,29]. We could clearly demonstrate that substitution at the 2-position of the purine ring of ATP with the BuS-, BuNH-, and BuO chain resulted in ATP derivatives capable of inducing Ca^{2+} responses at significantly decreased con-

centrations compared with the ATP concentrations required to induce a comparable response. The potency of 2-BuSATP was approximately equal to that of 2-MeSATP, whereas 8-substituted agonists showed a decreased potency in comparison to ATP. The steric influence and favorable hydrophobic interactions of C-2 substituents rather than an influence of the electron-donating groups [14,15] was confirmed here with this series of nucleotide analogues at the rP2Y_1 receptor in HEK 293 cells. 2-Substituted ATP analogues are more potent than ATP, both possessing an anti-conformation, probably because of a tighter fit of the ligand due to interaction of a C-2 substituent with a hydrophobic pocket in the receptor-binding cavity [15,31–33]. This effect induced by the C-2 butylthio group possibly compensates for the lack of the γ-phosphate group, since 2-BuSATP was as potent as 2-BuSADP.

In addition, the corresponding AMP analogues were assayed here to evaluate the importance of a triphosphate chain for receptor–ligand recognition as well as the ability of these AMP derivatives, with AMP generally not active at P2Y receptors, to stimulate P2Y_1 receptors. AMP and the corresponding AMP analogues had a low affinity for the receptor with the exception of 2-BuSAMP, which had an EC_{50} value similar to that of unsubstituted ATP. This agrees with the results of Boyer *et al*. [30], who found that 2-thioether derivatives of AMP are potent and selective P2Y purinoceptor agonists at different cells. Evidently, the β,γ-diphosphate group also plays an important role in the process of receptor activation, but the influence of a thioether substituent at C-2 even compensates for the lack of effect caused by the two phosphate groups missing in 2-BuSAMP. A detailed discussion of the molecular recognition of modified adenine nucleotides is given in [14].

At the rP2Y_1 receptor expressed at a high level in our system, ADP had an approximately 1000-fold greater potency than ATP [16]. The human P2Y_1 receptor is preferentially activated by ADP, with ATP being a partial agonist when expressed at high levels in 1321N1 cells [18]. Thus, species differences obviously exist between the human and rat P2Y_1 receptor regarding the potency of ATP. The different action of ATP at the same P2Y receptor subtype of different species is even more pronounced at the recombinantly expressed P2Y_4 receptor: ATP is a full and potent agonist at the rat P2Y_4 receptor, but is a competitive antagonist with moderate potency at the human P2Y_4 receptor [24].

The question arises as to whether the results obtained with endogenous receptors in natural systems are in agreement with those described for the recombinant expression system. The HEK 293 cell model is equivalent to other mammalian systems such as islet β-cells [29] and the rat superior cervical ganglion neurons [34], where 2-RSATP-α-S and 2-MeSADP, respectively, activate P2Y_1 receptors at the nanomolar range. Recombinant receptor-expressing systems have spare receptors that sensitize them towards agonists [26,35,36]. The maximally detectable agonist-evoked responses we found did not differ significantly, indicating that enhanced sensitivity is caused by a receptor reserve. Furthermore, the range of EC_{50} values derived from agonist-stimulated P2Y_1 receptors in turkey erythrocytes agrees with our data, but the potency is increased up to 10-fold in the transfected HEK 293 cells.

Concerning the question of possible cross-reactivity with P2Y_2 receptors, we previously showed that UTP, which activates P2Y_2 receptors with approximately equal potency as ATP, has an at least 100-fold decreased potency in transfected cells in comparison to ATP [16]. At NG108-15 neuroblastoma–glioma hybrid cells, which solely express the P2Y_2 receptor [37,38], even 10- to 100-μM concentrations of the same agonists as used in this study resulted in negligible responses [14]. Moreover, the fact that P2Y_2 receptors are not stimulated by C-2-substituted nucleotides at low concentrations [30] and that they are only responsive to triphosphates [39] and not activated by C-2-alkylthio-AMP compounds [30] strongly suggest that the analogs described here have no potency towards P2Y_2 receptors. The activation of α,β-methylene-ATP-sensitive P2X receptors can be excluded, because this compound does not evoke any response at the HEK cells (not shown). However, the P2Y_4 receptor and the P2Y_{11} receptor that are activated by 2-MeSATP [11] need to be tested in other systems with these adenine nucleotide compounds.

Based upon our results, we found that even a bulky 6-biotinylamido-hexylthio group linked to the 2-position of adenine does not hinder the proposed binding of the compound to the lipophilic pocket at the P2Y_1-binding site. The newly synthesized 2-[(6-biotinylamido)-hexylthio]ATP was 22-fold more potent in inducing Ca^{2+} release than ATP based on the corresponding EC_{50} values. Antagonists generally have some advantages over agonists for receptor isolation. However, antagonists reported for the P2Y_1 receptor are either of very low affinity, reduced specificity, or cannot be modified for affinity chromatography. Moreover, agonists with obviously high affinity should also be suitable for it. Thus, we used the biotinylated compound to enrich the heterologously expressed rP2Y_1–GFP receptor by affinity chromatography of the crude solubilized material. We could demonstrate the usefulness of this analogue for the purification of P2Y_1 receptors, whose definite authenticity has yet to be confirmed. If so, it will be suitable for a rapid advance in the field of protein biochemical analysis of this receptor type. Binding of biotin to avidin in immunoassays and other bioanalytical detection systems will provide another avenue for further receptor studies besides that opened by the availability of GFP-tagged P2Y_1 receptors [16].

In summary, we were able to obtain a clear affinity series for new P2Y_1 receptor-specific agents in a mammalian receptor, and we describe here a new promising P2Y_1 receptor-specific probe, which seems to be useful for P2Y_1 receptor isolation.

Acknowledgments

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References

[1] Burnstock G. The past, present and future of purine nucleotides as signalling molecules. Neuropharmacology 1997;36:1127–39.
[2] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.
[3] Centemeri C, Bolego C, Abbracchio MP, Cattabeni F, Puglisi L, Burnstock G, Nicosia S. Characterisation of the Ca^{2+} response evoked by ATP and other nucleotides in mammalian brain astrocytes. Br J Pharmacol 1997;12:1700–6.
[4] Bernstein M, Behnisch T, Balschun D, Reymann KG, Reiser G. Pharmacological characterisation of metabotropic glutamatergic and purinergic receptors linked to Ca^{2+} signalling in hippocampal astrocytes. Neuropharmacology 1998;37:169–78.
[5] Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y coupled to phospholipase C and adenylyl cyclase. J Biol Chem 1997;272:31969–73.
[6] Boyer JL, O'Tuel JW, Fischer B, Jacobson KA, Harden TK. Potent agonist action of 2-thioether derivatives of adenine nucleotides at adenylyl cyclase-linked P2Y purinoceptors. Br J Pharmacol 1995; 116:2611–6.
[7] Rathbone MP, Middlemiss JP, Kim JL, Gysbers JW, Deforge SP, Smith RW, Hughes DW. Adenosine and its nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. Neurosci Res 1992;13:1–17.
[8] Abbracchio MP, Ceruti S, Langfelder R, Cattabeni F, Saffrey MJ, Burnstock G. Effects of ATP analogues and basic fibroblast growth factor on astroglial cell differentiation in primary cultures of rat striatum. Int J Dev Neurosci 1995;13:185–93.
[9] Abbracchio MP, Burnstock G. Purinergic signalling: pathophysiological roles. Jpn J Pharmacol 1998;78:113–35.
[10] Barnard EA, Simon J, Webb TE. Nucleotide receptors in the nervous system. An abundant component using diverse transduction mechanisms. Mol Neurobiol 1997;15:103–29.
[11] Burnstock G. Current status of purinergic signalling in the nervous system. In: Illes P and Zimmermann H, editors. Progress in brain research 120. Nucleotides and their receptors in the nervous system. Amsterdam: Elsevier Science BV, 1999. p. 3–10.
[12] Moore D, Chambers J, Waldvogel H, Faull R, Emson P. Regional and cellular distribution of the $P2Y_1$ purinergic receptor in the human brain: striking neuronal localisation. J Comp Neurol 2000;421:374–84.
[13] Fischer B, Boyer JL, Hoyle CH, Ziganshin AU, Brizzolara AL, Knight GE, Zimmet J, Burnstock G, Harden TK, Jacobson KA. Identification of potent, selective P2Y purinoceptor agonists: structure–activity relationships for 2-thioether derivatives of adenosine 5′-triphosphate. J Med Chem 1993;36:3937–46.
[14] Halbfinger E, Major DT, Ritzmann M, Ubl J, Reiser G, Boyer JL, Harden TK, Fischer B. Molecular recognition of modified adenine nucleotides by the $P2Y_1$ receptor. 1. A synthetic, biochemical, and NMR approach. J Med Chem 1999;42:5325–37.
[15] Major DT, Halbfinger E, Fischer B. Molecular recognition of modified adenine nucleotides by the $P2Y_1$ receptor. 2. A computational approach. J Med Chem 1999;42:5338–47.
[16] Vöhringer C, Schäfer R, Reiser G. A chimeric rat brain $P2Y_1$ receptor tagged with green-fluorescent protein shows high-affinity ligand recognition of adenosine diphosphates and triphosphates and selectivity identical to that of the wild-type receptor. Biochem Pharmacol 2000; 59:791–800.
[17] Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK. Second messenger cascade specifity and pharmacological selectivity of the human $P2Y_1$-purinoceptor. Br J Pharmacol 1996;118:167–73.
[18] Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK. Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. Mol Pharmacol 1998;54:1118–23.
[19] Tokuyama Y, Hara M, Jones EM, Fan Z, Bell GI. Cloning of rat and mouse P2Y purinoceptors. Biochem Biophys Res Commun 1995; 211:211–8.
[20] Gendron FP, Halbfinger E, Fischer B, Duval M, D'Orleans-Juste P, Beaudoin AR. Novel inhibitors of nucleoside triphosphate diphosphohydrolases (NTPDases): chemical synthesis, biochemical and pharmacological characterizations. J Med Chem 2000;43:2239–47.
[21] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[22] Humphrey PA. The characterization and classification of receptors. J Recept Signal Transduct Res 1998;18:345–62.
[23] Zimmermann H. Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. Trends Pharmacol Sci 1999;20:231–6.
[24] Kennedy C, Qi AD, Herold CL, Harden TK, Nicholas RA. ATP, an agonist at the rat $P2Y_4$ receptor, is an antagonist at the human $P2Y_4$ receptor. Mol Pharmacol 2000;57:926–31.
[25] Ostrom RS, Gregorian C, Insel PA. Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. J Biol Chem 2000;275:11735–9.
[26] Kenakin T. The classification of seven transmembrane receptors in recombinant expression systems. Pharmacol Rev 1996;48:413–65.
[27] Boyer JL, Waldo GL, Harden TK. Molecular cloning and expression of an avian G protein-coupled P2Y receptor. Mol Pharmacol 1997; 52:928–34.
[28] Boyer JL, Delaney SM, Villanueva D, Harden TK. A molecularly identified P2Y receptor simultaneously activates phospholipase C and inhibits adenylyl cyclase and is nonselectively activated by all nucleoside triphosphates. Mol Pharmacol 2000;57:805–10.
[29] Fischer B, Chulklin A, Boyer JL, Harden TK, Gendron FP, Beaudoin AR, Chapal J, Hillaire-Buys D, Petit P. 2-thioether 5′-O-(thiotriphosphate)adenosine derivatives as new insulin secretagogues acting through P2Y receptors. J Med Chem 1999;42:3636–46.
[30] Boyer JL, Siddiqi S, Fischer B, Romero-Avila T, Jacobson KA, Harden TK. Identification of potent P2Y-purinoceptor agonists that are derivatives of adenosine 5′-monophosphate. Br J Pharmacol 1996;118:1959–64.
[31] Van Rhee MA, Fischer B, Van Galen PG, Jacobson KA. Modelling the P2Y purinoceptor using rhodopsin as template. Drug Des Discover 1995;13:133–54.
[32] Moro S, Guo D, Camaioni E, Boyer JL, Harden TK, Jacobson KA. Human $P2Y_1$ receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. J Med Chem 1998;41:1456–66.
[33] Tomkinson NP, Marriott DP, Cage PA, Cox D, Davis AM, Flower DR, Gensmantel NP, Humphries RG, Ingall AH, Kindon ND. P2T purinoceptor antagonists. A QSAR study of some 2-substituted ATP analogues. J Pharm Pharmacol 1996;48:206–9.
[34] Filippov AK, Brown DA, Barnard EA. The $P2Y_1$ receptor closes the N-type Ca^{2+} channel in neurons, with both adenosine triphosphates and diphosphates as potent agonists. Br J Pharmacol 2000;129:1063–6.
[35] Kenakin T. Agonist-specific receptor conformations. Trends Pharmacol Sci 1997;18:416–7.

[36] Kenakin T. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. Trends Pharmacol Sci 1997;18:456–64.
[37] Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci USA 1993;90:5113–7.
[38] Erb L, Lustig KD, Sullivan DM, Turner JT, Weisman GA. Functional expression and photoaffinity labeling of a cloned P2U purinergic receptor. Proc Natl Acad Sci USA 1993;90:10449–53.
[39] Nicholas RA, Lazarowski ER, Watt WC, Li Q, Boyer J, Harden TK. Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. J Auton Pharmacol 1996;16:319–23.