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Subtype specific internalization of $P2Y_1$ and $P2Y_2$ receptors induced by novel adenosine 5′-O-(1-boranotriphosphate) derivatives

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1 P2Y-nucleotide receptors represent important targets for drug development. The lack of stable and receptor specific agonists, however, has prevented successful therapeutic applications. A novel series of P-boronated ATP derivatives (ATP-α-B) were synthesized by substitution of a nonbridging O at P_α with a BH_3 group. This introduces a chiral center, thus resulting in diastereoisomers. In addition, at C2 of the adenine ring a further substitution was made (Cl- or methylthio-). The pairs of diastereoisomers were denoted here as A and B isomers.

2 Here, we tested the receptor subtype specificity of these analogs on HEK 293 cells stably expressing rat $P2Y_1$ and rat $P2Y_2$ receptors, respectively, both attached to the fluorescent marker protein GFP ($rP2Y_1$-GFP, $rP2Y_2$-GFP). We investigated agonist-induced receptor endocytosis, $[Ca^{2+}]_i$ rise and arachidonic acid (AA) release.

3 Agonist-induced endocytosis of $rP2Y_1$-GFP was more pronounced for the A isomers than the corresponding B counterparts for all ATP-α-B analogs. Both 2-MeS-substituted diastereoisomers induced a greater degree of agonist-induced receptor endocytosis as compared to the 2-Cl-substituted derivatives. Endocytosis results are in accordance with the potency to induce Ca^{2+} release by these compounds in HEK 293 cells stably transfected with $rP2Y_1$.

4 In case of $rP2Y_2$-GFP, the borano-nucleotides were very weak agonists in comparison to UTP and ATP in terms of Ca^{2+} release, AA release and in inducing receptor endocytosis. The different ATP-α-B derivatives and also the diastereoisomers were equally ineffective.

5 Thus, the new agonists may be considered as potent and highly specific agonist drug candidates for $P2Y_1$ receptors. The difference in activity of the ATP analogs at P2Y receptors could be used as a tool to investigate structural differences between P2Y receptor subtypes.
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Abbreviations: AA, [^{3}H]arachidonic acid; $[Ca^{2+}]i$, intracellular free calcium concentration; CLSM, confocal laser scanning microscope; DMEM, Dulbecco's modified Eagles' medium; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK 293, human embryonic kidney cells; 2-Me-S-ADP, 2-methlythio adenosine 5′-diphosphate; 2-Me-S-ATP, 2-methylthio adenosine 5′-triphosphate; PLL, poly-L-lysin

Introduction

P2 receptors are an important class of receptors, which are subdivided into ionotropic P2X receptors and metabotropic P2Y receptors. The family of P2Y receptors, members of the superfamily of G protein-coupled receptors (GPCRs), includes eight characterized subtypes, namely $P2Y_{(1,2,4,6,11,12,13,14)}$ (Dubyak, 2003). ATP exerts significant biological actions on various tissues and organs (Barnard *et al*., 1997; Boarder & Hourani, 1998). From the receptors in the P2Y family, $P2Y_1$ and $P2Y_{11}$ are selectively activated by adenine nucleotides (Webb *et al*., 1993; Communi *et al*., 1997; 1999). These different receptor subtypes that can be activated by ATP exhibit similar coupling in terms of phospholipase C activation, except that $P2Y_{11}$ additionally couples to G_s to activate adenylyl cyclase (Zambon *et al*., 2000). The $P2Y_4$ (Communi *et al*., 1995) and $P2Y_6$ receptors (Chang *et al*., 1995) are stimulated by pyrimidine nucleotides and the $P2Y_2$ receptor responds equipotently to the purine and pyrimidine triphosphates ATP and UTP (Lustig *et al*., 1993). In addition to these receptors, there are the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors, which are negatively coupled to adenylyl cyclase *via* the G_i protein (Müller, 2002). The human $P2Y_{12}$ receptor is stimulated by ADP, 2Me-S-ADP (Nicholas, 2001) and to a lesser extent by ATP. The pharmacological profile of the human $P2Y_{13}$ is similar to that of the $P2Y_{12}$, as ADP and ATP can also stimulate it. The latest member of the P2Y family, $P2Y_{14}$, is activated by UDP-glucose, while the other nucleotides like ATP, UTP, ADP or UDP do not stimulate this receptor (Chambers *et al*., 2000).

The P2Y receptors can be subdivided into two different subgroups depending on their structure and their phylogenetic origin. The first group includes $P2Y_{1,2,4,6,11}$, which have a similar putative nucleotide-binding motif on trans-membrane

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domains 6 (TM6) and TM7, and the second group includes $P2Y_{12,13,14}$ (Abbracchio *et al*., 2003). This indicates that there is a certain extent of difference in which the various receptor subtypes bind the nucleotides. Inspite of the difference in the putative binding motifs, ATP functions as an agonist at most of the P2Ys (Von Kügelgen & Wetter, 2000), except for $P2Y_{4,6,14}$. The P2Ys that are expressed in other species (canine, bovine, rat mouse, and turkey) exhibit a similar profile in terms of response to agonist.

The P2 receptors play an important role in different pathophysiological conditions and, thus, represent a class of receptors which are possibly promising targets for novel drug development for therapeutic application. A major problem encountered in the development of agonists specific for a nucleotide receptor was their stability and solubility (Guile *et al*., 2001). Synthesis of C2-substituted ATP analogs significantly increased the potency and the specificity of the agonist for the $P2Y_1$ receptor (Fischer *et al*., 1993; Halbfinger *et al*., 1999). It was observed with these compounds that steric effects rather than electronic configuration play a major role in ligand binding in case of $P2Y_1$ receptors. In line with these developments, novel agonists that are chemically and enzymatically more stable were developed to selectively stimulate $P2Y_1$ receptors. These agonists belong to the series of P-boronated ATP derivatives (ATP-α-B), synthesized by substitution of a nonbridging O with a BH_3 group, as shown in Figure 1 (Nahum *et al*., 2002). This group introduces a chiral center, thus resulting in pairs of diastereoisomers. In addition to the modification of the phosphate chain, the adenine ring was substituted by a Cl or an SMe group at position C2. These substitutions also affect the binding of the molecule in terms of the spatial conformation of the molecule.

It was observed that in case of both substitutions (2-Cl or 2-SMe) the A diastereoisomer was always more potent than the B counterpart. Therefore, the absolute configuration of the agonist determines the receptor subtype potency. The substitution of the adenine C2 with an SMe group also improved the potency of the agonist in terms of stimulating Ca^{2+} release in the $P2Y_1$ receptor-expressing cells (Nahum *et al*., 2002).

Stimulation of GPCRs with agonist is followed either by desensitization of the receptor or its internalization. Desensitization of the receptor is a fast process, which proceeds within seconds to minutes. Resensitization of the receptor is normally achieved by the removal of the agonist from the extracellular fluid (Böhm *et al*., 1996; Freedman & Lefkowitz, 1996). It has been shown that the recovery from desensitization of the $P2Y_2$ receptor-activated anion secretion takes less than 10 min after stimulation of the cells with UTP (Clarke *et al*., 1999). Long-term desensitization of GPCRs is due to endocytosis of the receptors involving protein complexes, which recruit a large number of adapter molecules, like arrestin (Barlic *et al*., 1999), dynamin (van Dam & Stoorvogel, 2002), clathrin (Luis Albasanz *et al*., 2002), or caveolin (Rybin *et al*., 2000). The complexes lead to the formation of vesicles and transport of the receptor from the plasma membrane to the cytosol. In the cytosol, the receptors are transported to endosomes, from where they can be recycled back to the plasma membrane (van Dam *et al*., 2002) or directed to the lysosomes. Targeting of receptor to lysosomes leads to degradation of the receptor. Even the endocytosed receptor can continue to transmit signals and thus help fine-tuning the initial response and induce responses like cell proliferation (Wang *et al*., 2002). Resensitization of P2Y receptors is affected by nitric oxide (Liu *et al*., 2002) or by growth hormone (Gutierrez *et al*., 2000). Thus, desensitization and endocytosis of the receptor are two different aspects of regulation of sensitivity to agonist.

We have already shown that the diastereoisomers of the novel boranophosphate ATP agonists exhibit clear differences in terms of Ca^{2+} release, which was induced in HEK 293 cells stably expressing the $rP2Y_1$ receptor (Nahum *et al*., 2002). Thus, the difference in absolute configuration around Pα leads to a stronger interaction between the receptor and one of the diastereoisomers. In the light of these findings, we decided to investigate the specificity and potency of the novel agonists on further physiological responses, such as (1) induction of arachidonic acid (AA) release and (2) internalization of the P2Y receptor. We performed these experiments in HEK 293 cells stably expressing either $rP2Y_1$ or $rP2Y_2$ tagged to GFP.

Methods

Materials

The following materials were used: geneticine (G418 sulphate) from Calbiochem; poly-L-lysin (PLL), ATP, UTP, 2-Me-S-ADP (Sigma, Deisenhofen); 2-Me-S-ATP (Biotrend, Köln); Ham's-F12, DMEM, penicillin/streptomycin (10,000/10,000 U ml^{-1}), trypsin/EDTA (0.05%/0.02%), fetal calf serum (FCS) (Seromed, Biochrom, Berlin); cell culture dishes (Nunc, Wiesbaden); coverslips (22 mm) (OmniLab); tritium-labeled AA ([^{3}H]AA) (Amersham Biosciences, Freiburg); Fura 2-AM (Biomol, Hamburg/Molecular Probes); Aquatex (Merck, Darmstadt, Germany).The novel agonists that were used in the study were synthesized as described (Nahum *et al*., 2002).

Cell culture and transfection

The $rP2Y_2$ receptor cDNA was kindly provided by Dr Rice (Rice *et al*., 1995). The $rP2Y_2$ receptor DNA was subcloned into pEGFPN2 (Clontech), with the GFP tag on the C-terminus. After subcloning, the DNA was sequenced to check for any mutations. HEK 293 cells were used for transfection, as these cells endogeneously express this receptor subtype. Cell

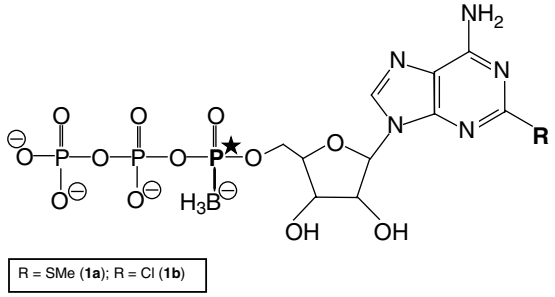

Figure 1 Structure of the novel ATP-α-B derivatives. The hydrogen at C2 (R) was replaced by either SMe or Cl. Secondly, a nonbridging O at the P_α was substituted by BH_3. The BH_3 group introduces a chiral center at P_α. The two substitutions result in the formation of two diastereoisomers for each analog. The compounds that contain the SMe and Cl substitution were termed 1a and 1b, respectively. The diastereoisomers in each case were called A and B.

culture and transfection of HEK 293 cells was carried out as described (Vöhringer *et al*., 2000). The HEK 293 cells were transfected with 5 μg ml^{-1} of DNA in serum-free medium using DOTAP for 8 h. After 8 h, the medium was replaced with growth medium and positive cells were selected with 1 mg ml^{-1} G418. Transfected cells were grown in medium consisting of DMEM/Ham's-F12 (1 : 1), supplemented with 10% FCS, 100 IU ml^{-1} penicillin and 100 IU ml^{-1} streptomycin in a 5% CO_2/95% air, humidified atmosphere at 37°C. The cells were plated at a density of 5×10^5 to 1×10^6 cells per dish (Ø = 50 mm) containing PLL (0.01%) pre-coated coverslips (Ø = 22 mm). HEK 293 cells expressing $P2Y_1$-GFP (Vöhringer *et al*., 2000) were handled in the same way. A549 cells were grown in DMEM medium supplemented with 10% FCS, 100 IU ml^{-1} penicillin and 100 IU ml^{-1} streptomycin in a 5% CO_2/95% air, humidified atmosphere at 37°C.

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

The cells were plated on PLL-coated plates and single-cell measument was carried out after 3 days, when the cells were 40–60% confluent. The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) changes were measured, as described before (Ubl *et al*., 1998) by preincubation of the cells with 2 μM Fura-2AM for 30 min in NaHBS (HEPES buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose and 20 mM HEPES/Tris pH 7.4) and then stimulating the cells under continuous superfusion of pre-warmed NaHBS at 37°C with different concentrations of UTP or the different agonist at 37°C. Fluorescence intensity was recorded alternatively at 340 and 380 nm excitation and 520 nm emission. Changes were monitored in single cells bathed in a perfusion chamber, which was placed on the microscope stage of a fluorescence imaging system from TILL Photonics with a $\times 40$ oil-immersion objective and a flow rate of 1 ml min^{-1} (Vöhringer *et al*., 2000).

Agonist-induced endocytosis and Confocal imaging

The cells were grown on PLL-coated coverslips for imaging of agonist-induced endocytosis of the receptor. The cells were stimulated with varying concentrations of agonist in medium at 37°C. Pictures were taken using a Zeiss inverted LSM 510 confocal laser scanning microscope (CLSM) equipped with a Plan-Apochromat $\times 63$ objective. Fluorescence of GFP was excited using a 488 nm argon/krypton laser, and emitted fluorescence was detected with 505–530 band pass filter. Images were processed with Zeiss confocal microscopy software, release 2.5.

Radiolabeling and assessment of AA release

HEK 293 cells expressing $P2Y_1$-GFP or $P2Y_2$-GFP or untransfected A549 cells were seeded in 12-well plates at a starting density 6×10^5 cells ml^{-1} (1 ml per well) in respective culture medium and grown at 37°C with 5% CO_2. The medium was changed after 2 days to culture medium containing [^{3}H]AA (0.25 μCi per well) for 16 h. Then, cells were washed twice with 1 ml of medium and were allowed to recover for 30 min in 1 ml of complete medium. After 30 min, agonist was added and then cells were incubated for 7.5 min at 37°C. In all, 250 μl of the supernatant was removed and the radioactivity was measured by liquid scintillation counting with 1 ml of 'Ultima Gold' scintillation cocktail (Packard Instruments, U.S.A.).

Data analysis

Unless stated otherwise, results are presented as means ± s.d. and statistical analysis was achieved by Student's unpaired *t*-test using Sigma Plot (Jandel Scientific, Erkrath, Germany).

Results

Functional heterologous expression of tagged $P2Y_2$ receptors in HEK 293 cells

The cells expressing $rP2Y_2$-GFP were directly visualized using a CLSM. It was observed that the receptor was localized only on the plasma membrane of the cells and not in any other compartment (see below Figure 4g), which is in accordance with the natural distribution of the endogeneous receptor. The presence of the GFP tag does not affect the localization of the receptor and the stable heterologous expression of the $P2Y_2$-GFP receptor is not toxic to the cells. Toxicity was found for GFP expressed in some cells (Liu *et al*., 1999). The functional expression of the receptor was confirmed by increase of intracellular Ca^{2+} after stimulation with UTP or ATP. This increase was observed after pre-incubation of the cells with 2 μM Fura-2AM for 30 min in NaHBS for dye loading, and then stimulating them with ATP or UTP at 37°C in NaHBS buffer (Vöhringer *et al*., 2000).

Evaluation of ATP-α-B analogs as P2Y receptor agonists

The structural differences of the novel agonists are shown in Figure 1. In the subsequent description, the SMe derivatives will be referred to as 1a and the Cl derivatives as 1b. The diastereisomers for both substitutions are referred to as A and B, keeping in line with the nomenclature that was used in the previous publication (Nahum *et al*., 2002). The novel agonists differ from each other in terms of Ca^{2+} release mediated by the $rP2Y_1$ receptor, because of the presence of varied substituents. The efficacy of the agonists 1a (2-MeS-ATP-α-B)A/B and 1b (2-Cl-ATP-α-B)A/B as more potent agonists at the $P2Y_1$ receptor compared to ATP, the naturally occurring agonist, has been shown by us previously in HEK 293 cells stably transfected with rat brain $P2Y_1$ receptor ($rP2Y_1$) (Nahum *et al*., 2002). The EC_{50} values in untransfected HEK 293 cells for ATP and UTP were 25 and 30 μM, respectively. Stably transfected HEK 293 cells, expressing rat $P2Y_2$ receptor tagged with GFP ($rP2Y_2$-GFP), were more sensitive to ATP and UTP, which is reflected as a reduction of the EC_{50} value to 200 and 250 nM for ATP and UTP, respectively.

HEK 293 cells stably expressing $rP2Y_2$-GFP were stimulated with 1a A/B and 1b A/B, and it was observed that these substances are very weak agonists compared to the natural agonist ATP (Figure 2a). This is different from the results observed with the $P2Y_1$ receptor, where we could demonstrate recently that the borano-nucleotides are agonists which are more potent than ATP and display stereo-selectivity (Nahum *et al*., 2002). The increased potency is obvious from the EC_{50} values obtained for the different agonists; for 1aA the EC_{50} value was 2.6×10^{-9} M, for 1aB it was 5.3×10^{-8} M, for 1bA

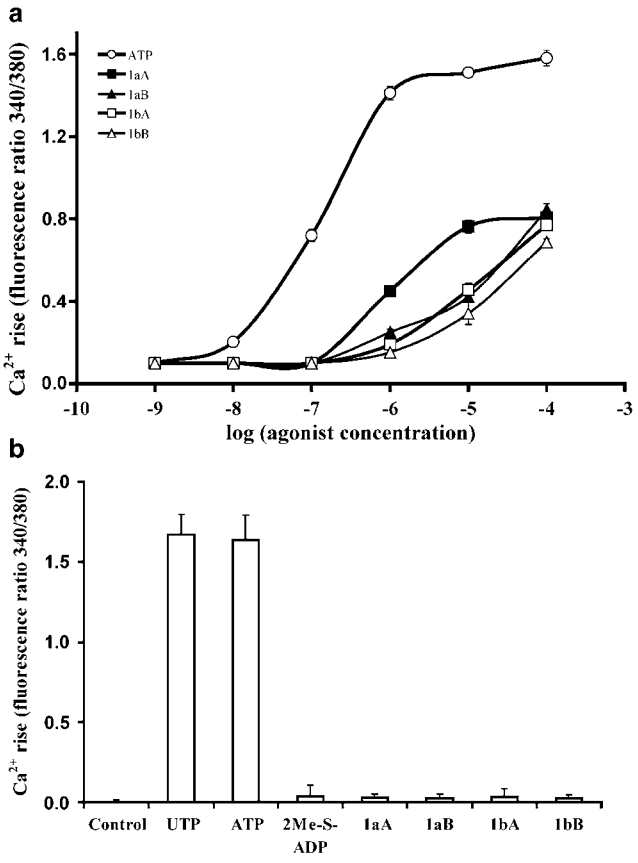

Figure 2 Concentration–response curves for Ca^{2+} rise induced by ATP-α B analogs in HEK 293 cells expressing the $P2Y_2$-GFP (a) and in A549 cells (b). HEK 293 cells stably expressing $rP2Y_2$-GFP were grown in serum-containing medium. For measurements, the cells were preloaded with Fura-2/AM and then superfused with NaHBS containing Ca^{2+} and stimulated for 1 min with different concentrations of agonist. The concentration-dependent rise in Ca^{2+} is measured as the ratio between the fluorescence obtained at 340 and 380 nm upon stimulation with different concentrations of ATP (open circles), 1aA (closed squares), 1aB (closed triangles), 1bA (open squares) and 1bB (open triangles). The data are from Ca^{2+} recordings in single cells and represent the mean ± s.d. from at least 50 cells for each value. (b) Ca^{2+} rise induced by ATP, UTP and ATP-αB diastereoisomers in A549 cells. A549 cells were loaded with Fura-2 and then stimulated as $rP2Y_2$-GFP cells, with 10 μM of the different agonists, and the rise in $[Ca^{2+}]_i$ was measured. The data are from single-cell measurements and represent the mean ± s.d. from at least 50 cells for each value.

4.5×10^{-9} M, or 1bB 3.6×10^{-8} M, and for ATP we obtained 2×10^{-7} M. In addition, it was observed that in case of both substitutions (2-Cl or 2-SMe) the A diastereoisomer was always more potent than the B counterpart. Therefore, the absolute configuration of the agonist determines the receptor subtype potency. In case of UTP, the natural agonist for the $P2Y_2$ receptor, which is equipotent with ATP, the calcium response curve is similar to that of ATP (data not shown). Upon stimulation of $rP2Y_2$-GFP cells with 100 μM of ATP-α-B, the calcium rise is only 51% of the maximum response elicited by 100 μM UTP. When the $rP2Y_2$-GFP cells are stimulated with the four substances at 10 μM concentrations, the calcium release is between 44 and 17% of that obtained with 10 μM ATP. At 1 μM the calcium response is between 25 and 0.04% of that obtained with 1 μM ATP. It is seen that in case of all four substances (1aA/1aB and 1bA/1bB), no calcium release can be observed at concentrations of 100 nM, whereas at 100 nM ATP the calcium response obtained with $rP2Y_2$ is 41% of the maximally induced response, which is obtained with 100 μM ATP. The EC_{50} value obtained for ATP was 200 nM. Thus, from these results it is clear that these four novel substances do not represent potent agonists at the $P2Y_2$ receptor.

To further confirm that the borano-nucleotides are specific agonists for the $P2Y_1$ receptor, we pre-incubated HEK 293 cells expressing $P2Y_1$-GFP with MRS-2179 (10, 30 and 100 μM) for 30 min. In these cells, we determined the Ca^{2+} rise (as described in Methods) induced by stimulation of the cells with 1 μM of 1aB, 1bA and 1bB. We observed that after pre-incubation of the cells with MRS 2179, a competitive and specific antagonist of the $P2Y_1$ receptor (Boyer *et al*., 1998), there is a decrease in the Ca^{2+} response (Table 1). The degree of reduction in the Ca^{2+} rise differs for the different borano-nucleotide analogs.

We next tested these four substances whether they induce Ca^{2+} release in A549 cells, which are lung epithelial cells. These cells were used, because they lack endogenous $P2Y_1$ receptors (Schäfer *et al*., 2003). It was observed that with 10 μM of ATP-α-Bs there was almost no Ca^{2+} response, in comparison to that observed when the cells were stimulated with ATP or UTP. ATP and UTP elicited a Ca^{2+} response of 1.63 and 1.6 times above basal level, respectively (Figure 2b). Thus, in comparison to the concentration–response curves obtained for the $rP2Y_1$ receptor (Nahum *et al*., 2002), it is clearly seen that the substances 1aA/B and 1bA/B are no agonists active at the $P2Y_2$ receptor in terms of calcium release.

Table 1 Effect of MRS-2179 on the Ca^{2+} response induced by the different agonists 1aB, 1bA and 1bB in HEK 293 cells expressing $P2Y_1$-GFP

Agonist	*Concentration of MRS-2179 (μM)*			
	0 (control)	*10*	*30*	*100*
	Response to agonist (fluorescence increase) *Inhibition (% of response in the absence of MRS-2179)*			
1aB	0.842 ± 0.0845 (0%)	0.412 ± 0.045 (51%)	0.148 ± 0.058 (82%)	0.01 ± 0.007 (98%)
1bA	1.36 ± 0.182 (0%)	0.915 ± 0.142 (30%)	0.650 ± 0.168 (52%)	0.325 ± 0.10 (76%)
1bB	1.03 ± 0.095 (0%)	0.294 ± 0.102 (71%)	0.121 ± 0.011 (88%)	0.01 ± 0.005 (99%)

The values for the response (mean ± s.d.) give the increases of the ratio of Fura-2 fluorescence (obtained at 340 and 380 nm) and the values in parenthesis indicate the % inhibition of the Ca^{2+} response in comparison to that obtained in the absence of MRS-2179 (control response).

AA release induced by stimulation with agonist

The next parameter used to test the selectivity of the agonists was their ability to release AA from the cells. The stimulation of HEK 293 cells expressing $rP2Y_1$-GFP with 10 μM of ATP resulted is a massive release of AA, which is 200% of the control value (data not shown). The $rP2Y_2$-GFP expressing cells were also stimulated with 10 μM of ATP and UTP, and the release of AA was 178 and 175% above the control (data not shown).

When the $rP2Y_2$-GFP cells were stimulated with 10 μM of the different ATP-α-Bs, there was no significant elevation of AA release over the control level (Figure 3a). When further increasing the concentration of the agonists to 100 μM, the release of AA from the $rP2Y_2$-GFP cells was only 50% higher than that of the control (data not shown). Thus, obviously these substances are very weak agonists at the $P2Y_2$ receptor. Similar results were observed after stimulation of A549 cells (Figure 3b) with the four ATP-α-B compounds. Substances 1aA/1aB and 1bA/1bB did not cause any release of AA. A549 cells were, however, equally responsive to ATP and UTP, due to the endogenous $P2Y_2$ receptors.

In $rP2Y_1$-GFP-expressing cells, all the ATP-α-B compounds exhibit at least a 270% increase in AA release above the control value (Figure 3a). Stimulation of the $P2Y_1$-GFP-expressing cells with a similar concentration of ATP achieved a smaller AA release (data not shown). These results further confirm our previous finding (Nahum *et al*., 2002) showing that the four substances 1aA/1aB and 1bA/1bB are more potent than ATP at the $P2Y_1$ receptor.

In case of the $P2Y_1$ receptor, 10 μM of both diastereoisomers is almost equipotent in evoking AA release. This is different from the correlation observed for Ca^{2+} rise, where at 10 μM the A form was more potent than the B form (Nahum *et al*., 2002). Thus, from the results obtained here it can be concluded that the ATP-α-B analogs are more potent than ATP in inducing AA release from HEK 293 cells expressing $rP2Y_1$-GFP. In the $rP2Y_2$-GFP-expressing cells, there is no significant stimulation of AA release, which strengthens the conclusion that these substances are specific agonists for the $P2Y_1$ receptor subtype.

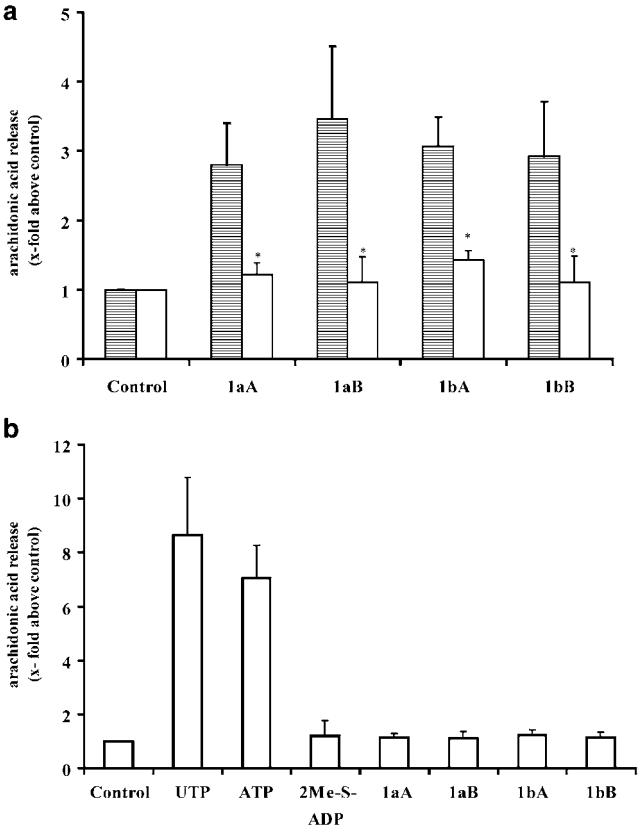

Figure 3 AA release from HEK 293 cells expressing $rP2Y_1$-GFP or $rP2Y_2$-GFP (a) and from untransfected A549 cells (b). Cells were grown under normal culture conditions. The cells were loaded with 0.25 μCi per well of AA for 16 h. For the release, the cells were washed twice with normal culture medium. After 30 min, cells were stimulated with 10 μM of the ATP-α-Bs and incubated at 37°C for 7.5 min, and then the radioactivity in the medium was measured, as described in Methods. Release without any addition (control) was normalized to 1. The cells expressing $rP2Y_1$-GFP (hatched bars) and $rP2Y_2$-GFP (open bars) were stimulated with 10 μM of the ATP-α-Bs. Values (mean $\pm$ s.d. from three independent experiments) are expressed as *x*-fold stimulation over basal AA release. The release of AA for cells expressing $rP2Y_1$-GFP and $rP2Y_2$-GFP cells was compared. *indicates $P<0.01$, calculated using paired Students *t*-test. (b) A549 cells were stimulated in the same way as HEK 293 cells and the AA release in the supernatant was measured. Values (mean $\pm$ s.d. of three independent experiments) are expressed as *x*-fold stimulation over basal AA release.

Agonist-induced endocytosis of $P2Y_1$ and $P2Y_2$ receptors

The $rP2Y_1$-GFP cells were stimulated with 10 μM of 2-Me-S-ATP or with ATP-α-B derivatives for 30 min at 37°C and the change in fluorescence intensity at the plasma membrane was determined. We observed that after stimulation of the cells with 10 μM agonist for 30 min there was a complete loss of fluorescence from the plasma membrane at the end of the 30 min incubation period (Figure 4b–f). The endocytotic pattern of the receptor after stimulation of the cells with either 10 μM 2-Me-S-ATP (Figure 4b) or with 10 μM ATP-α-B is completely different, both in terms of speed of endocytosis and the appearance of the endocytosed receptor. In case of substances 1aA (Figure 4c) and 1bA (Figure 4e), the receptor is endocytosed and is present in the cells as concentrated aggregates, whereas in the case of compound 1aB (Figure 4d) the receptor is present as loose aggregates, and with 1bB (Figure 4f) it is present as small vesicles that are diffusely distributed in the cytoplasm (as clearly depicted in the inset).

After stimulation of the cells with 10 μM 2-Me-S-ATP, a complete endocytosis of the $rP2Y_1$-GFP was observed; this coincided with a loss of fluorescence from the plasma membrane and a commensurate rise in the fluorescence intensity in the cytoplasm (data not shown). The changes in the fluorescence intensity were evaluated with Zeiss LSM software. The decrease in fluorescence intensity proceeds in a time-dependent manner (Figure 5a). In the case of the ATP-α-B substances, there is a complete loss of fluorescence from the plasma membrane at the end of 30 min, but the rate of loss of fluorescence is even faster for all the four ATP-α-B substances compared to 2-Me-S-ATP. Calculation of the rate of loss of fluorescence reveals that only 20% of the initial fluorescence remains on the plasma membrane at the end of 20 min in case of the boronated substances, whereas there is 40% of the initial fluorescence on the plasma membrane in the case of cells

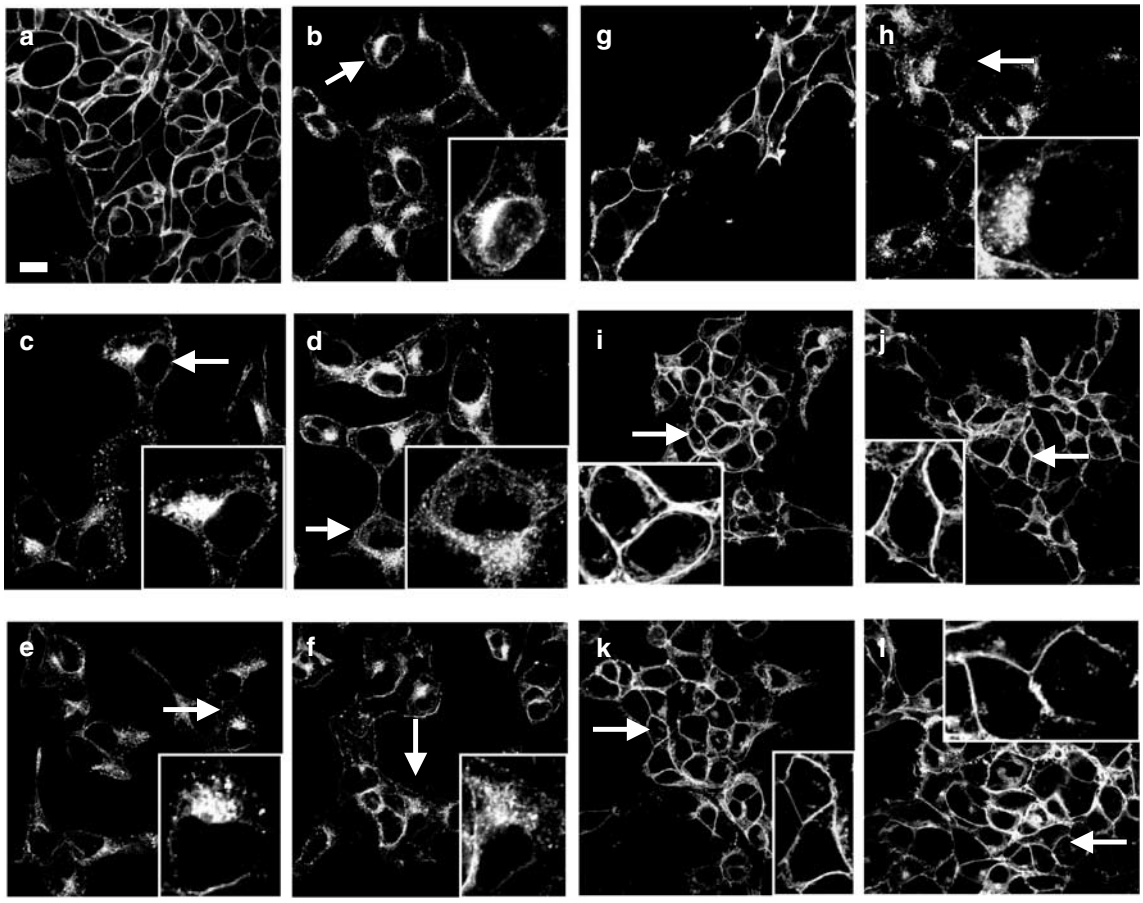

Figure 4 Visualization of $P2Y_1$ and $P2Y_2$ receptor endocytosis induced by ATP-α-B diastereoisomers in transfected cells. HEK 293 cells stably expressing $P2Y_1$-GFP or $P2Y_2$-GFP were stimulated with agonist for 30 min at 37°C. (a–f) Endocytosis of r$P2Y_1$-GFP upon stimulation with agonist. The localization of the receptor was observed in case of HEK 293 cells expressing $P2Y_1$-GFP without stimulation (a) and cells that were stimulated with 10 μM of 2-Me-S-ATP (b), 1aA (c), 1aB (d), 1bA (e) and 1bB (f). (g–l) Endocytosis of r$P2Y_2$ -GFP upon stimulation with agonist. The localization of the receptor was observed in case of HEK 293 cells expressing $P2Y_2$-GFP without stimulation (g) and cells that were stimulated with 10 μM ATP (h), 1aA (i), 1aB (j), 1bA (k) and 1bB (l). Pictures shown here are representative for three similar experiments. In (b–f) and (h), a cell marked by an arrow is shown enlarged in the respective inset, to show the intracellular structures with endocytosed receptor. Also in (i–l) insets are shown to demonstrate that there is only minimal change observed in the localization of the receptor (for details refer to results). Scale bar is 20 μm.

stimulated with 10 μM 2-Me-S-ATP. These results indicate that the analogs are more potent in inducing endocytosis of the $P2Y_1$ receptor in comparison to 2-Me-S-ATP. We observed that the A diastereoisomer in both ATP-α-B compounds was more potent in promoting endocytosis of the $P2Y_1$ receptor than the corresponding B diastereoisomer. This is reflected by the rate of decrease of the fluorescence intensity from the plasma membrane. Similarly, previously we observed that the A form was more potent than the B form to induce Ca^{2+} release (Nahum *et al*., 2002).

The r$P2Y_2$-GFP-expressing cells were also stimulated with 10 μM of ATP and ATP-α-B derivatives. After 30 min with 10 μM ATP, there was almost complete endocytosis of the receptor (Figure 4h). Stimulation of the cells with 10 μM ATP for 30 min resulted in 75% endocytosis of the receptor from the plasma membrane to the cytoplasm, which could be quantified as loss of fluorescence (Figure 5b). We observed a similar degree of endocytosis of the r$P2Y_2$-GFP with 10 μM UTP (data not shown). These results are in accordance with the fact that ATP and UTP are equipotent agonists at the $P2Y_2$ receptor (Sromek & Harden, 1998).

However, when the cells were stimulated with the ATP-α-B derivatives (Figure 4i–l), there was minimal endocytosis of the $P2Y_2$-GFP receptor. Small aggregates of green fluorescence in the cells, such as in Figure 4l, were present already at the beginning of the experiment and were not due to endocytosis of the receptor induced by the addition of agonist. The total amount of receptor that is expressed by the cells is not completely present on the plasma membrane at any given instance. There is always some continuous trafficking of the receptors. This is seen in the case of unstimulated cells too (Figure 4g). The effect of 100 μM of ATP and ATP-α-B derivatives was also compared quantitatively by calculating the degree of endocytosis of the receptor (Figure 5b). There was only 5% loss of fluorescence from the plasma membrane by 100 μM ATP-α-B derivatives, which indicates that there is very weak or negligible endocytosis of the receptor after stimulation. This is in contrast to 80% of endocytosis, which is observed after stimulation of the cells with 100 μM ATP. These data clearly confirm that the ATP-α-B substances preferentially stimulate the $P2Y_1$ receptor.

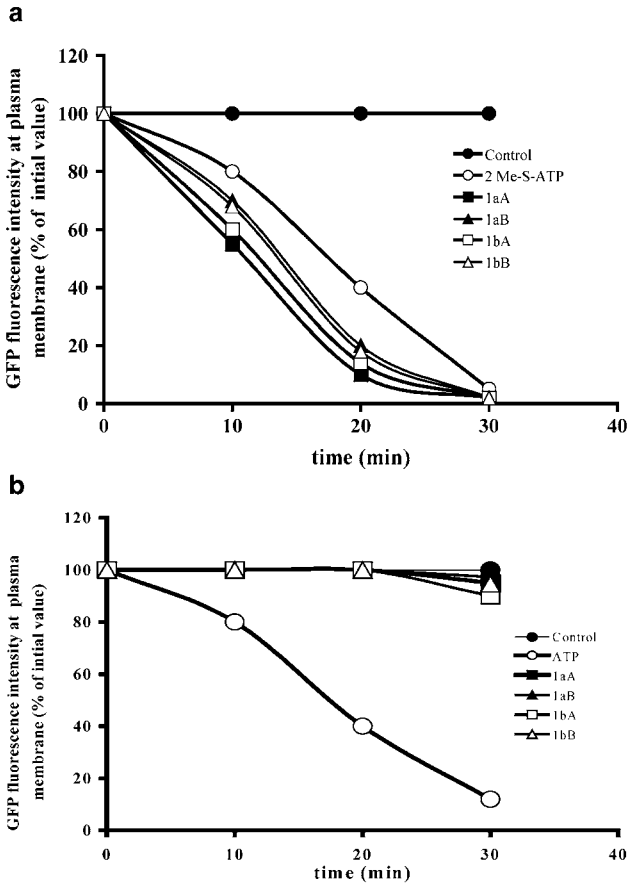

Figure 5 Endocytosis of $P2Y_1$ and $P2Y_2$ receptors induced by ATP-α-B diastereoisomers in transfected cells. HEK 293 cells stably expressing $P2Y_1$-GFP or $P2Y_2$-GFP were stimulated with agonist for 30 min at 37°C. The translocation of the receptor was observed using CLSM, and the fluorescence intensity changes on the plasma membrane and the cytoplasm were calculated using the Zeiss confocal microscope software. The data are from single-cell recordings and represent the mean ± s.d. from at least 30 cells for each value. (a) HEK $P2Y_1$-GFP cells were stimulated with 10 μM of 2-Me-S-ATP (open circles), 1aA (closed squares), 1aB (closed triangles), 1bA (open squares) and 1bB (open triangles). (b) HEK $P2Y_2$-GFP cells were stimulated with 100 μM of ATP (open circles), 1aA (closed squares), 1aB (closed triangles), 1bA (open squares) and 1bB (open triangles).

Discussion

The development of agonists specific for P2Y receptors is of great importance, because ATP functions as an agonist on several subtypes of human P2Y receptors, namely $P2Y_{1,2,11,12}$. These receptors elicit different responses in the cells on stimulation with the same agonist, although they are coupled to the same G proteins (Sak *et al*., 2001). Airway epithelial cells express different P2Y receptor subtypes on the apical and basolateral side, $P2Y_2$ and $P2Y_6$ apically and $P2Y_1$ and $P2Y_2$ basolaterally (Homolya *et al*., 2000). This necessitates the need for specific agonists for a given P2Y receptor subtype. The presence of ecto-nucleotidases adds a further degree of complexity to the possibilities of activating the receptors (Huang *et al*., 2001). Another way to activate specific subtypes is the use of subtype-specific antagonist. The antagonists that are commercially available, like suramin, reactive blue-2 or PPADS, are not specific for a given subtype of P2Y receptor (Von Kügelgen & Wetter, 2000). MRS 2179 is the only commercially available antagonist of the P2Y receptor family that specifically inhibits the $P2Y_1$ receptor. In addition, MRS 2279 is the other $P2Y_1$ receptor specific antagonist (Boyer *et al*., 1998; 2002).

Stimulation of most P2Y receptors leads to an increase in intracellular calcium *via* the inositol trisphosphate (IP_3) pathway, which is the initial step in the transmission of the signal to initiate further downstream cascades. The P2Y receptors are involved in activation of a number of downstream signal transduction pathways, like phospholipases, protein kinase C and MAP kinases (Ralevic & Burnstock, 1998). Stimulation of the $P2Y_2$ receptor in rat mesangial cells results in the production of AA and in the stimulation of the protein kinase B (PKB/Akt) (Huwiler *et al*., 2002). It is also known that ATP stimulates the release of AA from articular chondrocytes (Berenbaum *et al*., 2003) and rat brain astrocytes (Strokin *et al*., 2003). AA is an important mediator in inflammatory responses and is known to play a role in reactive astrogliosis (Brambilla & Abbracchio, 2001). For epithelial cells it has been demonstrated that P2 receptors mediate production of AA-derived products, namely PGE_2 (Insel *et al*., 2001).

In an attempt to generate subtype-specific agonists, borano-substituted ATP derivatives were synthesized. These P_α-boronated ATP derivatives have a chiral center, which is due to the substitution of the nonbridging O by a BH_3 group. In addition to this substitution, there is also a second substitution at the C-2 position of the adenine ring, where H was replaced with Cl or SMe. The two substitutions of the ATP molecule result in the production of four different derivatives of ATP. It has been shown in our previous study (Nahum *et al*., 2002) that the ATP-α-B derivatives were more potent than ATP, the naturally occurring agonist in stimulating Ca^{2+} release in HEK 293 cells transfected with $rP2Y_1$. The previous study also elucidated the fact that the substitution results in the production of diastereoisomers which have different potencies in stimulating the $rP2Y_1$ receptor in terms of the Ca^{2+} response. The study indicated that the A isomer was more potent than the B counterpart in case of both substitutions. 2-MeS-ATP-α-B isomers were more effective than the 2-Cl-ATP-α-B isomers. This fact highlights the presence of a high degree of stereoselectivity of the binding site of the receptor. It was also shown that these agonists are both chemically and enzymatically more stable than ATP (Nahum *et al*., 2002).

In the present work, we further evaluated the borano-substituted ATP derivatives for their receptor subtype specificity and used these derivatives to stimulate the corresponding downstream signaling pathways. We investigated the specificity of these novel ATP derivatives by stimulating Ca^{2+} release from HEK 293 cells expressing $rP2Y_2$-GFP. The $P2Y_2$ receptor is equipotently stimulated by ATP and UTP. The aim of testing these agonists on this cell line was to check the subtype specificity of these substances. HEK 293 cells transfected with $rP2Y_2$-GFP were used to increase the sensitivity of detection of specificity of these substances. The increased sensitivity of the transfected cells compared to the untransfected cells is seen by the decrease in the EC_{50} values for ATP and UTP. We observed that the novel derivatives elicited a very weak response at the $P2Y_2$ receptor, when determining the Ca^{2+} response. After stimulation with 100 μM of ATP-α-B derivatives, the Ca^{2+} response was equal to that elicited by 200 nM ATP. The ATP-α-B derivatives do not induce a Ca^{2+} response in HEK-$P2Y_2$-GFP cells at concentrations of 100 nM. In contrast, these

derivatives elicit a high response in HEK 293 cells stably transfected with $rP2Y_1$ receptor (Nahum *et al*., 2002).

The HEK 293 cells that we used to test the specificity of the borano-substituted ATP derivatives for the receptors, endogeneously express $P2Y_{1,2}$ (Van der Weyden *et al*., 2000; Schäfer *et al*., 2003). This could possibly explain the presence of the small Ca^{2+} response in $rP2Y_2$-GFP cells to the ATP-α-B derivatives. The small Ca^{2+} response that is observed can be attributed to the endogeneously expressed $P2Y_1$ receptor. It has been shown in our previous study (Nahum *et al*., 2002) that these novel ATP-α-B derivatives have a high affinity for the $P2Y_1$ receptor.

To check whether these substances stimulate a Ca^{2+} response *via* the $P2Y_2$ receptor, it is possible to stimulate cells in the presence of antagonists for the $P2Y_1$ receptor or to stimulate cells that endogeneously do not express $P2Y_1$ receptor. We tested the specificity of the borano-nucleotides by both methods. It is seen that after pre-incubating the cells for 30 min with MRS 2179, a specific $P2Y_1$ receptor antagonist (Boyer *et al*., 1998), there is a decrease in the Ca^{2+} response, which is obtained upon stimulating HEK 293 cells stably expressing $P2Y_1$-GFP. The decrease in the calcium response differs for the agonists tested (Table 1). This could be attributed to the difference in the potency of the agonists, which is reflected in the different EC_{50} values. We tested substances 1aB, 1bA and 1bB. It is seen that with substance 1aB, the least potent among the four analogs (EC_{50} value 5.3×10^{-8} M), there is complete inhibition of the calcium response after pre-incubation of the cells with 100 μM MRS 2179 for 30 min. This is similarly observed in the case of substance 1bB, where the inhibition of the Ca^{2+} response is 99%. In case of substance 1bA, the inhibition is 76%. Taking into consideration the results above, we did not test substance 1aA, which has an EC_{50} value of 2.6×10^{-9} M. Thus, the Ca^{2+} responses that are observed in the HEK 293 cells on stimulation with these agonists are specifically due to the stimulation of the $P2Y_1$ receptor. Furthermore, we can exclude that other types of P2Y receptors might be activated. From our previous data (Schäfer *et al*., 2003), it is clear that the level of expression of the $P2Y_4$ receptor, which can respond to ATP, is very weak compared to the expression level of the $P2Y_1$ receptor.

In the latter approach, these substances were tested in A549 cells, human lung epithelial cells, which endogenously do not express the $P2Y_1$ receptor (Schäfer *et al*., 2003). We observed that stimulation of these cells with 10 μM of the ATP-α-B derivatives caused no elevation of intracellular Ca^{2+}, while ATP and UTP at the same concentration in these cells induced a massive response (Figure 2b). Altogether, these facts indicate that the ATP-α-B derivatives specifically stimulate $P2Y_1$ receptor and not the $P2Y_2$ receptor.

Stimulation of A549 epithelial cells with nucleotides results in the release of AA. AA is one of the key players in inflammatory conditions and regulates a number of set points in the downstream signal transduction pathway (Xing *et al*., 1997; Ostrom *et al*., 2000). Thus, we decided to investigate the specificity of these derivatives on the release of AA from HEK 293 cells expressing $rP2Y_1$-GFP or $rP2Y_2$-GFP. It is seen that in case of $rP2Y_1$-GFP cells (Figure 3a), 10 μM of these substances specifically activated the release of AA. This occurred only in $rP2Y_1$-GFP-expressing HEK 293 cells and not in cells stably expressing $rP2Y_2$-GFP. Stimulation of A549 cells with a similar concentration of these substances results in no significant rise in release of AA in comparison to unstimulated cells, as seen in Figure 3b. These results indicate that these substances specifically stimulate the release of AA *via* the $P2Y_1$ receptor. The Ca^{2+} release induced by the stimulation of $P2Y_1$ receptor might result in the stimulation of PLA_2 and subsequent release of AA, as already shown before for other types of receptors (Handlogten *et al*., 2000; Sabourin *et al*., 2002).

Endocytosis of the receptor is one of the methods of termination of signal transduction (Tolbert & Lameh, 1996). Alternatively, there is desensitization of the receptor without endocytosis (Jockers *et al*., 1999). We also investigated the potency of these substances on the capacity to induce endocytosis of the different receptor subtypes. It is known that stimulation of the $P2Y_1$ receptor by ADP induces endocytosis of the receptor, which leads to the termination of the effect of the external stimulus on the cells (Baurand *et al*., 2000). Therefore, HEK 293 cells expressing $rP2Y_1$-GFP and $rP2Y_2$-GFP, respectively, were stimulated with 10 μM of these substances and also with 2-Me-S-ATP and ATP, respectively. These substances induced a complete endocytosis of the $P2Y_1$ receptor, whereas at 10 μM there was no endocytosis of the $rP2Y_2$ receptor. This demonstrates that these substances specifically induce complete endocytosis of the $P2Y_1$ receptor in comparison to the $P2Y_2$ receptor. The rank order of potency of these substances in eliciting an increase in $[Ca^{2+}]_i$ is 1aA > 1bA > 1bB > 1aB (Nahum *et al*., 2002). Here, we find that this rank order is also apparent in the potency of endocytosis induced by these substances. This indicates that endocytosis of the receptor is dependent on the potency of the agonist in stimulating the receptor (Kallal *et al*., 1998).

Thus, in the different signal transduction pathways, like Ca^{2+} release, AA release and endocytosis of the receptors, it is clearly observable that there is a difference in the selectivity and sensitivity of these derivatives for given receptor subtypes. Furthermore, these compounds are very stable under physiological conditions, as they undergo only 5% hydrolysis compared to ATP (Nahum *et al*., 2002). Thus, the results here show that these substances are highly potent and selective agonists of the $P2Y_1$-R, not only in stimulating Ca^{2+} release but also in further downstream signal transduction pathways.

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