



Hetero-oligomerization of the P2Y₁₁ and P2Y₁ receptor controls

receptor internalization and ligand selectivity of the P2Y₁₁ receptor

Denise Ecke, Theodor Hanck, Mohan Tulapurkar, Rainer Schäfer, Matthias Kassack, Rolf Stricker, Georg Reiser

► To cite this version:

Denise Ecke, Theodor Hanck, Mohan Tulapurkar, Rainer Schäfer, Matthias Kassack, et al.. Hetero-oligomerization of the P2Y₁₁ and P2Y₁ receptor controls receptor internalization and ligand selectivity of the P2Y₁₁ receptor. *Biochemical Journal*, 2007, 409 (1), pp.107-116. 10.1042/BJ20070671 . hal-00478810

HAL Id: hal-00478810

<https://hal.science/hal-00478810>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Hetero-oligomerization of the P2Y₁₁ with the P2Y₁ receptor controls receptor internalization and ligand selectivity of the P2Y₁₁ receptor

Denise Ecke, Theodor Hanck, Mohan E. Tulapurkar, Rainer Schäfer, Matthias Kassack[#], Rolf Stricker, and Georg Reiser*

Institut für Neurobiochemie, Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

[#]Institut für Pharmazeutische & Medizinische Chemie, Universität Düsseldorf

Short title: P2Y₁ - P2Y₁₁ receptor hetero-oligomers

* Author for correspondence: Prof. Dr. Georg Reiser, Otto-von-Guericke-Universität Magdeburg; Medizinische Fakultät, Institut für Neurobiochemie, Leipziger Str.44, 39120 Magdeburg; Germany

Tel: 49-391-6713088; Fax: 49-391-6713097; E-mail: georg.reiser@med.ovgu.de

Abstract

Nucleotides signal through purinergic receptors. The nucleotide P2 receptors are subdivided into ionotropic P2X receptors and metabotropic P2Y receptors. The tissue-specificity of nucleotide signaling requires diversity of function within the family of the purinergic receptors. In the present study, hetero-oligomerization between two metabotropic P2Y receptor subtypes is established. The P2Y₁ and P2Y₁₁ receptors were found to associate physically, when co-expressed in HEK293 cells. The association was detected by co-pulldown, immunoprecipitation and Fluorescence Resonance Energy Transfer (FRET) experiments. We found a striking functional consequence of the interaction between the P2Y₁₁ receptor and the P2Y₁ receptor. The interaction promotes agonist-induced internalization of the P2Y₁₁ receptor. This is remarkable because the P2Y₁₁ receptor by itself is completely endocytosis-reluctant. Co-internalization of these receptors was also seen in 1321N1 astrocytoma cells, co-expressing both P2Y₁₁ and P2Y₁ receptors, upon stimulation with ATP or the P2Y₁ receptor specific agonist 2-MeS-ADP. 1321N1 astrocytoma cells do not express endogenous P2Y receptors. Moreover, in HEK293 cells the P2Y₁₁ receptor was found to associate functionally with the endogenous P2Y₁ receptors. Treatment of HEK293 cells with siRNA directed against the P2Y₁ receptor diminished the agonist-induced endocytosis of the heterologously expressed P2Y₁₁GFP receptor. Pharmacological characteristics of the P2Y₁₁ receptor expressed in HEK293 cells were determined by recording Ca²⁺ responses after nucleotide stimulation. This analysis revealed a ligand-specificity, which was different from the agonist profile established in cells expressing the P2Y₁₁ receptor as the only metabotropic nucleotide receptor. Thus, the hetero-oligomerization of the P2Y₁ and P2Y₁₁ receptors allows novel receptor functions of the P2Y₁₁ receptor in response to extracellular nucleotides.

Key words: Purinergic receptor, hetero-oligomerization, pulldown experiment, Fluorescence Resonance Energy Transfer (FRET), endocytosis, calcium rise

Abbreviations

1321N1, human astrocytoma cell line; 2-MeS-ADP, 2-methylthio adenosine 5'-diphosphate; ATP, adenosine-5'-triphosphate; BzATP, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; DMEM, Dulbecco's modified Eagles' medium; FCS, fetal calf serum; G418, Geneticine; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; G protein, guaninenucleotide-binding protein; GSH, glutathion sepharose; IP, immunoprecipitation; MATra, Magnet Assisted Transfection; MRS2179, N⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate; NaHBS, HEPES buffered saline solution; NF157, 8,8'-[Carbonylbis[imino-3,1-phenylenecarbonylimino (4-fluoro-3,1-phenylene) carbonylimino]] bis-1,3,5-naphthalenetrisulfonic acid; PAR-2-AP, PAR-2-activating peptide; ROI, region of interest; siRNA, small interfering RNA; UDP, uridine-5'-diphosphate;

Introduction

The currently accepted notions of G protein-coupled receptor (GPCR) organization in the plasma membrane include the property that these proteins can oligomerize among each other. These oligomers link distinct signaling pathways and integrate receptor functions [1]. Visualization of rhodopsin dimers and higher order oligomers in native murine disc membranes by transmission electron and atomic force microscopy is an outstanding example of receptor oligomerization [2]. The possible functional consequences of hetero-oligomerization among GPCRs are manifold, including enhanced surface expression [3, 4], change in selectivity towards different G proteins [5] and alteration in the potency of receptor agonists [6]. Cross-internalization as a consequence of GPCR hetero-oligomerization may play an important role in regulating the desensitization of inter-connected signalling pathways. An influence on receptor endocytosis has been reported for several known GPCR hetero-oligomers [7-9]. In Parkinson's disease the hetero-dimerization of dopamine D₂ and adenosine A_{2A} receptors seems to accelerate the development of tolerance against L-DOPA, as increased adenosine levels in patients treated with L-DOPA lead to chronic activation of both receptors and thus to a greater extent of desensitization [1].

Hetero-oligomerization among metabotropic purinergic receptors originating from different subgroups has already been described. The purinergic receptor family consists of two different subgroups, the P1 and P2 receptors. P1 receptors (A₁, A_{2A}, A_{2B}, A₃) are responsive to adenosine, whereas the metabotropic P2 receptors (P2Y_{1,2,4,6,11-14}) are activated by nucleotides as natural agonists [10]. An interaction between members of both subgroups was found for the A₁ receptor in a heteromeric association with the P2Y₁ or P2Y₂ receptor, respectively [11, 12]. The A₁-P2Y₁ receptor hetero-oligomer was shown to create a P2Y-like adenosine receptor by creating ligand-binding sites with a A₁-P2Y₁ receptor hybrid selectivity. In contrast, the association of the A₁ receptor with the P2Y₂ receptor did not seem to affect the ligand selectivity at the receptors, but stimulation of the P2Y₂ receptor with UTP in the A₁-P2Y₂ receptor complex caused uncoupling of the A₁ receptor from G_i proteins [11].

Oligomerization between different P2Y nucleotide receptor subtypes has not yet been reported. However, this is an important issue, since nearly all tissues express more than one subtype of purinergic receptors which enables a functional crosstalk between them in response to nucleotide release and subsequent degradation. The P2Y₁ and P2Y₁₁ receptors are found to be co-expressed by several tissues, including specific parts of the brain [13], smooth muscle cells and cells of the immune system [14]. Both receptors respond to adenine nucleotides but prefer different derivatives [15]. Slight variations in the nucleotide binding

pocket of the P2Y₁₁ receptor as compared to that of the P2Y₁ receptor, might account for the difference in agonist selectivities [16].

We suspected that an interaction between the P2Y₁₁ receptor and other P2Y receptors might exist, as the ligand specificity of the endogenously expressed P2Y₁₁ receptors does not always agree with the profile found at the cloned and heterologously expressed P2Y₁₁ receptor. In the present study we provide evidence showing that the observed discrepancies might be due to the formation of hetero-oligomers between the P2Y₁ and P2Y₁₁ receptor. In addition, we found that the hetero-oligomer formation induces internalization of P2Y₁₁ receptor, which by itself is endocytosis-reluctant. This has an important impact on P2Y₁₁ receptor desensitization.

Material and Methods

Materials

Geneticine (G418 sulphate; Calbiochem, Darmstadt); 2-MeS-ADP, ATP, BzATP, MRS2179, UDP, UTP (Sigma, Deisenhofen); Rp/Sp-ATP- α -S (Biolog, Bremen); Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham's medium, Penicillin/Streptomycin (10 000/10 000 U/ml), Trypsin/EDTA (0.05%/0.02%), fetal calf serum (FCS), (Seromed, Biochrom, Berlin); cell culture dishes (Nunc, Wiesbaden); cover slips (22 mm) (OmniLab); fura 2-AM (Biomol, Hamburg/Molecular Probes); Complete protease inhibitor cocktail, DOTAP and Fugene 6 transfection reagents (Roche, Mannheim, Germany); Glutathione sepharose (GE Healthcare, München, Germany); cDNA plasmid for Cerulean Fluorescent Protein was a kind gift of Dr. David W. Piston (Vanderbilt University Medical Center, Nashville, TN, USA). NF157, a P2Y₁₁ receptor antagonists with nanomolar potency, was synthesized by M. U. K. by methods published previously [17]. The DNA sequence of the human P2Y₁₁ receptor (GenBank™/EBI accession number AF030335) was kindly provided by Dr. D. Communi (Brussels, Belgium).

Receptor constructs

The P2Y₁ receptor was constructed bearing a myc-epitope or a Cerulean fluorescent protein tag at its C-terminus. The P2Y₁ receptor DNA sequence was placed between the EcoRI/SmaI restriction sites of the pcDNA3.1/mycHis (A) vector (Invitrogen, Karlsruhe, Germany) or peGFPN1 vector (Clontech, Heidelberg, Germany). For generating the P2Y₁ receptor tagged with the Cerulean fluorescent protein, the GFP tag was replaced by the cDNA of Cerulean using PCR cloning. The P2Y₁₁ receptor tags (GST, GFP, Citrine fluorescent protein and the Cerulean-Citrine double tag) were all present at the C-terminus of the receptor. The GST tag was amplified from pGEX vector (GE Healthcare, München, Germany) using PCR cloning and placed downstream of the P2Y₁₁ receptor DNA sequence following a FXa cleavage site insert in a pcDNA3.1(+) vector. The fluorescent tagged receptor was generated by placing the P2Y₁₁ receptor sequence between the EcoRI/BamHI restriction sites of the peGFPN1 or peYFPN1 vector. The Q69M mutation in the YFP-tag to produce the Citrine-tag and the R268A mutation in the P2Y₁₁ receptor sequence were introduced using the site-directed mutagenesis QuikChange kit (Stratagene, La Jolla, CA, USA). For construction of a FRET positive control, DNA encoding for the Cerulean protein was ligated into the BamHI-digested P2Y₁₁Citrine vector by PCR cloning. The P2Y₄ and protease-activated receptor (PAR)-2 were present as HA fusion proteins. The N-terminal HA-tagged P2Y₄ receptor was cloned into the

pcDNA3.1(+) vector. The PAR-2 receptor was present in the pCMV-HA vector (Clontech) bearing the HA-epitope at the C-terminus of the receptor. All constructs were checked by sequencing analysis.

Cell culture and transfection

HEK293 cells were grown in DMEM/HAM's F12 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 IU/ml streptomycin in a 5% CO₂/ 95% air, humidified atmosphere at 37°C. Transient transfection of receptor constructs was performed using MATra-A (magnet-assisted transfection for adherent cells) reagent (IBA, Göttingen, Germany) as per manufacturer's protocol. Stable transfection of the cells was done using DOTAP transfection reagent as described previously [18]. 1321N1 human astrocytoma cells were cultured in high-glucose DMEM supplemented with 5% FCS, 100 U/ml penicillin, and 100 IU/ml streptomycin at 37°C in 10% CO₂. Transfection of the cells was done using Fugene 6 as described before [15]. A stable expression of the receptor constructs was achieved by keeping G418 throughout the culture period in the medium of both cell lines.

Co-Pulldown and Co-Immunoprecipitation

Transiently transfected (48 h) HEK293 cells were lysed in RIPA buffer (50 mM Tris, pH 8.1; 1% Igepal CA630; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM NaF; protease inhibitor cocktail), harvested by scraping, briefly sonicated and centrifuged at 14.000 x g for 15 min at 4°C. The whole cell lysate was collected from the resulting supernatant. The protein content was determined by the Bradford method [19]. For Co-Pulldown experiments whole cell lysates were diluted with RIPA-buffer to a final protein concentration of 500 µg/ml and incubated overnight on a rocker at 4°C with Glutathione Sepharose (GSH-beads). For Co-IP whole cell lysates were diluted with RIPA-buffer to a final protein concentration of 250 µg/ml and incubated with 1.5 µg anti-myc antibody (mouse monoclonal, Invitrogen) overnight as described above. Protein A/G Plus Agarose (Santa Cruz, Heidelberg, Germany) was added and mixed with the lysates for at least 2 h at 4°C. Pulldown fractions or immunoadsorbents were recovered by centrifugation and washed three times in lysis buffer. Samples were eluted in 10 µl 2X Laemmli buffer to prepare for loading on a SDS-PAGE.

Western Blot analysis

Samples were resolved by SDS-PAGE, followed by electro-transfer to nitrocellulose membrane. The membrane was blocked and probed with antibodies against the myc-epitope (Invitrogen, 1:5000), GST-tag (Santa Cruz, 1:20.000) and HA-epitope (Cell Signalling, 1:1000). After washing, the membrane was further incubated with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:20.000 or 1:10.000, resp.) and visualized using the

SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Membranes were stripped before reprobing.

Immunocytochemistry

Transiently transfected 1321N1 cells cultured on 22 mm coverglasses were fixed in 4% paraformaldehyde, blocked and permeabilised in FSBB buffer and incubated in mouse monoclonal anti-myc antibody (Invitrogen; 0.8 $\mu\text{g}/\mu\text{l}$) overnight at 4°C. After washing, the secondary antibody was applied (Alexa IgG Goat-anti-mouse A₅₅₅-1:200; Molecular Probes) for 2 h at room temperature. Cells were washed again and mounted in Vectashield mounting medium (Axxora, Grünberg, Germany). Visualization of the GFP fluorescence and immunostaining was done with a Zeiss LSM510 Confocal laser scanning microscope equipped with a Plan-Apochromat x63 objective. GFP was excited using a 488-nm argon/krypton laser, and the emitted fluorescence was detected with a 505-530-nm band pass filter. Alexa Fluor 555 was detected using a 543-nm helium/neon laser and a 560-nm long pass filter.

FRET assay

HEK293 cells transiently transfected (48 h) with the fluorescent tag receptors (P2Y₁Cerulean, P2Y₁₁Citrine or P2Y₁₁Cerulean-Citrine) or the fluorescent tags only were imaged on a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Apochromat x63 objective. For FRET analysis, emission spectra were measured at 458 nm excitation employing an argon/krypton laser and a beamsplitter NT80/20. The emitted fluorescence was detected from 473 nm to 580 nm in 10.6 nm steps using the lambda modus. The Cerulean protein showed an emission maximum at 483 nm and the emission maximum of the Citrine protein was at 526 nm. HEK293 cells co-transfected with Cerulean and Citrine fluorescent proteins served as negative control. The double tagged P2Y₁₁ receptor bearing a Cerulean tag that was connected to a Citrine tag by a 16 amino acid long linker was used as a FRET positive control. Fluorescence intensities (I) were normalized to the emission maximum of the Cerulean tag (donor) at 483 nm to display sample spectra in the graph. FRET ratios were

calculated using the following equation:
$$FRET_{ratio}(R) = \frac{I_{526nm}}{I_{483nm}}$$
.

Agonist-induced internalization

Agonist-induced internalization was measured as described previously [20]. Briefly, HEK293 cells stably expressing the P2Y₁₁GFP receptor were seeded on coverslips (30 mm diameter). Coverslips were placed in a Perfusion, Open and Closed (POC)-chamber (PeCon GmbH, Erbach, Germany) and incubated on stage in 5% CO₂ at 37°C in complete culture medium.

Images were taken on a Zeiss inverted LSM 510 META laser scanning confocal microscope. Cells were stimulated with various agonists and live-imaging was done for 60 min. Fluorescence intensities of confocal images were analyzed using the Zeiss LSM 510meta software histo macro. Regions of interest (ROI = ellipse 30-39 μm^2) were set in the cytosol of single cells and the average fluorescence intensity in the ROI was determined over time. The intensity values for 10-60 min were normalized to the starting value at 0 min.

siRNA

P2Y₁ receptor specific siRNA [sense r(CUC UCC UCU GAG GAG AAA A)dTdT] and non-silencing control siRNA were from Qiagen (Hilden, Germany). HEK-P2Y₁GFP cells were seeded on coverslips (30 mm diameter) pre-coated with 0.01% poly-L-lysine and transfected at 30-40% density with siRNA using MATra-A reagent as described above. Experiments and analysis of the P2Y₁ receptor knockdown were done 48 h post-transfection. For western blot analysis of the P2Y₁ receptor knock-down the nitrocellulose membrane was incubated with an anti-P2Y₁ antibody (Alomone, München, Germany) at a 1:500 dilution.

Calcium measurements

The cells were plated on coverslips (diameter = 22 mm), and single cell measurement was done after 3 days, when the cells were 30-50% confluent. The changes of free intracellular Ca²⁺ concentration ($[\text{Ca}^{2+}]_i$) were measured, as described before [21]. Briefly, cells were preincubated with 2 μM fura-2AM (for 1321N1 cells : plus 0.02% pluronic acid) at 37°C for 30 min in NaHBS (HEPES buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 25 mM glucose and 20 mM HEPES/ Tris pH 7.4). Stimulation of the cells with different concentrations of various agonists was done under continuous superfusion with a flow rate of 1 ml/min at 37°C [18]. Fluorescence intensity was recorded alternately at 340 and 380 nm excitation and 520 nm emission.

Data analysis

Statistical analysis of the data was done using Graph pad prism. Data were checked for gaussian distribution. In the case of gaussian distribution of data, further analysis was done using the one-way ANOVA test and the Tukey or Dunnett test as a post test. Significant difference was taken at a P value < 0.05.

Results and Discussion

Physical association of the P2Y₁ and P2Y₁₁ receptors in HEK293 cells

In the current study we investigated the possible interaction of two members of the P2Y receptor family. The P2Y₁ receptor and P2Y₁₁ receptor are close homologues in their receptor family. So far a crosstalk between both receptors has not been hypothesized. For the P2Y₁ receptor the ability to interact with other GPCRs became obvious in a study exploring the hetero-dimerization with the A₁ adenosine receptor [12]. In the present study, we analysed whether there is a physical interaction of the P2Y₁₁ receptor with the P2Y₁ receptor. For that purpose we carried out co-pulldown, co-immunoprecipitation, and FRET experiments.

The P2Y₁₁ receptor expressed as a GST fusion protein and the P2Y₁ receptor bearing a mycHis tag were co-expressed in HEK293 cells. This allowed us to check for their physical interaction by pulldown and immunoprecipitation experiments using whole cell lysates (Figs. 1 and 2). Western blot analysis of lysates from co-transfected cells revealed smearlike anti-myc reactive bands between 35 kDa and 75 to 100 kDa (Fig. 1A, lane 1). The smearlike staining probably represents several glycosylated forms of the receptor, as reported previously by Nakata and co-workers [12]. In addition, the homogenate may also contain receptor proteins, which are not fully processed, and higher molecular mass bands may correspond to oligomerized receptor. The main band of the P2Y₁ receptor protein is indicated by arrowhead in Fig. 1A. Western blot analysis applying a GST-antibody resulted in anti-GST reactive bands of the predicted molecular mass (~65 kDa) for the P2Y₁₁GST receptor (Fig. 1B, lane 1).

Pulldown experiments with lysates of cells co-expressing both receptors (Fig. 1A,B, lane 5) showed that the P2Y₁mycHis receptor (Fig. 1A, lane 5) was caught together with the P2Y₁₁GST receptor (Fig. 1B, lane 5) at the GSH beads. The prominent band marked by arrowheads, as well as a band below that, was greatly enriched at the GSH beads. The interaction of the P2Y₁ and P2Y₁₁ receptor was strong, as seen from the myc-staining in the pulldown sample. There the staining was much more intense than in the 10% input (Fig. 1A, compare lanes 5 and 1, respectively). The association seems to be constitutive because agonist addition did not alter the interaction of both receptors (data not shown).

To test for the specificity of the pulldown experiment, several control conditions were applied. The GST pulldown experiment with single transfected HEK293 cells, expressing either the P2Y₁mycHis or the P2Y₁₁GST receptor showed no myc-immunoreaction in the Western blot analysis (data not shown). Also, the P2Y₁mycHis receptor did not interact with the GST tag, which was examined using GST-preloaded GSH beads in a pulldown test (Fig. 1A, lane 6). This was further confirmed by cleaving the P2Y₁₁ receptor from the GST fusion

protein at the Factor Xa (FXa) cleavage site using this protease. The FXa treatment led to the disappearance of the myc-immunoreaction in the pulldown sample (Fig. 1A, lane 7). When we used a mixture of lysates from HEK293 cells transfected with a single receptor, HEK-P2Y₁mycHis or HEK-P2Y₁₁GST, the GST-pulldown fraction did not show any myc-immunoreaction (Fig. 1A, lane 4). This excludes that the formation of aggregates during the pulldown procedure might be a possible cause of the positive co-pulldown result [22].

We further investigated the specificity of the interaction between both receptors. The P2Y₁₁GST receptor was co-transfected with a non-related GPCR. Here we used the protease-activated receptor-2 (PAR-2) (bearing a HA tag). The PAR-2-HA protein showed a smear band (~37-70 kDa, Fig. 1C, lane 2) which is consistent with previous results [23]. The pulldown experiment in Fig. 1C, lane 8, revealed no HA-immunoreactivity, thereby excluding an interaction of the P2Y₁₁ receptor with the PAR-2 receptor. However, co-transfection of the P2Y₁₁GST receptor with the P2Y₄-HA nucleotide receptor (~37 kDa) resulted in a physical interaction of both receptors, as demonstrated by GST pulldown experiment and subsequent Western blot with an anti-HA antibody (Fig. 1C, lane 9). This implies the interesting possibility of P2Y receptor pooling in HEK293 cells.

The stability of the P2Y₁ and P2Y₁₁ receptor interaction was further examined by co-immunoprecipitation of the P2Y₁₁GST receptor by an anti-myc antibody from P2Y₁ myc / P2Y₁₁ GFP co-transfected cells. The P2Y₁₁GST receptor clearly co-immunoprecipitated together with the myc-tagged P2Y₁ receptor, as detected by Western blot analysis using a GST-antibody (Fig. 2A, lane 3). The signal was specific, as lysates precipitated with an unspecific IgG showed no anti-GST-reactive bands (Fig. 2A, lane 4). Moreover, like in the pulldown test, a mixture of lysates from single receptor-transfected HEK293 cells did not result in co-precipitation of the P2Y₁₁GST receptor (Fig. 2A, lane 2).

Furthermore, we analyzed the probability that the transiently overexpressed receptors were present in small membrane patches of HEK293 cells without direct physical interaction [22]. Therefore, the total lysates of double-transfected cells were extracted for 2 h at 4°C in lysis buffer and then centrifuged at 50.000 x g for 30 min to remove undissolved membrane patches [24]. After that procedure, the P2Y₁mycHis receptor (Fig. 3A, lane 1 and 2) and the P2Y₁₁GST receptor (Fig. 3B, lane 2) were present in the extract. When we performed a pulldown experiment with these extracts the extracted P2Y₁mycHis receptor (Fig. 3A, lane 4) could be found together with the P2Y₁₁GST receptor (Fig. 3B, lane 4) at the GSH beads. This confirmed the direct physical interaction of the two receptors.

Moreover, in a FRET assay we investigated the physical association of the P2Y₁ and P2Y₁₁ receptor in living HEK293 cells. As donor molecule we employed an improved variant of the cyan fluorescent protein named Cerulean which is especially useful for FRET [25]. Citrine fluorescent protein was used as acceptor molecule. This version of the yellow fluorescent protein shows enhanced pH and chloride resistance, thereby improving its usefulness in a FRET assay [26]. The P2Y₁ receptor bearing the Cerulean tag and the P2Y₁₁ receptor bearing the Citrine tag were transiently co-expressed in HEK293 cells and the fluorescence spectra were determined, as described in methods. Negative control conditions were mimicked by co-expressing the fluorescent tags only. A representative spectrum of the negative control is shown in Fig. 4A (dotted line) and the FRET ratio (n=6) was calculated as described in methods. Maximum FRET ratio (n=6) in our experiments was obtained by expressing the P2Y₁₁ receptor double-tagged with a Cerulean tag linked to a Citrine tag by a 16 amino acid linker (positive control) (Fig. 4B). Excitation of Cerulean at 458 nm resulted in an energy transfer to the linked Citrine protein leading to an emission maximum at the appropriate wavelength of 526 nm (Fig. 4A, line with dash-dots). The emission maximum of the positive control correlated with the fluorescence spectrum of Citrine alone (data not shown). Confocal imaging of HEK293 cells co-expressing the P2Y₁Cerulean and P2Y₁₁Citrine receptor also showed an emission maximum at 526 nm (Fig. 4A, continuous line). The FRET ratio (n=9) was smaller than in the positive control but clearly increased as compared to the negative control (Fig. 4B). This suggests a greater distance of the fluorophores in case of the hetero-oligomerized receptors than in case of the positive control, where both fluorescent proteins were closely connected by a linker of 16 amino acids.

Interaction of P2Y₁₁ and P2Y₁ receptors promotes agonist-induced internalization of the endocytosis-resistant P2Y₁₁ receptor

The functional consequence of the formation of P2Y₁-P2Y₁₁ receptor hetero-oligomers was studied by checking the impact on receptor endocytosis of the heterologously expressed receptors. For these studies we used 1321N1 astrocytoma cells, because this cell line lacks endogenous P2Y receptors [15, 27]. 1321N1 cells were firstly single transfected with the GFP-tagged P2Y₁ or P2Y₁₁ receptor to check for endocytosis properties of the respective individual receptor. Cells were imaged on a Zeiss inverted LSM 510 META laser scanning confocal microscope before and during agonist stimulation, as described in the methods section.

1321N1-P2Y₁GFP cells were stimulated with 10 μ M 2-MeS-ADP and the receptor trafficking was observed for 60 min. After 30 min of stimulation, receptor endocytosis could be clearly detected (data not shown) and was complete at the end of 60 min (Fig. 5, A2). This resembles the findings of a recent study [28], where the P2Y₁ receptor was found to be internalized with a similar kinetics. In contrast, 1321N1-P2Y₁₁GFP expressing cells did not show any sign of receptor endocytosis even after 60 min of stimulation with 100 μ M ATP (Fig. 5, B2). This substantiates the lack of agonist-induced receptor internalization of the P2Y₁₁ receptor. The P2Y₆ receptor behaves similarly and shows only slight reduction in cell surface expression after long-time exposure to agonists [29]. In contrast, the other members of the G_q-coupled P2Y receptor subgroup are able to undergo agonist-induced internalization, as shown in studies investigating the P2Y₁ receptor [30], the P2Y₂ receptor [20] or the P2Y₄ receptor [29].

However, co-transfected 1321N1 cells, expressing the P2Y₁mycHis and P2Y₁₁GFP receptor, yielded different results. Cells were stimulated with either 100 μ M ATP or 10 μ M 2-MeS-ADP and fixed after 60 min of stimulation. Fixed cells were stained with anti-myc antibody and visualized on a Zeiss inverted LSM 510 META laser scanning confocal microscope. The green fluorescence of the P2Y₁₁GFP receptor (Fig. 5, C1) and the red fluorescence of the Alexa₅₅₅ dye visualized myc-staining (Fig. 5, C2) co-localized nicely at the plasma membrane as shown in the merged image (Fig. 5, C3). Upon addition of 100 μ M ATP for 60 min, endocytosis of the P2Y₁mycHis receptor (Fig. 5, D2), and now also of the P2Y₁₁GFP receptor (Fig. 5, D1), was observed. Fig. 5, D3 shows that the receptors co-internalized to the same compartment (yellow colour) as well as to different endocytotic compartments. The zoomed pictures in Fig. 5, D3 emphasize the co-localization of the P2Y₁mycHis and P2Y₁₁GFP receptor in intracellular compartments. Similar observations were made after stimulating the co-transfected cells with the P2Y₁ receptor agonist 2-MeS-ADP at 10 μ M (Fig. 5, E1-E3). Again the myc-staining of the P2Y₁mycHis receptor and the green fluorescence of the P2Y₁₁GFP receptor co-localized in the same intracellular compartments (Fig. 5, E3). This was an important finding, as Communi et al. reported that 2-MeS-ADP has no activity at the P2Y₁₁ receptor [31]. Therefore, we can conclude that stimulation of P2Y₁ receptors by the selective agonist 2-MeS-ADP caused co-internalization of the P2Y₁-P2Y₁₁ receptor hetero-oligomers.

To further validate this conclusion, the interaction of the P2Y₁₁ receptor with an endogenously expressed P2Y₁ receptor was investigated. HEK293 cells endogenously express the P2Y₁ receptor [32]. Therefore, we stably transfected these cells with the P2Y₁₁ receptor

GFP fusion protein and observed receptor endocytosis by live imaging. Cells were stimulated on stage and imaged on a Zeiss inverted LSM 510 META confocal microscope. Stimulation of the HEK-P2Y₁₁GFP cells with ATP clearly induced receptor internalization (Fig. 6, A2-3). This endocytosis was blocked by the specific P2Y₁ receptor antagonist MRS2179 (Fig. 6, B2-3). Endocytosis of the P2Y₁₁GFP receptor could also be induced by the P2Y₁ receptor-specific agonist 2-MeS-ADP (Fig. 6, C2-3), whereas the potent P2Y₁₁ receptor agonist BzATP turned out to be completely ineffective (Fig. 6, D2-3). In addition, preincubation with a high concentration of NF157, a specific and highly potent P2Y₁₁ receptor antagonist [17], did not inhibit ATP-induced receptor internalization (Fig. 6, E2-3).

As 2-MeS-ADP was able to induce the endocytosis of the P2Y₁₁GFP receptor in HEK293 cells, whereas specific ligands of the P2Y₁₁ receptor were ineffective, we investigated whether an unresponsive P2Y₁₁ receptor was able to be co-internalized by P2Y₁ receptor activation. We have previously shown that the R268A-P2Y₁₁ receptor mutant (A₂₆₈P2Y₁₁-R) shows a 1000-fold reduced potency for ATP [16]. Therefore, we stably expressed the A₂₆₈P2Y₁₁ receptor in HEK293 cells. For detection of receptor endocytosis, the HEK_{A268}P2Y₁₁GFP cells were stimulated on stage with an ATP concentration (100 μM) that was not able to activate the mutant receptor, and cells were imaged by confocal microscopy. The results depicted in Fig. 6, F2-3 demonstrate that during the total time period of imaging (60 min) the GFP tagged A₂₆₈P2Y₁₁ receptor did not show any sign of internalization. The receptor remained at the plasma membrane. The same was true when we stimulated the cells with 2-MeS-ADP (data not shown). Real time PCR showed that HEK_{A268}P2Y₁₁GFP, mock transfected as well as HEK-P2Y₁₁GFP transfected cells all had comparable levels of P2Y₁ receptor mRNA (supplementary material). Therefore, the lack of internalization was not due to a lower P2Y₁ receptor level.

However, the A₂₆₈P2Y₁₁GST receptor expressed in HEK293 cells still displayed physical association with the co-transfected P2Y₁mycHis receptor, as analysed by pulldown experiments from cellular extract. The data from the co-pulldown experiment are shown in Fig. 3A, lane 3. Therefore, we conclude that a mutant P2Y₁₁ receptor with greatly reduced agonist affinity was not able to allow the functional interaction with the P2Y₁ receptor.

Interestingly, the induction of the endocytosis of the wild type P2Y₁₁GFP receptor in HEK293 cells was specific for P2Y₁ receptor agonists, because stimulation of the cells with UTP acting on the endogenous P2Y₂ receptor (Fig. 6, G2-3) was not able to induce endocytosis of the P2Y₁₁GFP receptor. Also, stimulation with PAR-2-activating peptide, which can specifically activate the PAR-2 receptor without proteolysis [33, 34], did not result

in endocytosis of the P2Y₁₁GFP receptor (Fig. 6, H2-3). Both stimuli elicited a clear calcium response in the cells (data not shown) and real time PCR analysis confirmed the expression of the P2Y₂ receptor and PAR-2 receptor in HEK-P2Y₁₁GFP cells (supplementary material).

To further validate the influence of the P2Y₁ receptor in the process of P2Y₁₁ receptor internalization, we attempted to knock-down P2Y₁ receptor expression in HEK293 cells. Oligonucleotides selected against a specific region in the coding sequence of the receptor were applied to HEK-P2Y₁₁GFP cells using magnet-assisted transfection (MATra) reagent. Cells treated with the P2Y₁ receptor siRNA showed approximately 50% knock-down in the mRNA level of the P2Y₁ receptor (data not shown). The decrease in P2Y₁ receptor expression, as confirmed on the protein level, was about 50% (Fig. 7, inset). Receptor expression remained unaffected when cells were treated with MATra reagent only or with control siRNA (data not shown). Treatment of the HEK-P2Y₁₁GFP cells with P2Y₁ receptor siRNA induced a significantly diminished P2Y₁₁GFP receptor endocytosis. The endocytosis was quantified by measuring the time course of the increase in GFP fluorescence in the cytosol of transfected cells upon agonist stimulation (Fig. 7). The time-dependent increase in GFP fluorescence intensity in the cytosol of ATP-stimulated cells was significantly smaller in P2Y₁-siRNA treated cells than in cells, which were not treated with P2Y₁-siRNA. The extent of reduction in P2Y₁ receptor mRNA transcripts correlated with the reduction in endocytosis of the P2Y₁₁ receptor. Cells treated with control siRNA or only transfection reagent behaved like control cells (Fig. 7). This verifies the specificity of the interaction of P2Y₁ and P2Y₁₁ receptors in the receptor endocytosis of the P2Y₁₁GFP receptor in HEK293 cells.

In conclusion, the phenomenon of co-internalization may be a very important functional consequence of GPCR hetero-oligomerization. This controls desensitization and resensitization of GPCRs [35]. Hetero-oligomerization among GPCRs can induce internalization of receptors which were previously considered reluctant to endocytosis. The somatostatin receptor (SSTR) SSTR1 failed to internalize upon agonist stimulation in several cell lines. However, the SSTR1 present as a heterodimer with the SSTR5 was found to display sufficient agonist-induced internalization [36].

Similar findings are presented here for the P2Y₁ and P2Y₁₁ receptor. The P2Y₁₁ receptor, when expressed alone, lacks any desensitization, as shown here and found previously by others [37]. This property of the the P2Y₁₁ receptor seems to be important for normal receptor function in the maturation process of dendritic cells (DC) which express P2Y₁₁ receptors. These antigen-presenting cells are trapped in the epicenter of inflammation due to activation of the P2Y₁₁ receptor by high extracellular ATP concentrations. This

prolongs exposure to maturation-inducing factors [38]. Thereby, the inability of the P2Y₁₁ receptor to desensitize seems to be important in overriding the chemotactic effects of nucleotides acting on other P2Y receptors. However, distinct DC subsets respond differently to ATP in terms of migration. DCs directly isolated from human blood show no arrest in migration in response to ATP treatment. Interestingly, these DC subpopulations display significant mRNA expression levels for the P2Y₁ receptor, opposed to a negligible PCR signal usually found in DCs (Langerhans cells, monocyte-derived DCs) which do not migrate in the presence of high ATP concentrations [38]. We hypothesize that the presence of the P2Y₁ receptor in DCs isolated from human blood enables the desensitization of the P2Y₁₁ receptor in response to ATP, and this controls the chemotactic behaviour and maturation of the cells.

Influence of the hetero-oligomerization on the ligand selectivity of the P2Y₁₁ receptor

The hetero-oligomerization of the P2Y₁₁GFP receptor with the endogenous P2Y₁ receptor seems to modify the activity of known agonists and antagonists at the receptor, as observed in the endocytosis experiments described above. BzATP was ineffective in inducing receptor-endocytosis and NF157 could not inhibit the ATP-induced internalization of the P2Y₁₁GFP receptor. So far there is already evidence that formation of hetero-oligomers can result in a change in pharmacology of GPCRs [39]. The A₁-P2Y₁ receptor hetero-dimer was found to have no significant affinity for MRS2179, whereas A₁ receptor antagonists did still bind. In contrast, the modified ligand binding pocket in the A₁-P2Y₁ heterodimer appears to bind the P2Y₁ receptor agonist ADPβS, but slightly less the A₁ receptor ligands [12]. Therefore, we also investigated receptor ligand selectivities. This was done by measuring the intracellular calcium rise in HEK293 cells expressing the P2Y₁₁GFP receptor. As described in methods, cells were subjected to single-cell calcium measurements using the calcium indicator fura-2.

P2Y₁₁GFP receptor-expressing HEK293 cells responded to the potent P2Y₁₁ receptor agonist BzATP, but the recently developed, highly potent P2Y₁₁ receptor antagonist NF157 [17] was not able to inhibit this response (Fig. 8A). However, when HEK-P2Y₁₁GFP cells were preincubated with the specific P2Y₁ receptor antagonist MRS2179, surprisingly the response to BzATP was inhibited (Fig. 8A). This was different when the P2Y₁₁GFP receptor was expressed singly in 1321N1 cells. There MRS2179 showed no effect on the activity of BzATP, but preincubation with NF157 totally abolished the activity of BzATP at the P2Y₁₁ receptor (Fig. 8C). MRS2179 was able to interfere with the BzATP-stimulated [Ca²⁺]_i rise and the ATP-induced internalization of the P2Y₁₁GFP receptor, whereas NF157 was not able to

inhibit any of these effects. Thus, the interaction of the P2Y₁₁GFP receptor with the endogenous P2Y₁ receptor in HEK293 cells apparently influences the ligand selectivity of the receptor, as measured by the induction of a [Ca²⁺]_i rise.

BzATP normally shows no activity at the P2Y₁ receptor [40]. Therefore, it can be concluded that BzATP and MRS2179 both bind to the hetero-dimer and that the ligand selectivity of both receptors is changed due to dimerization. However, there are some surprises regarding the activity of BzATP. This ligand could induce a [Ca²⁺]_i increase in HEK-P2Y₁₁GFP cells but was not able to stimulate receptor endocytosis. Such inconsistencies in the action of BzATP have also been found by other groups [41, 42]. Taken together, this indicates that BzATP can only induce specific receptor conformations in accordance with the probabilistic model of GPCR function [43].

The A₂₆₈P2Y₁₁GFP receptor did not functionally interact with the endogenous P2Y₁ receptor in HEK293 cells with regard to internalization. However, there was a physical interaction (Fig. 3). Therefore, we examined whether the ligand selectivity of this P2Y₁₁ receptor mutant was influenced by the interaction with the P2Y₁ receptor. We measured the intracellular calcium rise in HEK_{A268}P2Y₁₁GFP cells. The cells responded to 100 μM ATP in the same way as mock-transfected cells, confirming the decreased potency of ATP at the P2Y₁₁ receptor mutant (Fig. 8B). However, the more potent P2Y₁₁ receptor agonist BzATP elicited a significant response at a concentration of 100 μM at the R268A mutant (Fig. 8A). Pretreatment of the HEK_{A268}P2Y₁₁GFP cells with MRS2179 did not affect the action of BzATP at the receptor, in contrast to the observation made at the unmutated receptor. Therefore, this arginine seems to be a critical residue in the newly formed nucleotide binding pocket of the hetero-oligomer, as the physical interaction of both receptors was unaffected after mutation of this residue.

Two theories are currently discussed as to how GPCR oligomers are formed. One is the 'contact dimerization' and the other is the 'domain swapping' theory [1]. In the case of the P2Y₁-P2Y₁₁ receptor hetero-dimer our findings suggest that it is formed as a 'domain swapped' dimer because the change in the pharmacological profile of P2Y receptor ligands implies the creation of a newly formed binding pocket. In agreement with this suggestion is our finding that UDP induced a drastic [Ca²⁺]_i increase in HEK-P2Y₁₁GFP cells (data not shown). UDP is known to activate the P2Y₆ receptor, but this receptor was not expressed in our HEK293 cells. Interestingly, previous studies have reported that uridine nucleotides can activate the P2Y₁₁ receptor [44]. However, at the P2Y₁₁ receptor expressed in our batch of

1321N1 cells we could not confirm this finding. Our new findings in HEK293 cells suggest that the activity of uridine nucleotides might depend on the presence of the P2Y₁ receptor.

Conclusion

The present study provides convincing evidence for a physical interaction of the P2Y₁ and the P2Y₁₁ receptors in hetero-oligomers. This hetero-oligomerization enables the agonist-induced internalization of the P2Y₁₁ receptor. The P2Y₁₁ receptor expressed alone is reluctant to endocytosis and lacks desensitization. This property of the P2Y₁₁ receptor seems to be important for normal P2Y₁₁ receptor function. We hypothesize that the presence of P2Y₁ receptors in dendritic cells isolated from human blood enables the desensitization of the P2Y₁₁ receptor in response to ATP, whereby chemotactic behaviour and maturation of these cells are controlled. This is an important functional consequence of the heteromeric association of the P2Y₁₁ receptor leading to the alteration of its behaviour.

We have previously shown that the R268A-P2Y₁₁ receptor mutant (_{A268}P2Y₁₁) shows a 1000-fold reduced potency for ATP [16]. The _{A268}P2Y₁₁GFP receptor mutant did not functionally interact with the endogenous P2Y₁ receptor in HEK293 cells, since it did not co-internalize with the activated P2Y₁ receptor. Nevertheless, we found physical interaction between the _{A268}P2Y₁₁ receptor and the P2Y₁ receptor by co-pulldown.

In addition, we also found that the association of the P2Y₁ and the P2Y₁₁ receptor influences the ligand selectivity of the P2Y₁₁ receptor. Therefore we suggest that some discrepancies reported in the literature concerning the responsiveness of the P2Y₁₁ receptor to certain nucleotides could be due to a varying degree of co-expression of the P2Y₁ receptor.

The existence of GPCR homo- or hetero-oligomers is now largely accepted. The functional relevance of GPCR oligomers includes modulation of internalization processes or alteration of their agonist/antagonist potency. Both have to be considered regarding the development of subtype-specific drugs [6]. The importance of GPCR oligomerization becomes apparent, when we consider the organization of receptors and their signaling elements in specific microdomains. This clearly affects cellular communication processes [45]. The homo- or hetero-dimerization of purinergic GPCRs thereby allows fine tuning of the responses of cells to extracellular nucleotides.

Acknowledgements: We thank Ms. D. Terhardt for excellent technical help in the complete experimental study. We thank Dr. R. Hartig (Institute of Immunology, Medical Faculty

Magdeburg) for helpful suggestions in the FRET experiments. The study was supported by Bundesministerium für Bildung und Forschung (BMBF; grant NBL3; FKZ 01ZZ0407).

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20070671

References

- 1 Kroeger, K. M., Pflieger, K. D. and Eidne, K. A. (2003) G-protein coupled receptor oligomerization in neuroendocrine pathways. *Front Neuroendocrinol* **24**, 254-278
- 2 Suda, K., Filipek, S., Palczewski, K., Engel, A. and Fotiadis, D. (2004) The supramolecular structure of the GPCR rhodopsin in solution and native disc membranes. *Mol Membr Biol* **21**, 435-446
- 3 Hague, C., Uberti, M. A., Chen, Z., Hall, R. A. and Minneman, K. P. (2004) Cell surface expression of alpha1D-adrenergic receptors is controlled by heterodimerization with alpha1B-adrenergic receptors. *J. Biol. Chem.* **279**, 15541-15549
- 4 Margeta-Mitrovic, M., Jan, Y. N. and Jan, L. Y. (2000) A trafficking checkpoint controls GABA_B receptor heterodimerization. *Neuron* **27**, 97-106
- 5 Charles, A. C., Mostovskaya, N., Asas, K., Evans, C. J., Dankovich, M. L. and Hales, T. G. (2003) Coexpression of delta-opioid receptors with micro receptors in GH3 cells changes the functional response to micro agonists from inhibitory to excitatory. *Mol. Pharmacol.* **63**, 89-95
- 6 Milligan, G. (2006) G-protein-coupled receptor heterodimers: pharmacology, function and relevance to drug discovery. *Drug Discov Today* **11**, 541-549
- 7 Breit, A., Lagace, M. and Bouvier, M. (2004) Hetero-oligomerization between beta2- and beta3-adrenergic receptors generates a beta-adrenergic signaling unit with distinct functional properties. *J. Biol. Chem.* **279**, 28756-28765
- 8 Jordan, B. A., Trapaidze, N., Gomes, I., Nivarthi, R. and Devi, L. A. (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc. Natl. Acad. Sci. U S A* **98**, 343-348
- 9 Gregan, B., Jurgensen, J., Papsdorf, G., Furkert, J., Schaefer, M., Beyermann, M., Rosenthal, W. and Oksche, A. (2004) Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. *J. Biol. Chem.* **279**, 27679-27687
- 10 Burnstock, G. (2006) Purinergic signalling. *Br. J. Pharmacol.* **147 Suppl 1**, S172-181
- 11 Suzuki, T., Namba, K., Tsuga, H. and Nakata, H. (2006) Regulation of pharmacology by hetero-oligomerization between A1 adenosine receptor and P2Y2 receptor. *Biochem. Biophys. Res. Commun.* **351**, 559-565

- 12 Yoshioka, K., Saitoh, O. and Nakata, H. (2001) Heteromeric association creates a P2Y-like adenosine receptor. *Proc. Natl. Acad. Sci. U S A* **98**, 7617-7622
- 13 Moore, D. J., Chambers, J. K., Wahlin, J. P., Tan, K. B., Moore, G. B., Jenkins, O., Emson, P. C. and Murdock, P. R. (2001) Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. *Biochim. Biophys. Acta* **1521**, 107-119
- 14 Abbracchio, M. P., Burnstock, G., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., Knight, G. E., Fumagalli, M., Gachet, C., Jacobson, K. A. and Weisman, G. A. (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol. Rev.* **58**, 281-341
- 15 Ecke, D., Tulapurkar, M. E., Nahum, V., Fischer, B. and Reiser, G. (2006) Opposite diastereoselective activation of P2Y₁ and P2Y₁₁ nucleotide receptors by adenosine 5'-O-(α -boranotriphosphate) analogues. *Br. J. Pharmacol.* **149**, 416-423
- 16 Zylberg, J., Ecke, D., Fischer, B. and Reiser, G. (2007) Structure and ligand binding site characteristics of the human P2Y₁₁ nucleotide receptor deduced from computational modelling and mutational analysis. *Biochem. J.*, (in press)
- 17 Ullmann, H., Meis, S., Hongwiset, D., Marzian, C., Wiese, M., Nickel, P., Communi, D., Boeynaems, J. M., Wolf, C., Hausmann, R., Schmalzing, G. and Kassack, M. U. (2005) Synthesis and structure-activity relationships of suramin-derived P2Y₁₁ receptor antagonists with nanomolar potency. *J. Med. Chem.* **48**, 7040-7048
- 18 Vöhringer, C., Schäfer, R. and Reiser, G. (2000) A chimeric rat brain P2Y₁ 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. *Biochem. Pharmacol.* **59**, 791-800
- 19 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254
- 20 Tulapurkar, M. E., Schäfer, R., Hanck, T., Flores, R. V., Weisman, G. A., Gonzalez, F. A. and Reiser, G. (2005) Endocytosis mechanism of P2Y₂ nucleotide receptor tagged with green fluorescent protein: clathrin and actin cytoskeleton dependence. *Cell Mol Life Sci* **62**, 1388-1399

- 21 Ubl, J. J., Vöhringer, C. and Reiser, G. (1998) 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
- 22 Milligan, G. and Bouvier, M. (2005) Methods to monitor the quaternary structure of G protein-coupled receptors. *FEBS J.* **272**, 2914-2925
- 23 Luo, W., Wang, Y., Hanck, T., Stricker, R. and Reiser, G. (2006) Jab1, a novel protease-activated receptor-2 (PAR-2)-interacting protein, is involved in PAR-2-induced activation of activator protein-1. *J. Biol. Chem.* **281**, 7927-7936
- 24 Schäfer, R., Hartig, R., Sedehizade, F., Welte, T. and Reiser, G. (2006) Adenine nucleotides inhibit proliferation of the human lung adenocarcinoma cell line LXF-289 by activation of nuclear factor kappaB1 and mitogen-activated protein kinase pathways. *FEBS J.* **273**, 3756-3767
- 25 Rizzo, M. A., Springer, G. H., Granada, B. and Piston, D. W. (2004) An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol* **22**, 445-449
- 26 Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A. and Tsien, R. Y. (2001) Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J. Biol. Chem.* **276**, 29188-29194
- 27 Lazarowski, E. R., Watt, W. C., Stutts, M. J., Boucher, R. C. and Harden, T. K. (1995) Pharmacological selectivity of the cloned human P_{2U} -purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.* **116**, 1619-1627
- 28 Mundell, S. J., Luo, J., Benovic, J. L., Conley, P. B. and Poole, A. W. (2006) Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. *Traffic* **7**, 1420-1431
- 29 Brinson, A. E. and Harden, T. K. (2001) Differential regulation of the uridine nucleotide-activated P_{2Y_4} and P_{2Y_6} receptors. SER-333 and SER-334 in the carboxyl terminus are involved in agonist-dependent phosphorylation desensitization and internalization of the P_{2Y_4} receptor. *J. Biol. Chem.* **276**, 11939-11948
- 30 Tulapurkar, M. E., Zündorf, G. and Reiser, G. (2006) Internalization and desensitization of a green fluorescent protein-tagged P_{2Y} nucleotide receptor are differently controlled by inhibition of calmodulin-dependent protein kinase II. *J. Neurochem.* **96**, 624-634
- 31 Communi, D., Govaerts, C., Parmentier, M. and Boeynaems, J. M. (1997) Cloning of a human purinergic P_{2Y} receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.* **272**, 31969-31973

- 32 Schäfer, R., Sedehizade, F., Welte, T. and Reiser, G. (2003) ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **285**, L376-385
- 33 Wang, H. and Reiser, G. (2003) Thrombin signaling in the brain: the role of protease-activated receptors. *Biol. Chem.* **384**, 193-202
- 34 Rohatgi, T., Sedehizade, F., Reymann, K. G. and Reiser, G. (2004) Protease-activated receptors in neuronal development, neurodegeneration, and neuroprotection: thrombin as signaling molecule in the brain. *The Neuroscientist* **10**, 501-512
- 35 Prinster, S. C., Hague, C. and Hall, R. A. (2005) Heterodimerization of G protein-coupled receptors: specificity and functional significance. *Pharmacol. Rev.* **57**, 289-298
- 36 Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C. and Patel, Y. C. (2000) Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J. Biol. Chem.* **275**, 7862-7869
- 37 Communi, D., Robaye, B. and Boeynaems, J.-M. (1999) Pharmacological characterization of the human P2Y₁₁ receptor. *Br. J. Pharmacol.* **128**, 1199-1206
- 38 Schnurr, M., Toy, T., Stoitzner, P., Cameron, P., Shin, A., Beecroft, T., Davis, I. D., Cebon, J. and Maraskovsky, E. (2003) ATP gradients inhibit the migratory capacity of specific human dendritic cell types: implications for P2Y₁₁ receptor signaling. *Blood* **102**, 613-620
- 39 Carrillo, J. J., Padiani, J. and Milligan, G. (2003) Dimers of class A G protein-coupled receptors function via agonist-mediated trans-activation of associated G proteins. *J. Biol. Chem.* **278**, 42578-42587
- 40 von Kugelgen, I. (2006) Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther.* **110**, 415-432
- 41 Feng, C., Mery, A. G., Beller, E. M., Favot, C. and Boyce, J. A. (2004) Adenine nucleotides inhibit cytokine generation by human mast cells through a Gs-coupled receptor. *J Immunol* **173**, 7539-7547
- 42 Lee, H., Jun, D. J., Suh, B. C., Choi, B. H., Lee, J. H., Do, M. S., Suh, B. S., Ha, H. and Kim, K. T. (2005) Dual roles of P2 purinergic receptors in insulin-stimulated leptin production and lipolysis in differentiated rat white adipocytes. *J. Biol. Chem.* **280**, 28556-28563
- 43 Kenakin, T. (2004) Principles: receptor theory in pharmacology. *Trends Pharmacol. Sci.* **25**, 186-192

- 44 White, P. J., Webb, T. E. and Boarder, M. R. (2003) Characterization of a Ca^{2+} response to both UTP and ATP at human P2Y_{11} receptors: evidence for agonist-specific signaling. *Mol. Pharmacol.* **63**, 1356-1363
- 45 Woolf, P. J. and Linderman, J. J. (2004) An algebra of dimerization and its implications for G-protein coupled receptor signaling. *J Theor Biol* **229**, 157-168

Stage 2(a) POST-PRINT

Figure legends

Fig. 1: Co-Pulldown (PD) of the P2Y₁mycHis and P2Y₁₁GST receptors from HEK293 cells. For the pulldown, total lysates of transfected HEK293 cells expressing the indicated receptors were incubated overnight with GSH beads at 4°C, as described in methods. Western blot (WB) analysis was performed using anti-myc (A), anti-GST (B) or anti-HA (C) antibodies. **Lane 1-3:** Input signals from total lysates (10% of PD samples) of co-transfected HEK293 cells. **Lane 4:** Mixture of lysates from P2Y₁mycHis and P2Y₁₁GST single transfected cells and subsequent incubation with GSH beads. **Lane 5:** Co-pulldown of the P2Y₁mycHis and P2Y₁₁GST receptor from lysates of co-transfected cells (compare with lane 1). **Lane 6:** Lysate of P2Y₁mycHis single transfected cells incubated with GST-preloaded GSH beads. **Lane 7:** P2Y₁mycHis/P2Y₁₁GST receptor co-pulldown, beads after incubation with FXa. **Lane 8:** Lysates of PAR-2-HA/P2Y₁₁GST co-transfected cells incubated with GSH beads. **Lane 9:** Co-pulldown of the P2Y₄HA and P2Y₁₁GST receptor from lysates of co-transfected cells. Approximate molecular mass: P2Y₁mycHis ~ smearlike staining between 35 and 100 kDa; P2Y₁₁GST ~ 65 kDa; PAR-2-HA ~ smearlike bands from 37-70 kDa; P2Y₄HA ~ 37 kDa. Here and in Figs. 2 and 3, Western blots shown are representative of at least two independent experiments analyzing the various receptors and testing the different conditions. * indicates positive pulldown signal. The arrowheads point to the main band of the P2Y₁mycHis protein.

Fig. 2: Co-Immunoprecipitation (IP) of the P2Y₁mycHis and P2Y₁₁GST receptor from HEK293 cells. Total lysates of differently transfected HEK293 cells were incubated first overnight with anti-myc antibody and then with Agarose Plus A/G beads at 4°C, as described in methods. Western blot (WB) analysis was performed using anti-GST (A) or anti-myc (B) antibodies. **Lane 1:** Input signal from total lysate (10% of IP samples). **Lane 2:** Mixture of lysates from P2Y₁mycHis and P2Y₁₁GST single transfected cells immunoprecipitated with anti-myc antibody. **Lane 3:** Co-IP of P2Y₁mycHis and P2Y₁₁GST receptor from lysates of co-transfected cells (compare with lane 1) using anti-myc antibody (arrow points to P2Y₁₁GST protein). **Lane 4:** Precipitation of lysates from P2Y₁mycHis/P2Y₁₁GST cells using unspecific mouse IgG (negative control). Molecular mass for receptors is given in legend to Fig. 1. The arrowhead indicates a signal corresponding to Protein A. * the indicated band represents non-reduced IgG due to unboiled samples.

Fig. 3: Cellular extracts of differently transfected HEK293 cells were incubated overnight with GSH beads at 4°C, as described in methods. Western blot (WB) analysis was performed

using anti-myc (A) or anti-GST (B) antibodies. **Lane 1-2:** Input signals from extracts (10% of pulldown samples) of co-expressing cells. **Lane 3-4:** Co-pulldown of the P2Y₁mycHis receptor with the A₂₆₈P2Y₁₁GST receptor (lane 3) or the wild type P2Y₁₁GST receptor (lane 4) from lysates of co-transfected cells. Molecular mass of receptors is given in legend to Fig. 1. The arrowheads point to the main band of the P2Y₁mycHis protein.

Fig. 4: Analysis of the hetero-oligomerization of P2Y₁Cerulean and P2Y₁₁Citrine by FRET experiments. HEK293 cells transiently transfected with the fluorescent constructs were imaged by confocal microscopy to detect the fluorescence spectra at 458 nm excitation, as described in methods. **(A)** The graph displays representative emission spectra normalized to fluorescence intensity at 483 nm. **(B)** Analysis of FRET ratios. FRET ratios (R) were calculated using the following equation as explained in the methods section:

$$FRET_{ratio}(R) = \frac{I_{526nm}}{I_{483nm}}. \text{ Statistical analysis was done using the one-way ANOVA test and the}$$

Dunnett test as a post test. ** p < 0.001

Fig. 5: Confocal imaging of internalisation of P2Y₁GFP and P2Y₁₁GFP receptors in transfected 1321N1 cells. **(A)** 1321N1 cells expressing the P2Y₁GFP receptor were stimulated with 10 μM 2-MeS-ADP on stage at 37°C (5% CO₂) and living cells were imaged with a LSM510meta confocal microscope for up to 60 min. Pictures show unstimulated (US) cells (A1) and cells after 60 min of agonist stimulation (A2). Scale bar indicates 5 μm. **(B)** 1321N1 cells stably expressing the P2Y₁₁GFP receptor were stimulated with 100 μM ATP and measured in the same way as P2Y₁GFP expressing cells. **(C-E)** Transiently co-transfected 1321N1 cells expressing the P2Y₁mycHis and the P2Y₁₁GFP receptor were stimulated with nucleotides for 60 min, fixed with 4% PFA and stained with anti-myc antibody as described in methods. Images display the GFP fluorescence (C1, D1, E1) and myc-staining (C2, D2, E2) separately, as well as a merged picture (C3, D3, E3). White lines in the merged picture illustrate the x-z or y-z section-plane displayed in the zoomed pictures on top and besides the image. Scale bar indicates 10 μm. Pictures shown are representative for at least three independent experiments.

Fig. 6: HEK-P2Y₁₁GFP cells were visualized with a LSM510meta confocal microscope and stimulated with various agonists for up to 60 min at 37°C (5% CO₂) as described in methods. Pictures show unstimulated (US) cells (A1-H1) and cells after 30 min (A2-H2) and 60 min

(A3-H3) of agonist stimulation. (A) Stimulation with 100 μM ATP (n=5). (B) Cells preincubated with 100 μM MRS2179 and stimulated with 100 μM ATP (n=3). (C) Stimulation with 10 μM 2-MeS-ADP (n=3). (D) Stimulation with 100 μM BzATP (n=3). (E) Cells preincubated for 30 min with 10 μM NF157 and stimulated with 100 μM ATP (n=3). (F) Stimulation with 100 μM UTP (n=4). (G) Stimulation with 100 μM PAR-2-AP (n=3). (H) HEK_{A268}P2Y₁₁GFP cells stimulated with 100 μM ATP (n=3). Results shown are representative for n independent experiments analysing 20-30 cells in each case. Scale bar represents 5 μm .

Fig. 7: Kinetics of receptor endocytosis analyzed by the increase in fluorescence intensity of the P2Y₁₁GFP receptor in the cytosol of siRNA transfected HEK293 cells. Images of HEK-P2Y₁₁GFP cells transfected with siRNA and stimulated with 100 μM ATP for up to 60 min were taken on a Zeiss LSM510 meta confocal microscope, 48 hours after transfection, and fluorescence intensities were analyzed using the Zeiss software. The average fluorescence intensity was normalised to the value at 0 min as described in methods. Displayed are curves for untreated cells (n=16), MATra transfection reagent treated cells (n=19), control siRNA treated cells (n=18) and P2Y₁ receptor siRNA (n=21) treated cells. Asterisks indicate significance (* p<0.05) compared to untreated cells, analysed by one way ANOVA and Tukey's test. The inset shows a representative blot depicting the 50% decrease in P2Y₁ receptor protein expression, as determined by western blot analysis using a P2Y₁ receptor antibody.

Fig. 8: Influence of hetero-oligomerization on the ligand selectivity of the receptors. Cells were stimulated with 100 μM of agonists and preincubated with 100 μM of antagonists, unless the subscript indicates treatment with the agent at 1 μM concentration. Bar graphs show peak values ($\Delta\text{F}_{340\text{nm}}/\text{F}_{380\text{nm}}$) from $[\text{Ca}^{2+}]_i$ measurements using fura-2. Data represent the mean \pm s.e.m obtained in at least three independent experiments. (A) Measurements in HEK293 cells, which were mock transfected (blank bars), P2Y₁₁GFP transfected (black bars) or A₂₆₈P2Y₁₁GFP transfected (cross-hatched bars). Cells were stimulated with BzATP. Preincubation of the cells with MRS2179 and NF157 are indicated. (B) Measurements in transfected HEK293 cells using ATP (100 μM) as stimulating agonist. (C) Intracellular calcium rise measured in 1321N1 cells, stably expressing the P2Y₁₁GFP receptor, evoked by the agonists indicated.

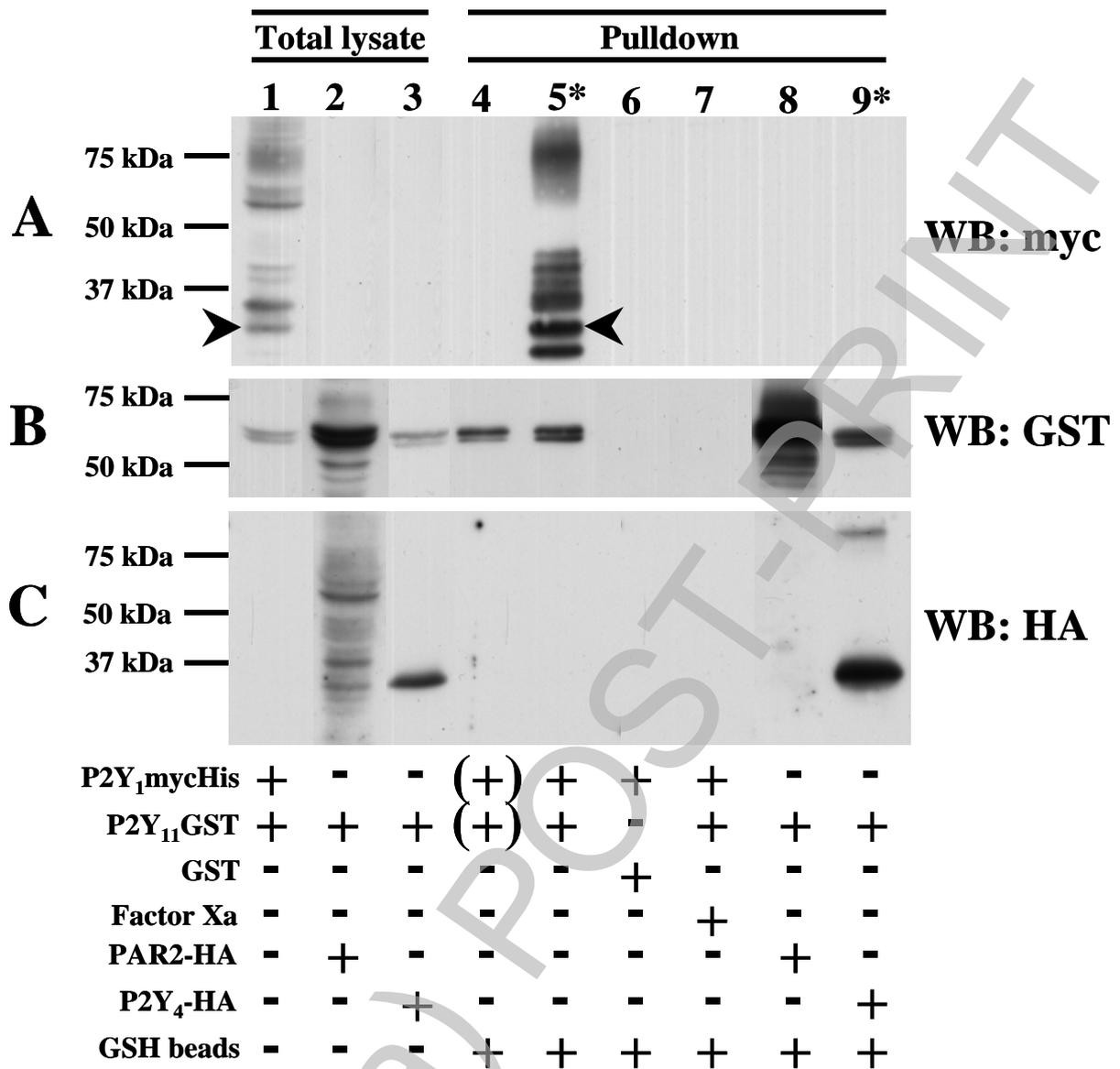


Figure 1

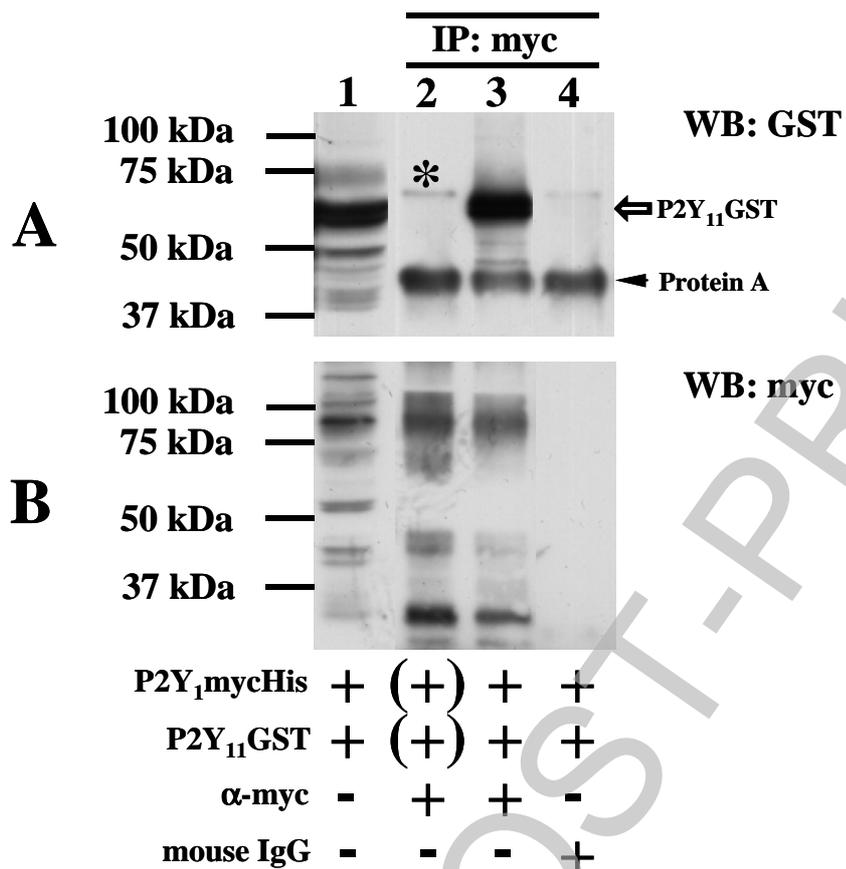


Figure 2

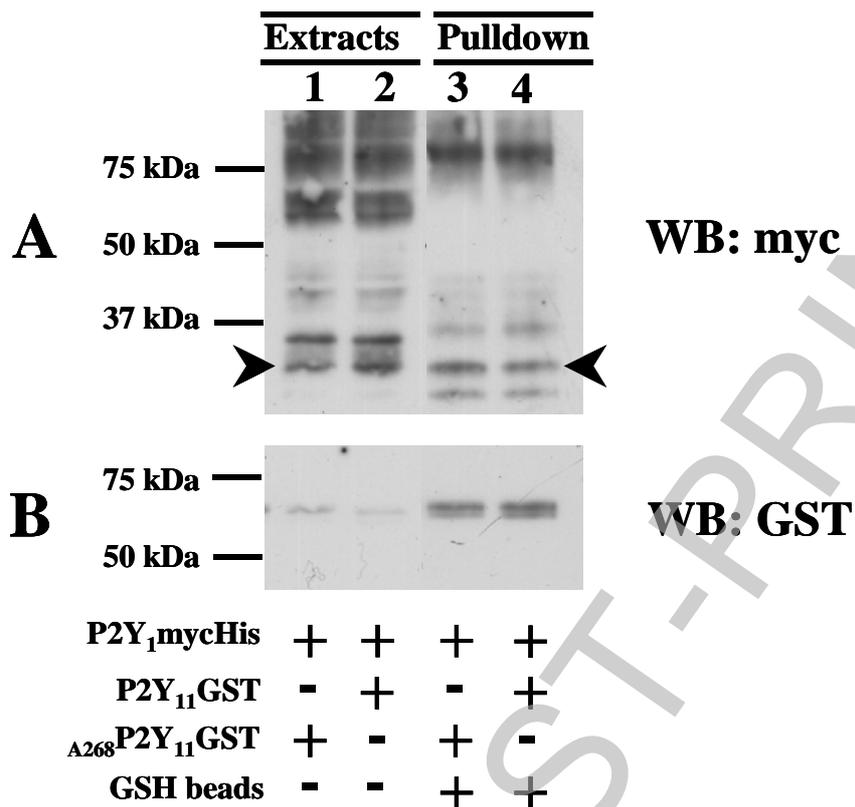


Figure 3

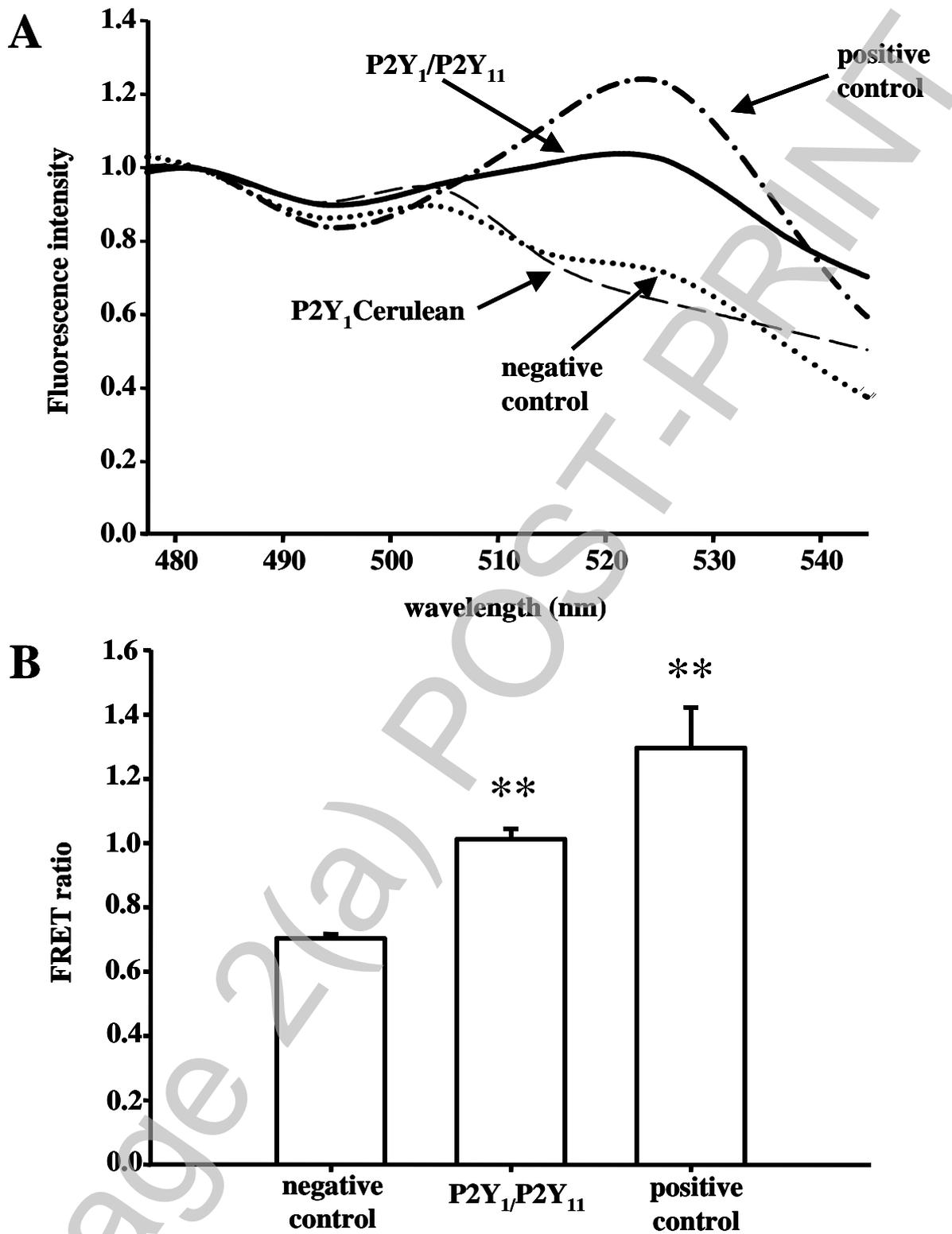


Figure 4

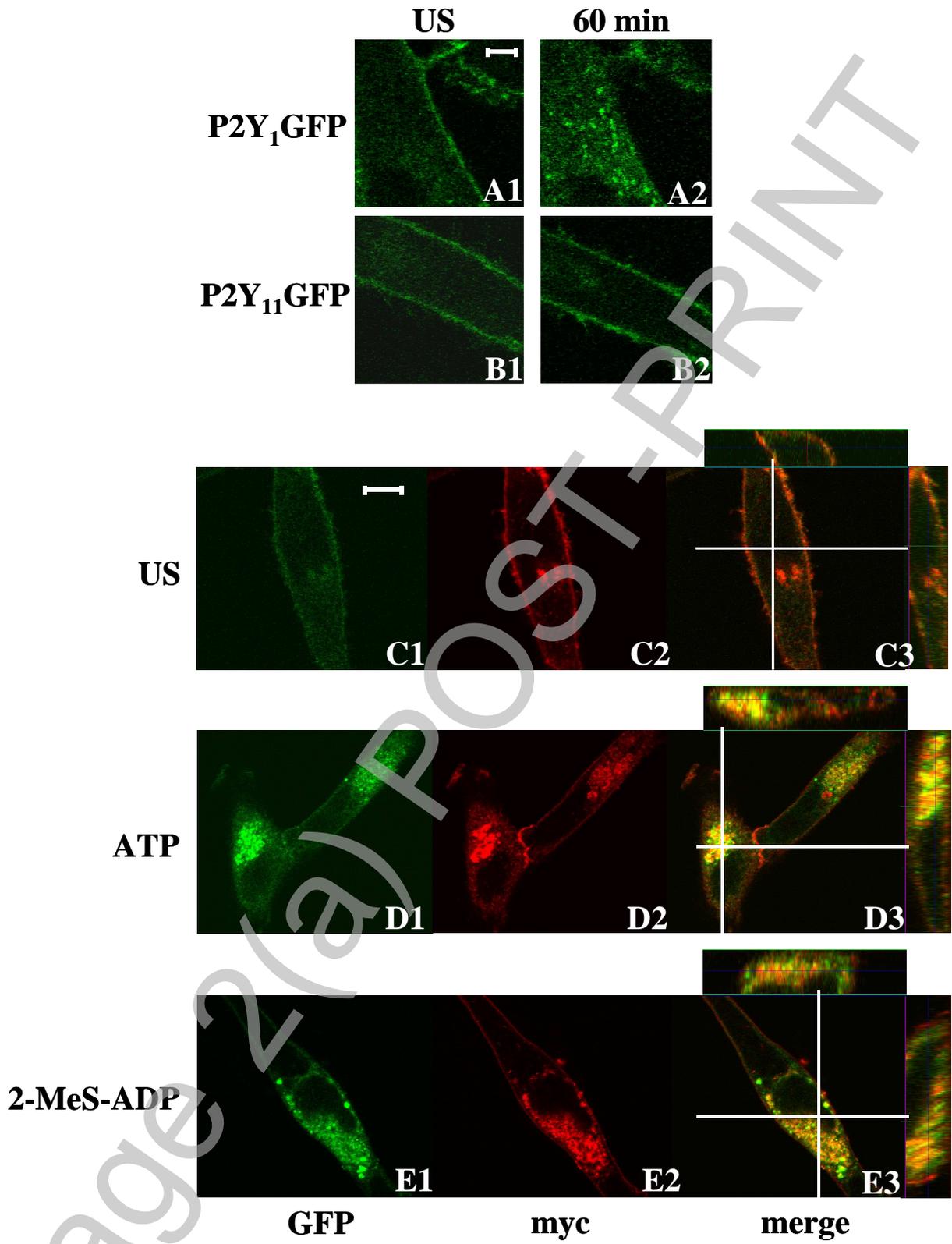


Figure 5

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20070671

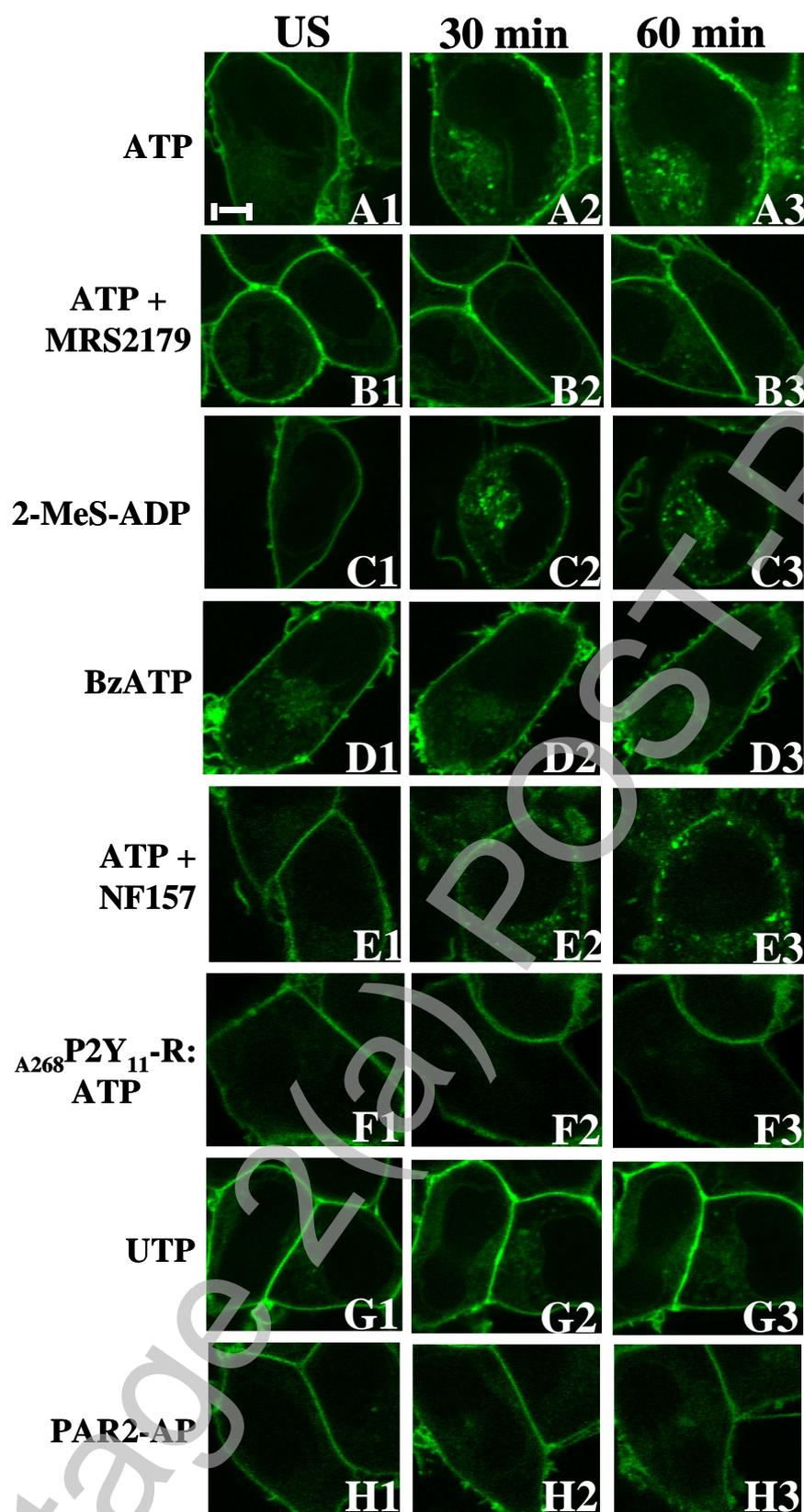


Figure 6

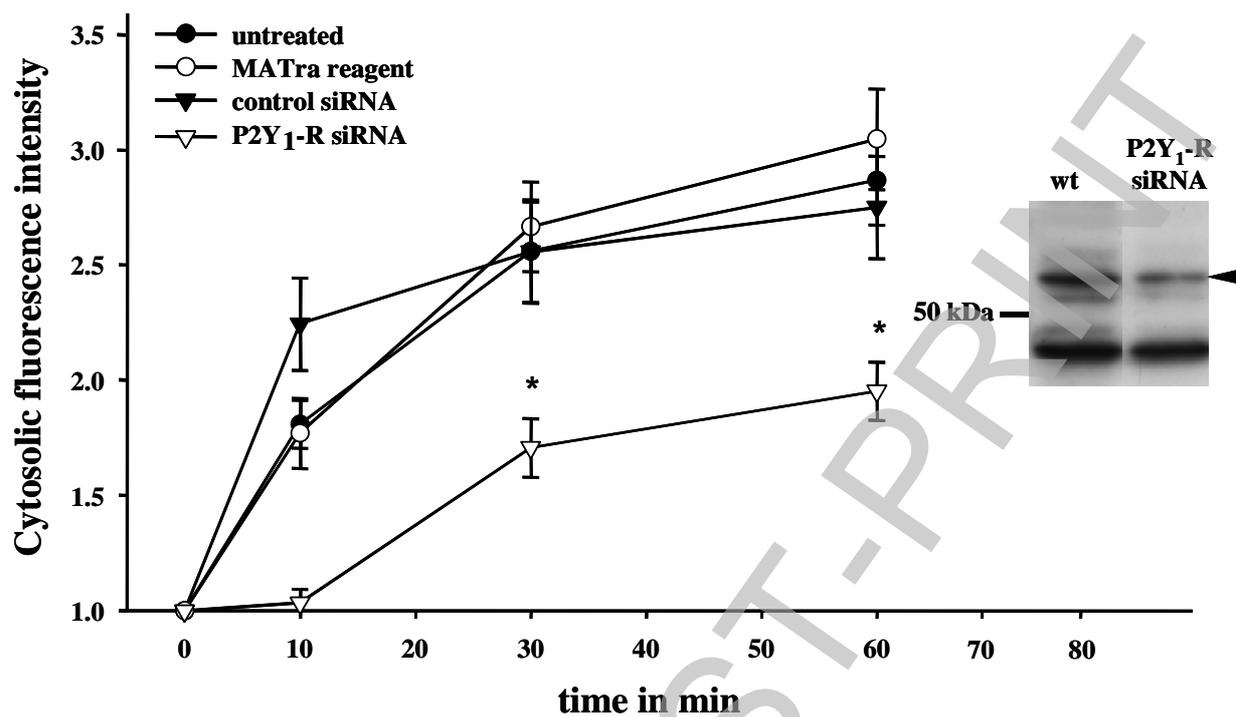


Figure 7

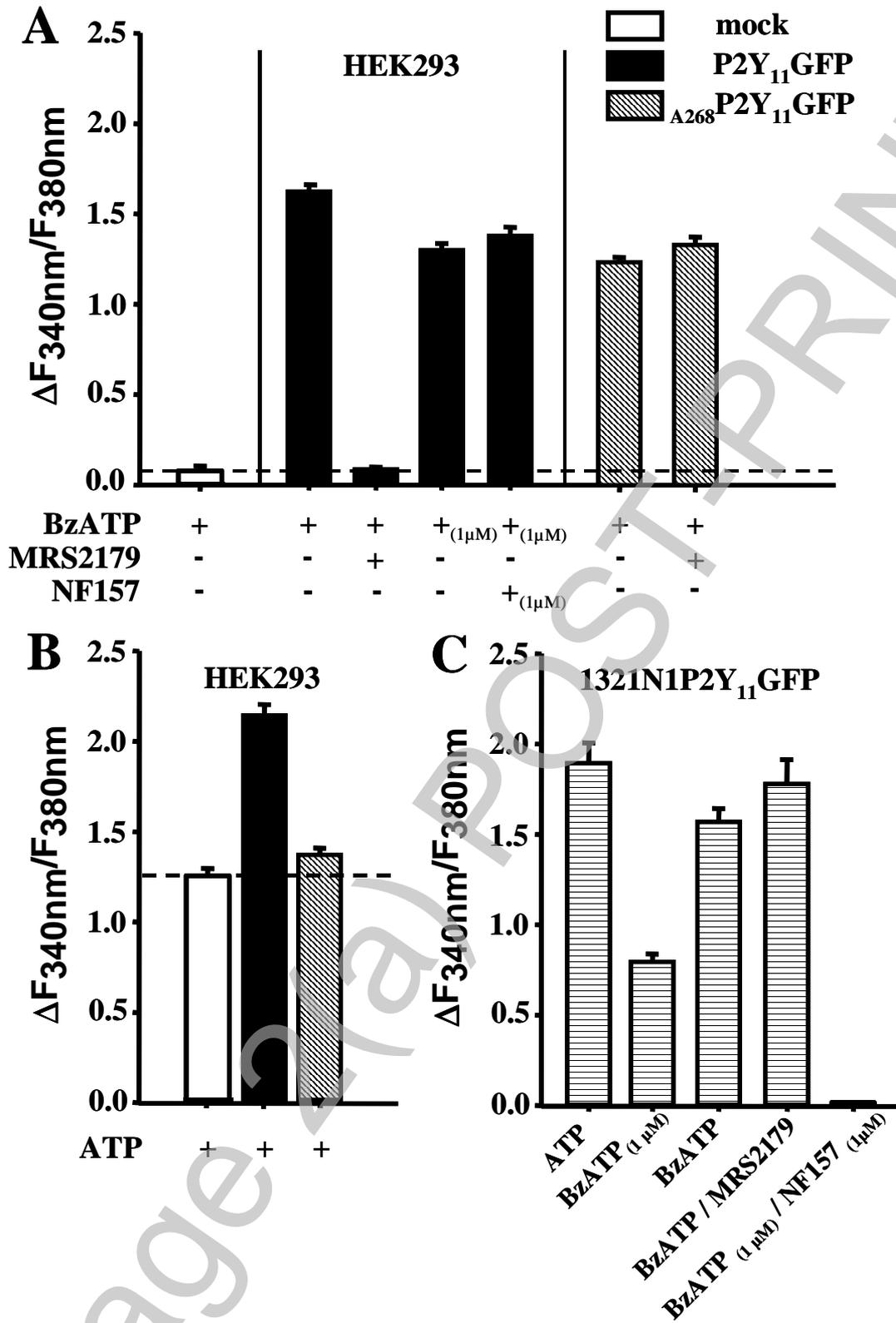


Figure 8