Nucleotides signal through purinergic receptors such as the P2 receptors, which are subdivided into the ionotropic P2X receptors and the metabotropic P2Y receptors. The diversity of functions within the purinergic receptor family is required for the tissue-specificity of nucleotide signalling. In the present study, hetero-oligomerization between two metabotropic P2Y receptor subtypes is established. These receptors, P2Y1 and P2Y11, were found to associate together when co-expressed in HEK293 cells. This association was detected by co-pull-down, immunoprecipitation and FRET (fluorescence resonance energy transfer) experiments. We found a striking functional consequence of the interaction between the P2Y11 receptor and the P2Y1 receptor where this interaction promotes agonist-induced internalization of the P2Y11 receptor. This is remarkable because the P2Y11 receptor by itself is not able to undergo endocytosis. 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.

INTRODUCTION

The current position on GPCR (G-protein-coupled receptor) organization in the plasma membrane includes the discovery that these proteins can oligomerize among each other. These oligomers link distinct signalling 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 alterations 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 heterodimerization of dopamine D2 and adenosine A2A 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, A2A, A2B and A3) are responsive to adenosine, whereas the metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) 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 P2Y2 or P2Y6 receptor respectively [11,12]. The A1–P2Y receptor hetero-oligomer was shown to create a P2Y-like adenosine receptor by creating ligand-binding sites with a A1–P2Y receptor hybrid selectivity. In contrast, the association of the A1 receptor with the P2Y2 receptor did not seem to affect the ligand selectivity of the receptors, but stimulation of the P2Y2 receptor with UTP in the A1–P2Y2 receptor complex caused uncoupling of the A1 receptor from G, 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.
MATERIAL AND METHODS

Materials

Geneticine (G418 sulphate) was from Calbiochem. 2-MeS-ADP (2-methylthio-ADP), ATP, BzATP [\(2'(3')\)-O-(4-benzoylbenzoyl)ATP], MRS2179 (N\(^6\)-methyl-2-deoxyadenosine-3',5'-bisphosphate), UDP and UTP were from Sigma. Rp Sp ATP [\(\alpha\)S] (adenosine 5'-[\(\alpha\)-thio]triphosphate, Rp- or Sp-isomer) was from Biolog, Germany. DMEM (Dulbecco’s modified Eagle’s medium), DMEM/Ham’s medium, penicillin/streptomycin (10 000/10 000 units/ml), trypsin/EDTA (0.05:0.02, v/v) and FCS (foetal calf serum) were from Seromed. Cell culture dishes were from Nunc and 22 mm coverslips were from OmniLab. Fura 2/AM (fura 2 acetoxymethyl ester) was from Biomol/Molecular Probes and complete protease inhibitor cocktail, DOTAP (dioleoyltrimethylammonium propane) and FuGENE™ 6 transfection reagents were from Roche. Glutathione–Sepharose was from GE Healthcare and cDNA plasmid for Cerulean fluorescent protein reagents were from Roche. Polyclonal and monoclonal antibodies were from Santa Cruz, Promega, Cell Signaling, 1:100, 1:500 and 1:1000 respectively.

Receptor constructs

The P2Y\(_1\) receptor was constructed bearing a Myc-epitope or the pEGFPN1 vector (Clontech). For generating the P2Y\(_4\) receptor tagged with the Cerulean fluorescent protein, the GFP (green fluorescent protein) tag was replaced by the cDNA of Cerulean using PCR cloning. The P2Y\(_{11}\) receptor tags [GST (glutathione transferase), 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 the pGEX vector (GE Healthcare) using PCR cloning and placed downstream of the P2Y\(_{11}\) receptor DNA sequence following a Factor Xa cleavage-site insert in a pcDNA3.1(+)- vector. The fluorescent-tagged receptor was generated by placing the P2Y\(_{11}\) receptor sequence between the EcoRI/BamHI restriction sites of the pEGFPN1 or pEYFPN1 vector. The Q69M mutation in the YFP (yellow fluorescent protein)-tag to produce the Citrine-tag and the R268A mutation in the P2Y\(_{11}\) receptor sequence were introduced using the site-directed mutagenesis QuickChange kit (Stratagene). For construction of a FRET (fluorescence resonance energy transfer)-positive control, DNA encoding for the Cerulean protein was ligated into the BamHI-digested Citrine–P2Y\(_{11}\) vector by PCR cloning. The P2Y\(_4\) and PAR (protease-activated receptor)-2 were present as HA (haemagglutinin) fusion proteins. The N-terminal HA-tagged P2Y\(_4\) receptor was cloned into the pcDNA3.1(+) vector. The PAR-2 (protease-activated receptor-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 units/ml penicillin, and 100 international units/ml streptomycin in a humidified atmosphere (CO\(_2/\)air, 1:19) at 37 °C. Transient transfection of receptor constructs was performed using the MATra-A (magnet-assisted transfection for adherent cells) reagent (IBA GmBH) 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 units/ml penicillin, and 100 international units/ml streptomycin at 37 °C in 10 % CO\(_2\). Transfection of the cells was done using FuGENE™ 6 as described in [15]. A stable expression of the receptor constructs was achieved in the presence of G418 in the medium of both cell lines throughout the culture period.

Co-pull-down and co-immunoprecipitation

Transiently transfected (48 h) HEK293 cells were lysed in RIPA buffer [50 mM Tris/HCl (pH 8.1); 1 % Igepal CA630; 0.25 % sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM NaF; protease inhibitor cocktail], harvested by scraping, briefly sonicated and centrifuged at 14 000 \(\times\) 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-pull-down experiments, whole cell lysates were diluted with RIPA buffer to a final protein concentration of 500 \(\mu\)g/ml and incubated overnight with rocking at 4 °C with glutathione–Sepharose. For co-immunoprecipitation, whole cell lysates were diluted with RIPA buffer to a final protein concentration of 250 \(\mu\)g/ml and incubated with 1.5 \(\mu\)g anti-Myc antibody (mouse monoclonal, Invitrogen) overnight as described above. Protein A/G plus agarose (Santa Cruz) was added and mixed with the lysates for at least 2 h at 4 °C. Pull-down fractions or immunoadsorbents were recovered by centrifugation and washed three times in RIPA buffer. Samples were eluted in 10 \(\mu\)l of 2× Laemmli buffer to prepare for loading on to SDS/PAGE.

Western blot analysis

Samples were resolved by SDS/PAGE, followed by transfer on to nitrocellulose membrane. The membrane was blocked and probed with antibodies against the Myc-epitope (Invitrogen, 1:5000), GST-tag (Santa Cruz, 1:20000) and HA-epitope (Cell Signaling, 1:1000). After washing, the membrane was further incubated with peroxidase-conjugated anti-rabbit IgG or antimouse IgG (1:20000 or 1:10000 respectively) and visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce). Membranes were stripped before reprobing.
Immunocytochemistry

Transiently transfected 1321N1 cells cultured on 22 mm coverglasses were fixed in 4% paraformaldehyde, blocked and permeabilized in FSBB [Foetal Serum Blocking Buffer; 17% (v/v) FCS, 20 mM Na₂HPO₄, 450 mM NaCl, 0.3% Triton X-100] and incubated in mouse monoclonal anti-Myc antibody (Invitrogen; 0.8 µg/µl) overnight at 4°C. After washing, the secondary antibody was applied (Alexa Fluor® 555 goat anti-mouse IgG antibody at 1:200; Molecular Probes) for 2 h at 25°C. Cells were washed again and mounted in Vectashield mounting medium (Axxora). Visualization of the GFP fluorescence and immunostaining was done with a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Apochromat ×63 objective. For FRET analysis, emission spectra were measured at 458 nm excitation employing an argon/krypton laser and a beam splitter NT80/20. 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-tagged receptors (Cerulean–P2Y₁, Citrine–P2Y₁₁, or Cerulean–Citrine–P2Y₁₁) or the fluorescent tags only were imaged on a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Apochromat ×63 objective. For FRET analysis, emission spectra were measured at 458 nm excitation employing an argon/krypton laser and a beam splitter 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 P2Y₁ receptor bearing a Cerulean tag that was connected to a Citrine tag by a 16-amino-acid 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:

$$\text{FRET ratio} (R) = \frac{I_{526}}{I_{483}}$$

**Agonist-induced internalization**

Agonist-induced internalization was measured as described previously [20]. Briefly, HEK293 cells stably expressing the GFP–P2Y₁ receptor were seeded on coverslips (30 mm diameter). Coverslips were placed in a POC (perfusion, open and closed)-chamber (PeCon GmbH, Erbach, Germany) and incubated on a 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 analysed using the Zeiss LSM 510meta software histo macro. ROI (Regions of interest; ellipse 30–39 µm²) 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 (small interfering RNA)**

P2Y₁ receptor-specific siRNA [sense r(CUC UCC UCU GAG GAG AAA A)dTdT] and non-silencing control siRNA were from Qiagen. HEK-293 cells expressing GFP–P2Y₁₁ were seeded on coverslips (30 mm diameter) pre-coated with 0.01% poly-L-lysine and transfected at 30–40% density with siRNA using MTAra-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) at 1:500 dilution.

**Calcium measurements**

The cells were plated on coverslips (22 mm diameter), and single cell measurement was done after 3 days when the cells were 30–50% confluent. The changes in [Ca²⁺], (intracellular free Ca²⁺ concentration) were measured as described previously [21]. Briefly, cells were preincubated with 2 µM fura 2/AM (with 0.02% pluronic acid for 1321N1cells) 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.

**Statistical analysis**

Statistical analysis of the results was done using GraphPad Prism. Results were checked for Gaussian distribution. In the case of Gaussian distribution of results, 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 of less than 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, which are close homologues in their receptor family. So far, crosstalk between the two 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 Aₐ adenosine receptor [12]. In the present study, we analysed whether there is an interaction between the P2Y₁ receptor and the P2Y₁₁ receptor. For this purpose we carried out co-pull-down, co-immunoprecipitation and FRET experiments with the two proteins expressed together.

The P2Y₁ receptor expressed as a GST-fusion protein and the P2Y₁₁ receptor bearing a MycHis tag were co-expressed in HEK293 cells. This allowed us to check for their physical interaction by pull-down and immunoprecipitation experiments using whole cell lysates (Figures 1 and 2). Western blot analysis of lysates from co-transfected cells revealed smearlike anti-Myc reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1). The smearlike staining probably represents several reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1). The smearlike staining probably represents several reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1). The smearlike staining probably represents several reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1). The smearlike staining probably represents several reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1). The smearlike staining probably represents several reactive bands between 35 kDa and 75–100 kDa (Figure 1A, lane 1).
Total lysates of transfected HEK293 cells expressing the indicated receptors were incubated overnight with glutathione sepharose at 4 °C, as described in the Materials and methods section. Western blot (WB) analysis was performed using anti-Myc (A), anti-GST (B) or anti-HA (C) antibodies. Lanes 1–3: input signals from total lysates (10% of pull-down samples) of co-transfected HEK293 cells. Lane 4: a mixture of lysates from cells, transfected with only MycHis–P2Y1 or GST–P2Y11, incubated with glutathione–Sepharose. Lane 5: co-pull-down of the MycHis–P2Y1 and GST–P2Y11 receptor from lysates of co-transfected cells (compare with lane 1). Lane 6: lysate of cells transfected with MycHis–P2Y1 after incubation with GST-preloaded glutathione sepharose. Lane 7: MycHis–P2Y1/GST–P2Y11 receptor co-pull-down, beads after incubation with Factor Xa. Lane 8: lysates of HA–PAR-2/GST–P2Y11, co-transfected cells incubated with glutathione Sepharose. Lane 9: co-pull-down of the HA–P2Y4 and GST–P2Y11 receptor from lysates of co-transfected cells. Approximate molecular mass: MycHis–P2Y1, smearlike staining between 35 and 100 kDa; GST–P2Y11, ~65 kDa; HA–PAR-2, smearlike bands from 37–70 kDa; HA–P2Y4, ~57 kDa. In this Figure and in Figures 2 and 3, Western blots shown are representative of at least two independent experiments analysing the various receptors and testing the different conditions. * indicates positive pull-down signal. The arrowheads point to the main band of the MycHis–P2Y1 protein.

Mychis–P2Y1 receptor (Figure 1A, lane 5) was pulled down together with the GST–P2Y11 receptor (Figure 1B, lane 5) with glutathione Sepharose. This created a prominent band, as well as a band below that, upon isolation with glutathione sepharose. The interaction of the P2Y1 and P2Y11 receptor was strong, as seen from the Myc-staining in the pull-down sample. There, the staining was much more intense than in the 10% input (Figure 1A, compare lanes 5 and 1, respectively). The association seems to be constitutive because agonist addition did not alter the interaction between the two receptors (results not shown).

To test for the specificity of the pull-down experiment, several control conditions were applied. The GST pull-down experiment with HEK293 cells, expressing either the MycHis–P2Y1 or the GST–P2Y11 receptor, showed no Myc-immunoreaction in the Western blot analysis (results not shown). Also, the MycHis–P2Y1 receptor did not interact with the GST tag, which was examined using GST-preloaded glutathione–Sepharose in a pull-down test (Figure 1A, lane 6). This was further confirmed by cleaving the P2Y11 receptor from the GST fusion protein at the Factor Xa cleavage site using this protease. The Factor Xa treatment led to the disappearance of the Myc-immunoreaction in the pull-down sample (Figure 1A, lane 7). When we used a mixture of lysates from HEK293 cells transfected with MycHis–P2Y1 or GST–P2Y11, the GST-pull-down fraction did not show any Myc-immunoreaction (Figure 1A, lane 4). This excludes that the formation of aggregates during the pull-down procedure might be a possible cause of the positive co-pull-down result [22].

We further investigated the specificity of the interaction between both receptors. The GST–P2Y11 receptor was co-transfected with a non-related GPCR. Here we used the PAR-2 bearing a HA tag. The HA–PAR-2 protein showed a smeared band (~37–70 kDa, Figure 1C, lane 2) which is consistent with previous results [23]. The pull-down experiment in Figure 1C, lane 8, revealed no HA-immunoreactivity, thereby excluding an interaction of the P2Y11 receptor with PAR-2. However, co-transfection of the GST–P2Y11 receptor with the HA–P2Y11 nucleotide receptor (~37 kDa) resulted in a physical interaction of both receptors, as demonstrated by GST pull-down experiment and subsequent Western blot with an anti-HA antibody (Figure 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 GST–P2Y11 receptor by an anti-Myc antibody from Myc–P2Y1–GFP–P2Y11 co-transfected cells. The GST–P2Y11 receptor clearly co-immunoprecipitated together with the Myc-tagged P2Y11 receptor, as detected by Western blot analysis using a GST-antibody (Figure 2A, lane 3). The signal was specific, as lysates precipitated with an unspecific IgG showed no anti-GST-reactive bands (Figure 2A, lane 4). Moreover, as in the pull-down test, a mixture of lysates from HEK293 cells transfected with only one of

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the receptors did not result in co-precipitation of the GST–P2Y11 receptor (Figure 2A, lane 2).

Furthermore, we analysed 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 RIPA buffer and then centrifuged at 50 000 g for 30 min to remove undissolved membrane patches [24]. After that procedure, the MycHis–P2Y1 receptor (Figure 3A, lanes 1 and 2) and the GST–P2Y11 receptor (Figure 3B, lane 2) were still present in the extract. When we performed a pull-down experiment with glutathione Sepharose on these extracts, MycHis–P2Y1 receptor (Figure 3A, lane 4) was still present together with the GST–P2Y11 receptor (Figure 3B, lane 4). This confirmed the direct physical interaction of the two receptors.

Moreover, in the 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 the 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 the Materials and methods section. Negative control conditions were mimicked by co-expressing the fluorescent tags only. A representative spectrum of the negative control is shown in Figure 4(A) (dotted line) and the FRET ratio (n = 6) was calculated as described in the Materials and methods section. 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) (Figure 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 (Figure 4A, line with dash-dots). The emission maximum of the positive control correlated with the fluorescence spectrum of Citrine alone (results not shown). Confocal imaging of HEK293 cells co-expressing the Cerulean–P2Y1 and Citrine–P2Y11 receptor also showed an emission maximum at 526 nm (Figure 4A, line with dash-dots). The FRET ratio (n = 9) was smaller than the positive control but clearly increased when compared with the negative control (Figure 4B). This suggests a greater distance of the fluorophores in the case of the hetero-oligomerized receptors than in the 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 investigating 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
Figure 5. Confocal imaging of the internalization of GFP–P2Y₁, and GFP–P2Y₁₁ receptors in transfected 1321N1 cells

(A) Live 1321N1 cells expressing the GFP–P2Y₁ receptor were stimulated with 10 μM 2-MeS-ADP on a stage at 37°C (5% CO₂) and imaged with a LSM510 META laser scanning 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 GFP–P2Y₁ receptor were stimulated with 100 μM ATP and measured in the same way as GFP–P2Y₁ expressing cells. (C–E) Transiently co-transfected 1321N1 cells expressing the MycHis–P2Y₁ and the GFP–P2Y₁₁ receptor were fixed with 4% paraformaldehyde and stained with anti-Myc antibody as described in the Materials and methods section. (D) and (E) were stimulated with nucleotides for 60 min before fixation. Images display the GFP fluorescence of the MycHis–P2Y₁ receptor and the green fluorescence of the GFP–P2Y₁₁ receptor in intracellular compartments. Similar observations were made after stimulating the co-transfected cells with the P2Y₁ receptor agonist 2-MeS-ADP at 10 μM (Figure 5, E1–E3). Again, the red Myc-staining of the MycHis–P2Y₁ receptor and the green fluorescence of the GFP–P2Y₁₁ receptor co-localized in the same intracellular compartments (Figure 5, E3). This was an important finding, as Communi et al. [31] reported that 2-MeS-ADP has no activity on the P2Y₁₁ receptor. 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-oligomer.

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 GFP-tagged P2Y₁₁ receptor and observed receptor endocytosis by live imaging. Cells were stimulated on a stage and imaged using a Zeiss inverted LSM 510 META confocal microscope. Stimulation of the GFP–P2Y₁₁-transfected HEK293 cells with ATP clearly induced receptor internalization (Figure 6, A2–A3). This endocytosis was blocked by the specific P2Y₁ receptor antagonist MRS2179 (Figure 6, B2–B3). Endocytosis of the GFP–P2Y₁ receptor could also be induced by the P2Y₁₁ receptor-specific agonist 2-MeS-ADP (Figure 6, C2–C3), whereas the potent P2Y₁₁ receptor agonist BzATP was completely ineffective (Figure 6, D2–D3). 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 (Figure 6, E2–E3).

As 2-MeS-ADP was able to induce the endocytosis of the GFP–P2Y₁₁ 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 P2Y₁₁ receptor mutant at R268A (A268P2Y₁₁) has 100-fold less potency for ATP [16]. Therefore, we stably expressed the A268P2Y₁₁ receptor in HEK293 cells. For detection of receptor endocytosis, the GFP–A268P2Y₁₁-transfected HEK293 cells were stimulated on a stage with an ATP concentration (100 μM) that was unable to activate the mutant receptor, and cells were imaged by confocal microscopy. The results depicted in Figure 6 (F2–F3) demonstrate that during the total time period of imaging (60 min), the GFP-tagged A268P2Y₁₁ receptor did not show any sign of internalization and the receptor remained at the plasma.
2-MeS-ADP

BzATP

ATP + NF157

\( \text{A}268 \text{P}2\text{Y}_{11} \)-R: ATP

UTP

PAR2-AP

Figure 6  Effect of stimulation of HEK293 cells with various agonists on P2Y_{11} localization

HEK293 cells expressing GFP–P2Y_{11} were visualized with a LSM510 meta confocal microscope and stimulated with various agonists for up to 60 min at 37°C (5% CO_{2}) as described in the Materials and methods section. Pictures show unstimulated (US) cells (A–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) GFP–\( \text{A}_{268} \text{P}2\text{Y}_{11} \)–transfected HEK293 cells stimulated with 100 µM ATP (n = 3). (G) Stimulation with 100 µM UTP (n = 4). (H) Stimulation with 100 µM PAR-2 activating peptide (n = 3). Results shown are representative for n independent experiments analysing 20–30 cells in each case. Scale bar represents 5 µm.

membrane. The same was true when we stimulated the cells with 2-MeS-ADP (results not shown). Real time PCR showed that GFP–\( \text{A}_{268} \text{P}2\text{Y}_{11} \)–transfected HEK293 cells, mock transfected and GFP–P2Y_{11}–transfected HEK293 cells, all had comparable levels of P2Y_{1} receptor mRNA (see Supplementary material, http://www.BiochemJ.org/bj/409/bj4090107add.htm). Therefore, the lack of internalization was not due to a lower P2Y_{1} receptor level.

However, the GST–\( \text{A}_{268} \text{P}2\text{Y}_{11} \)–receptor expressed in HEK293 cells still displayed a physical association with the co-transfected MycHis–P2Y_{1} receptor, as analysed by pull-down experiments from cellular extracts. The results from the co-pull-down experiment are shown in Figure 3(A), lane 3. Therefore we concluded that the mutant \( \text{A}_{268} \text{P}2\text{Y}_{11} \) receptor, with greatly reduced agonist affinity, was unable to functionally interact with the P2Y_{1} receptor.

Interestingly, the induction of endocytosis of the wild-type GFP–P2Y_{11} receptor in HEK293 cells was specific for P2Y_{1} receptor agonists, because stimulation of the cells with UTP, which can activate the PAR-2 receptor without proteolysis [33,34], did not result in endocytosis of the GFP–P2Y_{11} 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 GFP–P2Y_{11} receptor (Figure 6, G2–G3). This both stimuli elicited a calcium response in the cells (results not shown) and real time PCR analysis confirmed the expression of the P2Y_{2} receptor and PAR-2 receptor in HEK293 cells transfected with GFP–P2Y_{11} (see Supplementary material).

To further validate the influence of the P2Y_{1} receptor in the process of P2Y_{11} receptor internalization, we attempted to knock-down P2Y_{1} receptor expression in HEK293 cells. Oligonucleotides selected against a specific region in the coding sequence of the receptor were applied to GFP–P2Y_{11}–transfected HEK293 cells using MA Tra reagent. Cells treated with the P2Y_{1} receptor siRNA showed approx. 50% knock-down in the mRNA level of the P2Y_{1} receptor (results not shown). This decrease in P2Y_{1} receptor expression was confirmed at the protein level as also approx. 50% (Figure 7, inset). Receiver expression remained unaffected when cells were treated with MA Tra reagent only or with control siRNA (results not shown). Treatment of the GFP–P2Y_{11}–transfected HEK293 cells with siRNA for the P2Y_{1} receptor induced a significantly diminished GFP–P2Y_{11} 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 (Figure 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 that 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 (Figure 7). This verifies the specificity of the interaction of P2Y1 and P2Y11 receptors in the receptor endocytosis of the GFP–P2Y11 receptor in HEK293 cells.

In conclusion, the phenomenon of co-internalization may be a very important functional consequence of P2Y hetero-oligomerization as it is known to control desensitization and resensitization of GPCRs [35]. Hetero-oligomerization among GPCRs can induce internalization of receptors which were previously considered unable to endocytose. The somatostatin receptor 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 P2Y11 and P2Y1 receptor. The P2Y11 receptor, when expressed alone, lacks any desensitization, as shown here and found previously by others [37]. This property of the P2Y11 receptor seems to be important for normal receptor function in the maturation process of DCs (dendritic cells) that express P2Y11 receptors. These antigen-presenting cells are trapped in the epicentre of inflammation due to the 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, as 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 GFP–P2Y11 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 GFP–P2Y11 receptor. There is evidence that formation of hetero-oligomers can result in a change in the pharmacology of GPCRs [39]. The A1–P2Y1 receptor heterodimer was found to have no significant affinity for MRS2179, whereas A1 receptor antagonists still bound. In contrast, the modified ligand binding pocket in the A1–P2Y1 heterodimer appears to bind the P2Y1 receptor agonist ADP$\beta$S, but slightly less than the A1 receptor ligands [12]. Therefore, we also investigated receptor ligand selectivities by measuring the intracellular calcium rise in HEK293 cells expressing the GFP–P2Y11 receptor. As described in the Materials and methods section, cells were subjected to single-cell calcium measurements using the calcium indicator fura-2/AM.

GFP–P2Y11 receptor-expressing HEK293 cells responded to the potent P2Y11 receptor agonist BzATP, but the recently developed highly potent P2Y1 receptor antagonist NF157 [17] was not able to inhibit this response (Figure 8A). However, when HEK293 cells expressing GFP–P2Y1 were preincubated with the specific P2Y1 receptor antagonist MRS2179, surprisingly the response to BzATP was inhibited (Figure 8A). This was not the case when the GFP–P2Y11 receptor was expressed alone in 1321N1 cells. Then MRS2179 showed no effect on the activity of BzATP, but preincubation with NF157 totally abolished the activity of BzATP on the P2Y11 receptor (Figure 8C). MRS2179 was able to interfere with the BzATP-stimulated [Ca$^{2+}$]i rise and the ATP-induced internalization of the GFP–P2Y11 receptor, whereas NF157 was not able to inhibit any of these effects. Thus the interaction of the GFP–P2Y11 receptor with the endogenous P2Y1 receptor in HEK293 cells apparently influences the ligand...
selectivity of the receptor, as measured by the induction of a [Ca\(^{2+}\)]\(_i\) rise.

BzATP normally shows no activity at the P2Y\(_1\) receptor [40]. Therefore, it can be concluded that BzATP and MRS2179 both bind to the heterodimer 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 [Ca\(^{2+}\)]\(_i\) increase in HEK293 cells expressing GFP–P2Y\(_1\) 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 GFP–A268P2Y\(_{11}\) receptor did not interact functionally with the endogenous P2Y\(_1\) receptor in HEK293 cells with regard to internalization. However, there was a physical interaction (Figure 3). Therefore, we examined whether the ligand selectivity of the P2Y\(_{11}\) receptor mutant was influenced by the interaction with the P2Y\(_1\) receptor. We measured the intracellular calcium rise in HEK293 cells expressing GFP–A268P2Y\(_{11}\). The cells responded to 100 \(\mu\)M ATP in the same way as mock-transfected cells, confirming the decreased potency of ATP at the P2Y\(_{11}\) receptor mutant (Figure 8B). However, the more potent P2Y\(_{11}\) receptor agonist BzATP elicited a significant response at a concentration of 100 \(\mu\)M with the R268A mutant (Figure 8A). Pretreatment of the HEK293 cells expressing GFP–A268P2Y\(_{11}\) 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 P2Y\(_1–P2Y_{11}\) receptor heterodimer our findings suggest that it is formed as a ‘domain swapped’ dimer because the change in the pharmacological profile of the P2Y\(_1\) receptor ligands implies the creation of a newly formed binding pocket. In agreement with this suggestion is our finding that UDP induced a drastic [Ca\(^{2+}\)]\(_i\) increase in HEK293 cells expressing GFP–P2Y\(_{11}\) (results not shown). UDP is known to activate the P2Y\(_{11}\) receptor [44], but this receptor was not expressed in our HEK293 cells. Interestingly, previous studies have reported that uridine nucleotides can activate the P2Y\(_{11}\) receptor [44]. However, with the P2Y\(_{11}\) 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 P2Y\(_1\) receptor.

CONCLUSION

The present study provides convincing evidence for a physical interaction between the P2Y\(_1\) and the P2Y\(_{11}\) receptors to form hetero-oligomers. This hetero-oligomerization enables the agonist-induced internalization of the P2Y\(_{11}\) receptor. The P2Y\(_1\) receptor expressed alone is unable to endocytose and lacks desensitization. This property of the P2Y\(_1\) receptor seems to be important for normal P2Y\(_{11}\) receptor function. We hypothesize that the presence of P2Y\(_1\) receptors in DCs isolated from human blood enables the desensitization of the P2Y\(_{11}\) 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 P2Y\(_{11}\) receptor leading to the alteration of its behaviour.

We have previously shown that A268P2Y\(_{11}\) shows a 1000-fold reduced potency for ATP [16]. The GFP–A268P2Y\(_{11}\), receptor mutant did not interact functionally with the endogenous P2Y\(_1\) receptor in HEK293 cells, since it did not co-internalize with the activated P2Y\(_1\) receptor. Nevertheless, we found physical interaction between the A268P2Y\(_{11}\) receptor and the P2Y\(_1\) receptor by co-pull-down.

In addition, we also found that the association of P2Y\(_1\) with the P2Y\(_{11}\) receptor influences the ligand selectivity of the P2Y\(_{11}\) receptor. Therefore we suggest that some discrepancies reported in the literature concerning the responsiveness of the P2Y\(_{11}\) receptor to certain nucleotides could be due to a varying degree of co-expression of the P2Y\(_1\) 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 signalling elements in specific microdomains. This clearly affects cellular communication processes [45]. The homo- or heterodimerization of purinergic GPCRs thereby allows fine tuning of the responses of cells to extracellular nucleotides.

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REFERENCES


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