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Constitutive interaction of the $P2Y_2$ receptor with the hematopoietic cell-specific G protein $G_{\alpha16}$ and evidence for receptor oligomers☆

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Abstract

Hematopoietic cells uniquely express $G_{\alpha16}$, a G protein α-subunit of the G_q-type. $G_{\alpha16}$ is obligatory for $P2Y_2$ receptor-dependent Ca^{2+}-mobilization in human erythroleukemia cells and induces hematopoietic cell differentiation. We tested whether $P2Y_2$ receptors physically interact with $G_{\alpha16}$. Receptor and G protein were fused to cyan (CFP) and yellow (YFP) variants of the green fluorescent protein (GFP), respectively. When expressed in K562 leukemia cells, the fusion proteins were capable of triggering a Ca^{2+}-signal upon receptor stimulation, demonstrating their functional integrity. In fluorescence resonance energy transfer (FRET) measurements using confocal microscopy, a strong FRET signal from the plasma membrane region of fixed, resting cells was detected when the receptor was co-expressed with the G protein as the FRET acceptor, as well as when the CFP-tagged receptor was co-expressed with receptor fused to YFP. We conclude that, under resting conditions, $G_{\alpha16}$ and $P2Y_2$ receptors form constitutive complexes, and that the $P2Y_2$ receptor is present as an oligomer.
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Keywords: G protein-coupled receptor; $P2Y_2$ receptor; G protein; $G_{\alpha16}$; Protein–protein interaction; Oligomerization; Fluorescence resonance energy transfer (FRET); Hematopoietic cells

1. Introduction

G protein-coupled receptors (GPCRs) signal by activating G proteins, which in turn stimulate downstream effector systems. Activation of the G_q family of G proteins results in upregulation of phospholipase C as a primary consequence and then to two diverging signalling branches that proceed via Ca^{2+}-mobilization and DAG production. Recent publications suggest that GPCRs may initiate additional, G protein independent signalling pathways. Among these are the transactivation of growth factor receptor tyrosine kinases, and association with src-familiy tyrosine kinases [1], as well as the activation of small G proteins [2,3], possibly by direct interaction via guanine nucleotide exchange factors. Hence, GPCR signalling appears to involve more molecular pathways than previously anticipated [4]. Finally, many GPCRs are now recognized to be able of forming homo- or hetero-oligomeric assemblies. In view of the increasingly appreciated complexity of GPCR signalling, detailed analyses of protein–protein interactions between GPCRs and G proteins are becoming highly relevant. However, only in a few instances, the physical relationship between the two signalling partners has been examined by direct methods. Novel methods such as fluorescence resonance energy transfer (FRET) make it now possible to directly measure

Abbreviations: CFP, GFP, YFP, cyan, green, yellow fluorescent protein, respectively; CRFR1, corticotropin releasing factor receptor 1; fluo-3/AM, fluo-3 acetoxymethylester; FRET, fluorescence (Förster) resonance energy transfer; GPCR, G protein-coupled receptor; HEK cells, human embryonic kidney cells; HEL cells, human erythroleukemia cells; ROI, region of interest.

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physical proximity and to detect potential changes during the activation cycle [5].

Hematopoietic cells specifically express $G_{\alpha16}$, a G protein of the G_q-type that is not expressed in other cell types. $G_{\alpha16}$ appears to play a rather distinct role because most haematopoietic cell lineages terminate the expression of this unique G protein in later stages of differentiation [6,7]. The ability to regulate its expression seems to be indispensable for acquisition of functional competence [8]. In a previous study, we demonstrated that $G_{\alpha16}$ promoted erythroid differentiation when expressed in the leukemia cell line, MB-02 [9]. In human erythroleukemia (HEL), cells blocking $G_{\alpha16}$ expression results in a complete loss of $P2Y_2$ receptor-dependent Ca^{2+}-mobilization [10]. This is not due to a lack of $G_{\alpha q}$ in these cells but rather is a consequence of an interruption of selective functional coupling of the $P2Y_2R$ to $G_{\alpha16}$, while G_i-dependent signalling of the receptor is not affected. The selectivity in $P2Y_2R$-$G_{\alpha16}$ signalling in HEL cells seems to contradict the propensity of $G_{\alpha16}$ to interact with a large number of structurally diverse GPCRs—exploited in functional assays to screen for potential ligands of orphan receptors [11–13]. However, results from gene knockout studies indicate that promiscuous coupling is not limited to the hematopoietic cell specific $G_{\alpha16}$ but also occurs with other G proteins of the G_q-class [14]. Specificity of receptor-G protein coupling may therefore be rather relative than absolute, depending on the cellular context and on the expression level of the various G proteins.

The $P2Y_2$ receptor, initially termed P_{2U} receptor for its feature of being potently activated by UTP in addition to ATP, is detectable in most tissues [15]. Extracellular UTP appears to be the most important natural ligand [16]. P2Y metabotropic nucleotide receptors assume various physiological roles in non-hematopoietic cells comprising developmental processes [17], tissue homeostasis [18,19] and secretory processes. The latter currently represent the clinically most important aspects of $P2Y_2$ receptor signalling, due to the receptor's role in the therapy of cystic fibrosis [20]. Future applications of $P2Y_2$ receptor pharmacology are likely to include the therapy of dry eye disease [21], and potentially also of cancer, because activation of $P2Y_2$ receptors in some cancer forms results in inhibition of cell proliferation [22,23].

Evidence for a role of $P2Y_2$ receptors in hematopoietic cells is growing rapidly. Within the P2Y subfamily, the $P2Y_2$ receptor is unique in that it is the most highly expressed member that is found in both, lymphocytes and monocytes, and shows very high expression in $CD34^+$ stem cells [24,25]. In monocytes, the receptor is functionally involved in the activation of Ca^{2+}-dependent and -independent pathways such as the activation of ERK1/2 [26]. Recently, UTP was discovered to potently stimulate growth factor action in $CD34^+$ human hematopoietic stem cells as well as in $CD34^-$ progenitor cells [27]. The potentiating effect could be attributed to the presence of $P2Y_1$ and $P2Y_2$ receptors on these cells.

The pharmacology and the distal effector systems of the $P2Y_2$ receptor are understood in fair detail for hematopoietic as well as non-hematopoietic cells [26,28,29]. The $P2Y_2$ receptor appears to engage the G_q- and G_i-types of G proteins for signal propagation. However, the role of the potential interaction of the $P2Y_2$ receptor with the hematopoietic cell-specific $G_{\alpha16}$ in growth and differentiation has not been examined so far. Since overexpression of wild-type and—even more pronounced—of activated $G_{\alpha16}$ may induce differentiation in leukemic cells [9], a more detailed understanding of the interaction between the $P2Y_2$ receptor and $G_{\alpha16}$ is of importance, because the $P2Y_2R$-$G_{\alpha16}$ axis presents a potential signalling system that could limit uncontrolled growth of hematopoietic tumor cells.

Here, we sought to demonstrate a physical and constitutive interaction of $G_{\alpha16}$ with $P2Y_2$ receptors using FRET-based measurements in non-hematopoietic (HEK 293) and hematopoietic (K562) cells. Ca^{2+}-measurements in live cells first showed that the fluorescent fusion proteins used in the FRET study had retained their functional integrity. Confocal microscopy of single, fixed cells was then used to examine the spatial relationship between these two proteins in the physiologically relevant region of the plasma membrane. We demonstrated a constitutive, physical interaction between the $P2Y_2$ receptor and $G_{\alpha16}$ in resting cells, and analyzed the specificity of the interaction. In both, non-hematopoietic and hematopoietic cells, the $P2Y_2$ receptor appeared to form homo-oligomers in the absence of $G_{\alpha16}$. Our data provide the basis for a more systematic examination of constitutive G protein–GPCR interactions and for screening systems that are based on FRET.

2. Experimental procedures

2.1. Cell culture media and reagents

Tissue culture media and media supplements were purchased from Life Technologies (Basel, Switzerland). DOTAP, ATP and UTP were from Boehringer Mannheim (Mannheim, Germany); G418 sulfate was from Promega (Catalys, Wallisellen, Switzerland). Fura-2/AM and fluo-3/AM were from Molecular Probes (Juro, Supplies, Lucerne, Switzerland). If not otherwise stated, all other reagents were purchased from Sigma, Fluka (Buchs, Switzerland) or Merck (Dietikon, Switzerland).

2.2. Cell culture

K562 cells, a cell line derived from an acute-phase chronic myeloid leukemia patient [30], were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI based medium as previously described [31]. MB-02 cells were maintained in RPMI/HS medium, which was composed of RPMI 1640, 5 ng/ml GM-CSF (Werthenstein-Chemie, Schachen, Switzerland), 2 mM

Glutamax 1, 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin, and 10% human serum which was prepared as described previously [9]. HEK 293 cells were maintained in DMEM medium [DMEM, 10% fetal bovine serum, 2 mM Glutamax 1 (Life Technologies), 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin].

2.3. Plasmids

A cDNA encoding the human $P2Y_2$ purinoceptor [15] was a kind gift from Dr. S. Sromek [32]. Using standard PCR cloning strategy, the entire $P2Y_2$ receptor coding region was linked at its 5′ end to the *Eco*RI and at its 3′ end to the *Bam*H1 site of EGFP-N1 (Clontech, Palo Alto, CA, USA), resulting in the expression plasmid denoted as $P2Y_2$R-GFP. The nucleotide sequence between the 3′ end of the $P2Y_2$ receptor coding region and the 5′ end of EGFP encoded a linker peptide with the sequence Ala-Pro-Ala-Pro-Pro-Val-Ala-Thr, which does not contain known structural motifs according to the ProSite database (Version 18.34). From EYFP-N1 and ECFP-N1, the EYFP and ECFP genes were retrieved using the flanking *Bam*HI and *Not*I sites and then substituted for the EGFP gene of $P2Y_2$R-GFP, resulting in the plasmids encoding the $P2Y_2$-receptor tagged with EYFP ($P2Y_2$R-YFP) and ECFP ($P2Y_2$R-CFP), respectively. The $G_{\alpha 16}$ cDNA [33] was retrieved from the Baculovirus transfer vector pVL1393 and inserted into the unique *Xba*I site of the pEYFP-C3 and pECFP-C3 multiple cloning sites. The extra nucleotide sequences originating from the multiple cloning sites encode the linker peptide (Tyr-Ser-Asp-Leu-Glu-Leu-Lys-Leu-Arg-Ile-Leu-Gln-Ser-Thr-Val-Pro-Arg-Ala-Arg-Asp-Pro-Pro-Asp-Leu-Asp-Arg-Thr), which contains no known structural motifs according to the ProSite database (version 18.34) except for a potential tyrosine sulfation site at position 1 and a casein kinase 2 phosphorylation site at position 2 (both representing motifs with a high probability of occurrence). $G_{\alpha 16}$ fusions with variants of green fluorescent protein (GFP) were transiently expressed in HEK 293 cells. Lysates of such transfected cells were analyzed by Western blotting with anti-GFP antibody, and showed bands of approximately 70 kDa, which is consistent with the predicted size (71 kDa) of the fusion proteins (not shown). The construct CRFR1-CFP-YFP, denoting the corticotropin releasing factor-receptor 1 bearing a concatemer of ECFP and EYFP at its carboxy-terminus, was a kind gift from Oliver Krätke, Research Institute of Molecular Pharmacology (FMP), Berlin, Germany. The plasmid β1AR-GFP, encoding a fusion of the β1-adrenergic receptor with EGFP at its carboxy-terminus, was described earlier [34].

2.4. Stable transfection of MB-02 cells

MB-02 cells (2×10^7 cells at a density of 1×10^6 cells/ml) were transfected in Opti-MEM (Life Technologies) with 30 μg of the plasmid $P2Y_2$R-GFP, using DOTAP as transfection reagent as described previously [10]. Cells were seeded into two 96-well dishes and selected for G418 resistance (400 μg/ml). Twenty-five days after transfection, 6 wells contained viable, G418 sulfate resistant clones, which showed continuous proliferation and were expanded. Two clonal cell lines (denoted as clones A and B) were finally selected and used in the experiments.

2.5. Transient transfections

K562 cells were transfected using Geneporter 1, according to manufacturer's protocol (Gene Therapy Systems, Axon Lab AG, Baden-Dättwil, Switzerland). HEK 293 cells were transfected using the Ca^{2+}-phosphate co-precipitation protocol [35].

2.6. Ratiometric measurements of intracellular free $[Ca^{2+}]$

Cells were loaded with fura-2/AM (9 μM) as described previously [10]. Ca^{2+}-changes were recorded with a Perkin Elmer LS50 spectrofluorimeter that was equipped with an excitation filter wheel for fast ratiometric measurements. Intensity ratios were converted to intracellular Ca^{2+}-concentrations using the instrument's FL data manager software (version 3.5). Conversions were based on an apparent K_d of 224 nM for the fura-2·Ca^{2+} complex [36].

2.7. FRET measurements

HEK cells ($6000/cm^2$) were grown on cover slips that were placed in 12-well multiwell dishes. Cells were then transfected with the plasmids as indicated in the figures. Total amount of DNA per transfection was kept constant (1.2 μg), as well as the absolute amounts of the CFP (0.4 μg) and the YFP (0.8 μg) constructs; if necessary, vector DNA without insert was added. Forty-eight hours later, cells were fixed in ice cold 4% paraformaldehyde for 20 min. After washing the cover slides twice with PBS, they were mounted on microscopic glass slides using SlowFade mounting solution (Molecular Probes). For FRET measurements a confocal LSM510 instrument equipped with an inverted Axiovert 100M microscope (Carl Zeiss) was used. Individual cells were examined with a 63×, 1.4 N.A. Zeiss oil immersion objective. Twelve-bit images of 512×512 pixels were recorded, representing 58.7 μm in each dimension. The CFP-linked fusion protein (donor) was excited at 458 nm and fluorescence emission was detected using a 470–490-nm bandpass filter. The YFP-linked fusion protein (acceptor) was excited at 514 nm and fluorescence emission was detected using a 560-nm longpass filter. For FRET measurements in single cells, an acceptor photobleaching method was used, recording donor fluorescence before and after bleaching of YFP as the potential acceptor [37]. In each cell, a single region of interest (ROI 1) encompassing a well-defined membrane

region was bleached using the 514 laser line at maximum intensity. The time for bleaching ranged between 15 and 30 s depending on the size of ROI 1. Two images each were collected before and after the bleaching step to control for stability of the fluorescence signal. The change in FRET efficiency as the percentage of total donor fluorescence in the absence of the acceptor (Ef) was calculated using the formula: $Ef=(I_3-I_2)/I_3\times100$, where I_n is the donor intensity in ROI 1 of the nth image. Similar calculations were performed in a non-bleached region (ROI 2) of the image to obtain the parameter $Cf=(I_3-I_2)/I_3\times100$. Cf represents an internal control value that under ideal conditions should be zero.

2.8. Immobilization of non-adherent cells to glass cover slides

K562 cells were immobilized on glass cover slides for live cell microscopy as follows: cover slides were precoated with an antibody to β_2-microglobulin (Serotec, Düsseldorf, Germany) at 10 μg/ml in PBS for 30 min. Excess solution was removed, the slides were washed with PBS and then dried. Transiently transfected K562 cells were resuspended in RPMI medium without serum, seeded at a density of 25,000 cells/cm^2 onto the coated cover slides that were attached to a plexiglass incubation chamber, and then incubated for 30 min at room temperature. Finally, cover slides were rinsed with fresh medium to remove non-adhering cells and immersed in PBS supplemented with glucose (20 mM) as required for subsequent experiments.

2.9. Single cell Ca^{2+}-measurements

For Ca^{2+}-measurements cells were incubated for 30 min with the Ca^{2+}-indicator fluo-3/AM (10 μM) during the immobilization procedure (see above). After 30 min, the supernatant containing excess fluo-3/AM and non-adhering cells was removed and PBS supplemented with glucose (20 mM) was added. For Ca^{2+}-measurements, cells were placed in a thermostatted (37 °C) chamber on the LSM510 microscope. In order to identify transfected cells, the CFP-linked fusion proteins were excited at 458 nm and fluorescence emission was detected using a 470–490-nm bandpass filter. Fluo-3·Ca^{2+} was excited at 514 nm and fluorescence emission was detected using a 560-nm long-pass filter. Images were collected with a 40×, 1.3 N.A. Zeiss oil immersion objective. Single plane eight-bit images of 256×256 pixels representing 325.7 μm in each dimension were recorded at intervals of maximally 6 s. After starting the recording process, an equal volume of medium supplemented with the agonist was added, yielding a final concentration of agonist as indicated in the figures. From the resulting image series, signal intensities were calculated using the LSM510's built-in quantification software (Zeiss 510, version 2.5). Each transfected cell (identified by CFP fluorescence) in the image was analyzed by manually defining a spherical region of interest (ROI) that enclosed the entire cell.

2.10. Data analysis

Statistical analyses were performed using the software package GraphPad Prism, version 4.0c (GraphPad Software, San Diego, CA). Error values refer to the S.E.

3. Results and discussion

FRET analysis of the receptor–G protein interaction required fusion of both proteins to cyan and yellow variants of GFP. In a first series of experiments, the fluorescently tagged versions of $P2Y_2R$ and $G_{\alpha16}$ were tested for functional integrity. In a second series, constitutive protein–protein interactions between the tagged proteins were analyzed using confocal microscopy and FRET measurements of single, fixed cells.

3.1. The $P2Y_2$ receptor retains its function when fused to green fluorescent protein

As a reference system for the functional properties of overexpressed the $P2Y_2$ receptor, we first created a stable cell line expressing the $P2Y_2R$ that did not bear a florescent tag. To that end the leukemia cell line MB-02 was stably transfected with the $P2Y_2R$. MB-02 cells express endogenous $G_{\alpha16}$ but not $P2Y_2R$ [9]. The stably transfected cell line (PB-10) showed large changes in intracellular Ca^{2+}-concentrations when stimulated with UTP, while parental cells (MB-02) did not respond (Fig. 1A). From dose–response analyses of PB-10 cells, an EC_{50} value of 0.2 μM was estimated (Fig. 1B). The EC_{50} was lower than for UTP-induced Ca^{2+}-changes observed previously in human HEL cells (EC_{50}=3 μM and Ref. [10]), apparently a consequence of receptor overexpression as opposed to the lower level of endogenously expressed $P2Y_2Rs$ in HEL cells.

Next, the $P2Y_2R$ was tagged at its carboxy-terminus with GFP, or with the GFP variants, CFP and YFP. In Western blots of HEK cells which transiently expressed fluorescently tagged $P2Y_2Rs$, a distinctive band of ~70 kDa was detected that was not present in HEK cells expressing vector alone, and indicated expression of the fusion constructs with a predicted molecular mass of 71 kDa (Fig. 1C). A second, diffuse band of 80–100 kDa most likely represented glycosylated forms of the tagged receptor proteins. Representative of all three fluorescently tagged versions of the $P2Y_2R$, the $P2Y_2R$-GFP was stably transfected into the MB-02 cells and tested for its ability to stimulate a Ca^{2+}-change. Two clonal cell lines were isolated and characterized further. The EC_{50} values from UTP dose–response measurements for clones A and B were 1.2 and 0.4 μM, respectively (Fig. 1E,F). These values were similar to those obtained in PB-10 cells (0.2 μM) and suggested that the GFP-tagged receptors

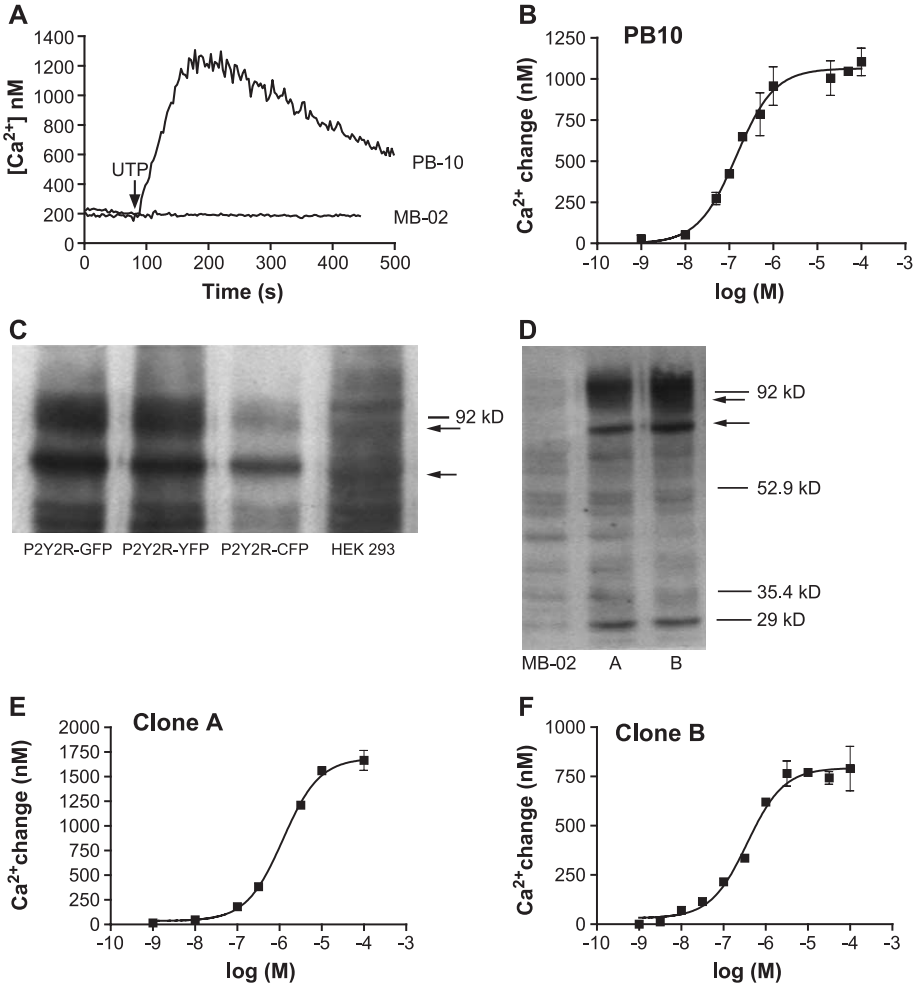

Fig. 1. Functional expression of $P2Y_2R$ fused to fluorescent proteins. (A) Ratiometric Ca^{2+}-measurements with stably transfected MB-02 cells that express the $P2Y_2R$ (PB-10), or with the parental cell line (MB-02) were performed using fura-2/AM as the Ca^{2+}-indicator. Representative traces of Ca^{2+}-changes in transfected and parental cells that were challenged with UTP (100 μM) are shown. (B) Dose–response relationship of peak Ca^{2+}-changes in PB-10 cells that were challenged with UTP at concentrations as indicated in the figure. (C) Lysates of transiently transfected HEK 293 cells expressing $P2Y_2R$ fused to GFP or its yellow and cyan variants ($P2Y_2R$-YFP or $P2Y_2R$-CFP, respectively) showed a ~70-kDa band as predicted (71 kDa, lower arrow). A second specific, but more diffuse band of ~90 kDa in each of the transfectants appears to correspond to glycosylated forms of tagged receptors (upper arrow). (D) Cellular lysates of two stably transfected MB-02 cell lines that express the $P2Y_2R$ fused to GFP (clones A and B) were separated by SDS gel electrophoresis and immunoblotted with anti-GFP antibody. The bands of ~70 (lower arrow) and ~90 kDa (upper arrow) correspond to the nonglycosylated and glycosylated forms of receptors fused to GFP, respectively. (E, F) Clones A and B were analyzed for their Ca^{2+}-responses upon stimulation with UTP using fura-2 ratiometric measurements as in panel B.

were capable of signalling through endogenously expressed G proteins, such as $G_{\alpha16}$. Western blots from cellular lysates of clones A and B showed bands of ~70 and 80–100 kDa, which is consistent with the overexpression of the GFP-tagged receptor (Fig. 1D).

3.2. Fluorescently tagged fusions of the $P2Y_2$ receptor and $G_{\alpha16}$ proteins are capable of productive functional interactions

We then examined whether the $P2Y_2R$-CFP was capable of coupling to $G_{\alpha16}$ that was fluorescently labeled at its amino-terminus using the fluorescent Ca^{2+}-indicator fluo-3/AM. We hypothesized that if both signalling components were functionally intact, co-expression of $P2Y_2R$-CFP with fluorescently tagged $G_{\alpha16}$ should lead to an increase in agonist-dependent Ca^{2+}-signalling when compared to cells that express the receptor alone. To optimally fit the experimental conditions for such experiments, the leukemia cell line K562 was used. K562 cells do not endogenously express $G_{\alpha16}$ [6,10] and also lack $P2Y_2$ receptors [24], a prerequisite that allowed to attribute the expected signals to the transfected constituents. K562 cells were transiently transfected with $P2Y_2R$-CFP in the presence or absence of tagged $G_{\alpha16}$. Since excitation and emission spectra of GFP or YFP would have overlapped with the emission spectrum of fluo-3 · Ca^{2+}, $G_{\alpha16}$-CFP was used in these experiments. Fluo-3 · Ca^{2+} fluorescence was then followed by recording time-series of images on a confocal microscope after agonist stimulation of the receptor.

As an initial assessment of the reliability of the method, individual, non-transfected K562 cells were examined for their responses to U46619, a thromboxane A_2 (TxA_2) receptor agonist acting through the endogenous TxA_2 receptor, one of the few endogenous receptors in these cells that prompt a Ca^{2+}-signal. Indeed, strong, transient Ca^{2+}-signals were observed, showing complete restoration to levels measured prior to stimulation (Fig. 2A). UTP did not elicit a Ca^{2+}-response in $G_{\alpha16}$-transfected cells, while a subsequent challenge with U46619 proved that the cells were properly loaded and responsive, in principle (Fig. 2B). To measure Ca^{2+}-signals in transfected cells, individual, cyan fluorescent cells (expressing CFP labeled receptor and G protein) were selected for analysis. Following transfection with $P2Y_2R$-CFP, or with $P2Y_2R$-CFP and $G_{\alpha16}$-CFP, single cells consistently responded to UTP with transient Ca^{2+}-changes (Fig. 2C and D, respectively). On the average, cells transfected with the $P2Y_2R$ and stimulated with UTP showed a considerable, but smaller Ca^{2+}-response than cells that were stimulated with U46619 (Fig. 3). Non-transfected

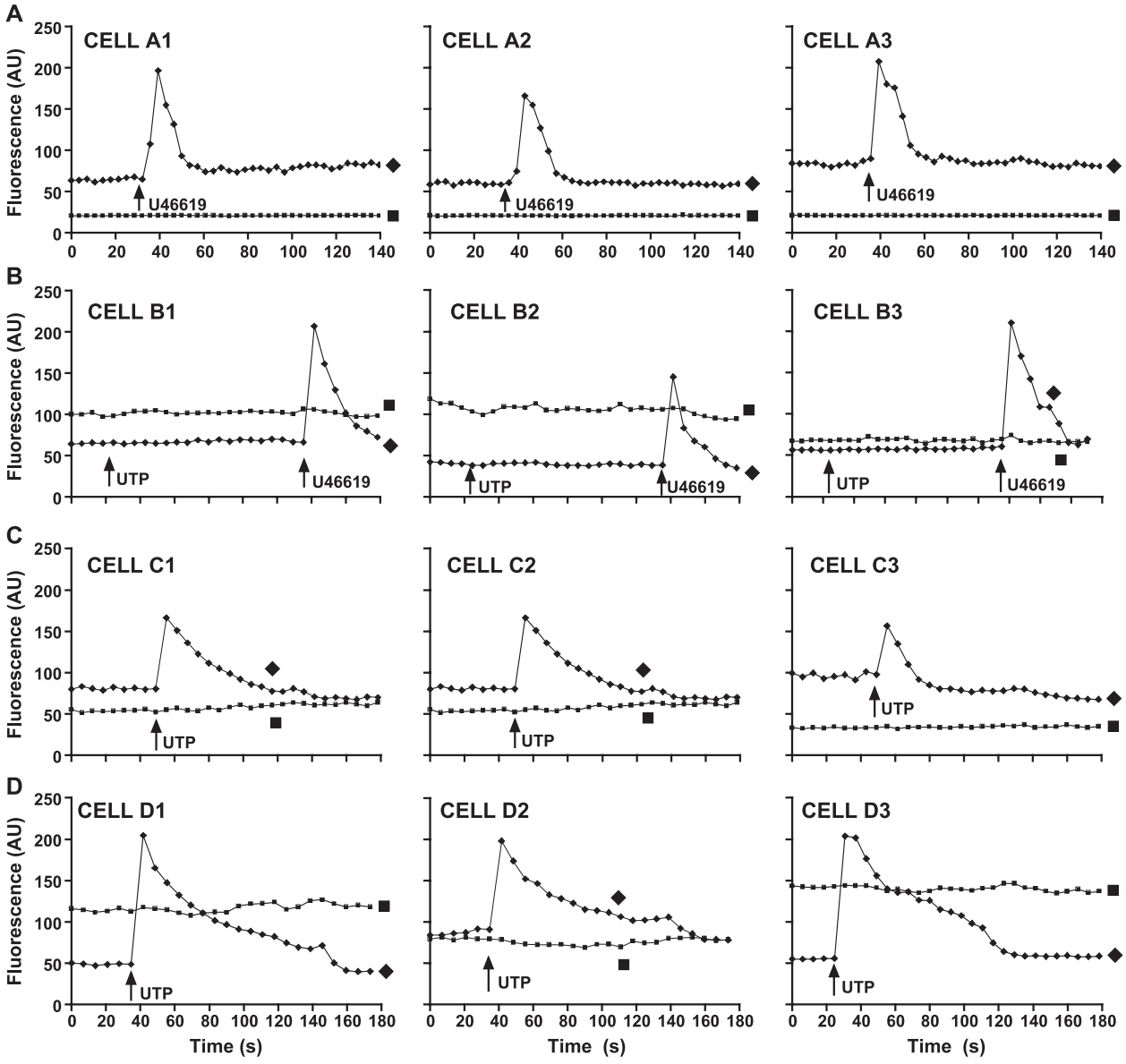

Fig. 2. Single cell Ca^{2+}-measurements by confocal microscopy in immobilized K562 cells. Immobilized K562 cells were loaded with the Ca^{2+}-indicator fluo-3/AM and analyzed using confocal microscopy as described under Section 2. Ca^{2+}-measurements of three individual cells per condition are presented in the figure. Individual traces show fluorescence intensities in the fluo-3 channel (♦), or in the CFP channel (■). (A) K562 cells that were not transfected were stimulated with the TxA_2 receptor agonist U46619 (10 μM). (B) Cells were transiently transfected with $G_{\alpha16}$-CFP and stimulated sequentially with UTP (100μM) and U46619 (10 μM), as indicated in the figure. (C) Cells transfected with the $P2Y_2R$-CFP were stimulated with UTP. (D) Cells co-transfected with $P2Y_2R$-CFP and $G_{\alpha16}$-CFP were stimulated with UTP. From such recordings Ca^{2+}-changes were determined as the difference between the baseline prior to the challenge and the peak value. The arbitrary units representing the mean grey value of a circular mask covering the entire cell were used for aggregate calculations such as in Fig. 3.

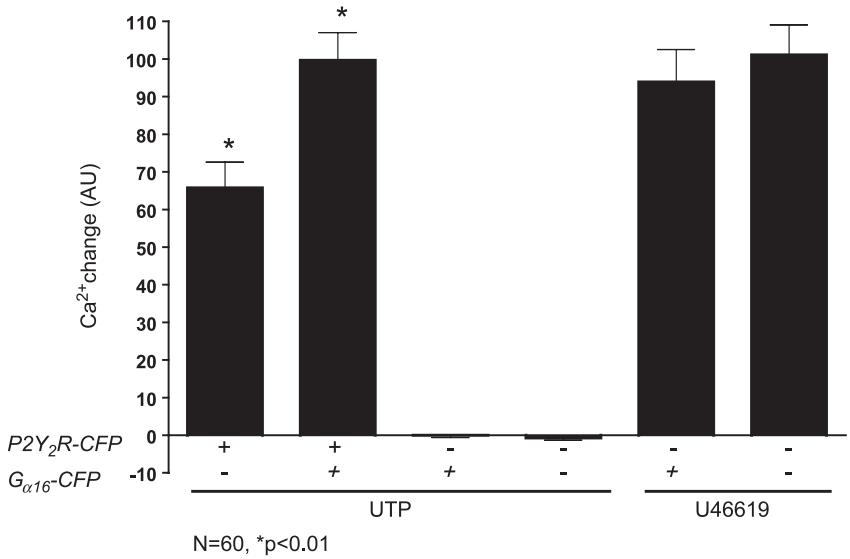

Fig. 3. Functional integrity of fluorescently tagged $P2Y_2R$ and $G_{\alpha16}$ as detected by single cell Ca^{2+} measurements. Ca^{2+}-changes in transfected K562 cells were estimated from single cell recordings using fluo-3/AM as the Ca^{2+} indicator (see also Fig. 2). Cells were stimulated with UTP (100 μM) or with the TxA_2 receptor agonist, U46619 (10 μM). Individual transfected cells (positive for CFP fluorescence) were selected and fluo-3 fluorescence was followed. The figure shows average fluorescence intensities given as arbitrary units (grey values of the image) of 60 cells that were analyzed per condition. One of three experiments with similar results is shown. The difference between the two columns denoted by asterisks is statistically highly significant ($p<0.01$).

cells or cells that were transfected with $G_{\alpha16}$-CFP alone failed to show a response. Apparently, the $P2Y_2R$-CFP was able to productively couple to an endogenous G protein, while no endogenous UTP receptor was available that could trigger a Ca^{2+}-response. However, co-expression of $G_{\alpha16}$-CFP along with the $P2Y_2R$-CFP significantly enhanced the UTP-induced Ca^{2+}-transients by 1.54±0.10-fold when compared to cells that expressed $P2Y_2R$-CFP alone (Fig. 3). The increase in signal strength was not due to an overall improvement of Ca^{2}-signalling in $G_{\alpha16}$-CFP expressing cells, because TxA_2 receptor activation remained unchanged by the overexpression of the G protein. The data demonstrate that fluorescently tagged versions of the $P2Y_2R$ and $G_{\alpha16}$ proteins are functionally intact entities and are capable of transmitting a Ca^{2+}-signal upon agonist-induced receptor stimulation.

3.3. Constitutive, physical interaction between receptor and G protein in resting cells as detected by FRET

The above experiments showed that fluorescently tagged $P2Y_2R$ and $G_{\alpha16}$ functionally interact upon agonist binding. Using the fusion constructs $P2Y_2R$-CFP and $G_{\alpha16}$-YFP a series of experiments were performed to probe for molecular proximity of the $P2Y_2R$ with $G_{\alpha16}$ under resting conditions, i.e. in the absence of agonist-induced receptor stimulation. A FRET method in combination with confocal microscopy and exhaustive photobleaching of the fluorescence resonance acceptor (YFP) in specific regions of interest (ROIs) was applied.

The FRET-method was first established using fixed HEK cells that transiently expressed the control construct CRFR1-CFP-YFP, denoting the corticotropin releasing factor-receptor 1 bearing CFP and YFP entities in a concatenated configuration at its carboxy-terminus. Using CRFR1-CFP-YFP, high ratios of FRET in clearly dissectable membrane regions were observed in regions that were bleached (ROI 1), while in non-bleached regions (ROI 2) the donor signal remained unchanged (Fig. 4A). On the average, FRET ratios in photobleached regions (Ef) reached more than 30% of the unquenched donor fluorescence (Fig. 5A). When HEK cells were co-transfected with $P2Y_2R$-CFP and $G_{\alpha16}$-YFP, the FRET signal amounted to 13% (Figs. 4B and 5A), which represented approximately 40% of the maximum signal that was observed with the CRFR1-CFP-YFP control construct. To exclude that any endogenous component other than the transfected YFP fusion protein could have served as a photobleachable acceptor cells were transfected with $P2Y_2R$-CFP in the absence of $G_{\alpha16}$-YFP. No increase in CFP fluorescence was detected after the photobleaching cycle, indicating that indeed no other acceptor beside $G_{\alpha16}$-YFP was present in the fixed cells (Fig. 5B). From these results, we conclude that the FRET signal observed in cells co-expressing $P2Y_2R$-CFP and $G_{\alpha16}$-YFP arose from the simultaneous presence of the tagged proteins and that the tagged $P2Y_2R$ and $G_{\alpha16}$, therefore, must be in molecular proximity to each other.

HEK 293 cells are optimized for high level transgene expression under the control of the cytomegalovirus promoter of the pcDNA3 vector, which was also used in our experiments. Physical proximity of the two fusion proteins might therefore have resulted from high level protein expression and random associations. Moreover, $G_{\alpha16}$ is exclusively expressed in hematopoietic cells [6]. For these two reasons, the potential for interaction was also examined in the hematopoietic cell line K562. In K562

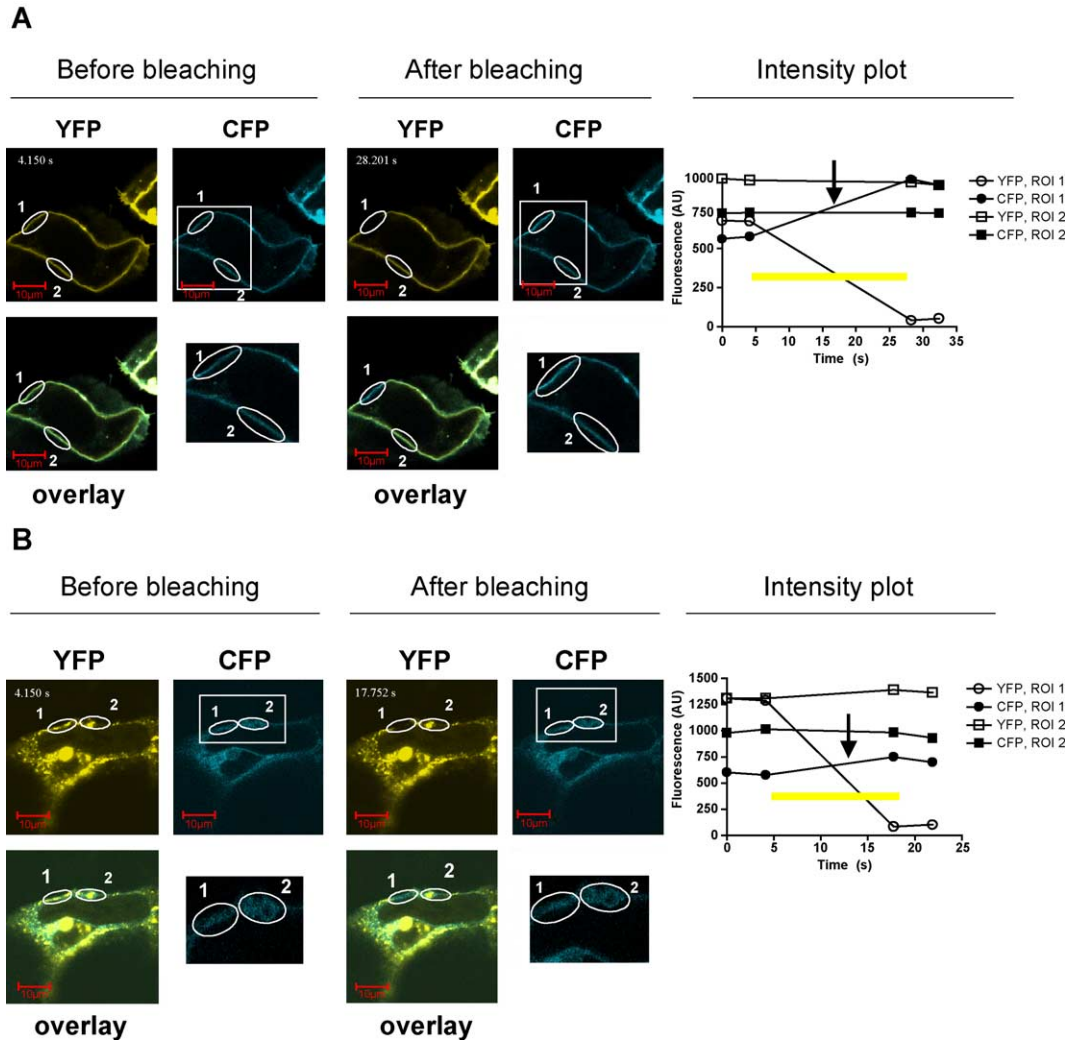

Fig. 4. FRET between fluorescently tagged $P2Y_2R$ and $G_{\alpha16}$. HEK 293 cells were transiently transfected with fluorescently tagged proteins, fixed with paraformaldehyde solution and recorded on a confocal microscope in two channels for CFP and YFP fluorescence (for details, see Section 2). The fluorescence images show the recordings immediately before and after the photobleaching cycle, and an overlay of the two channels. The circled areas mark two regions of interest (ROIs) selected in each image: ROI 1 denotes a membrane region where YFP was bleached and ROI 2 denotes a similar region of the same cell, which was not subjected to the bleaching cycle. The latter served as an internal reference. The plot on the right hand side shows the fluorescence intensities in the two regions for each channel. Two images, each, of fixed cells were recorded before and after the bleaching cycle. The duration of the bleaching cycle is indicated by the yellow bar in the intensity plots. The arrows mark the characteristic increase in donor fluorescence due to photobleaching of the acceptor. (A) HEK 293 cells transiently transfected with the CRFR1-CFP-YFP construct as a positive control. (B) HEK cells that were transiently co-transfected with $P2Y_2R$-CFP and $G_{\alpha16}$-YFP. For better visibility of the donor fluorescence in ROI 1 and ROI 2, enlarged images of the boxed regions in the CFP channels are shown below each panel of the CFP channel. Scale bar=10 μm.

cells, the FRET signal between $P2Y_2R$-CFP and $G_{\alpha16}$-YFP amounted to ~8% (Fig. 5B). The FRET signal was highly significant, confirming that also in K562 cells $P2Y_2R$ and $G_{\alpha16}$ formed constitutive complexes. Moreover, the signal was significantly higher (2.7-fold) when compared to control cells transfected with the $P2Y_2R$-YFP along with the β1AR-CFP as an alternative donor that was not expected to interact with the $P2Y_2R$.

Taken together, we conclude that the $P2Y_2$ receptor and $G_{\alpha16}$ are in close proximity in the strongly overexpressing HEK cell system as well as in the physiologically relevant environment for $G_{\alpha16}$, the hematopoietic cell. While the functional tests show such interactions upon agonist binding, the FRET measurements demonstrate that receptor–G protein complexes preexist under resting conditions.

3.4. Evidence for constitutive $P2Y_2$ receptor homo-oligomers

GPCRs may form oligomeric complexes with new pharmacological properties [38,39]. Of the P2Y receptor family members, the $P2Y_1$ receptor may form oligomers and the $P2Y_2$ receptor can associate with the adenosine A_1 receptor (Refs. [40,41], respectively). However, it is not clear whether the $P2Y_2R$ also may form homodimers or higher order homo-oligomeric assemblies. Using the FRET

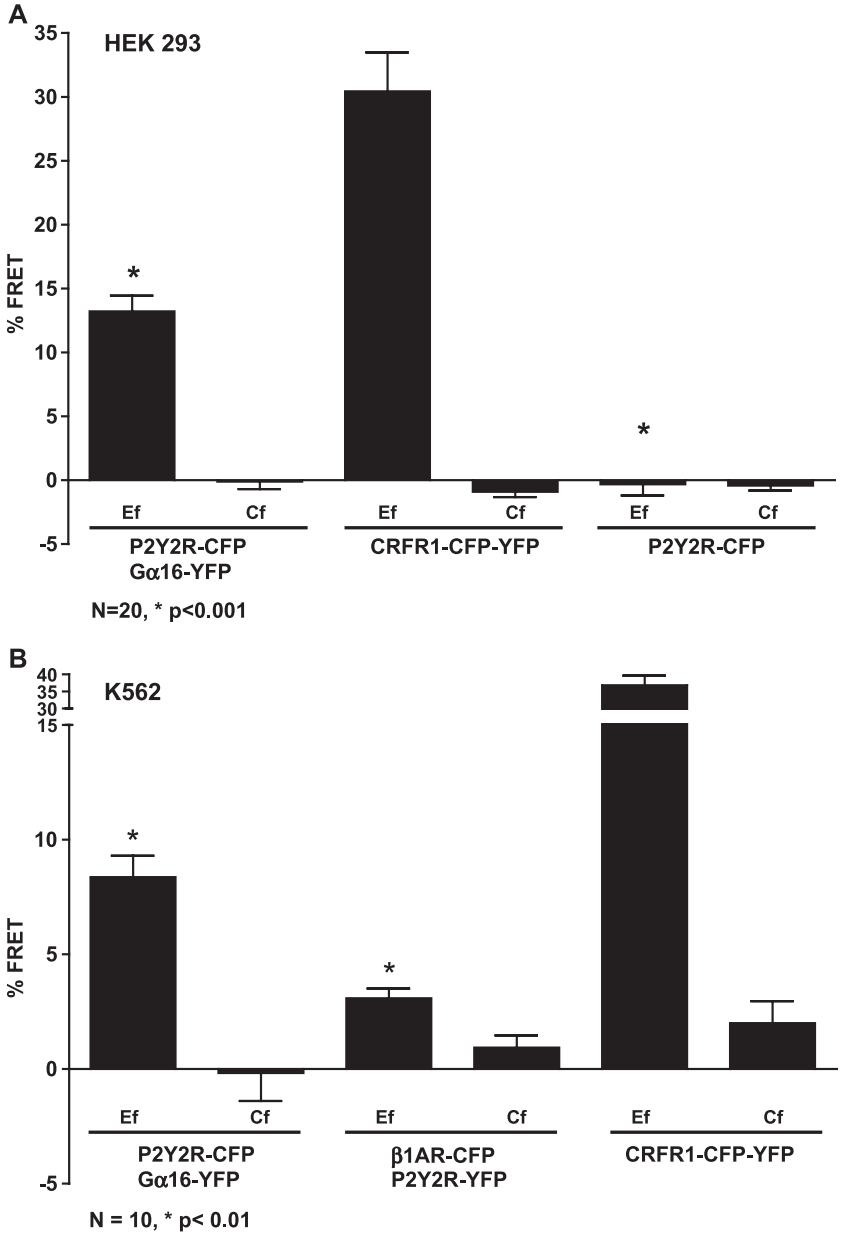

Fig. 5. High FRET efficiencies between $P2Y_2R$-CFP and $G_{\alpha 16}$-YFP when transiently transfected into HEK 293 and K562 cells. FRET efficiencies (Ef) are represented as the differences of CFP fluorescence before and after acceptor (YFP) photobleaching in defined regions (ROI 1, see Fig. 4), that are normalized to the CFP fluorescence after bleaching of the acceptor. Similar calculations were done in a non-bleached region (ROI 2) of comparable intensity of the same cell to calculate the control value Cf. The panels show a representative experiment out of at least four similar experiments. (A) FRET efficiencies in HEK 293 cells. (B) FRET efficiencies in K562 cells that were transiently transfected with the constructs as indicated in the figures. In each experiment, N=20 (A) and N=10 (B) cells were recorded and analyzed per condition. *The differences between the columns marked by asterisks are highly significant (p<0.01). The differences between the paired Ef and Cf values in HEK cells co-expressing $P2Y_2R$-CFP and $G_{\alpha 16}$-YFP, or the tandem control construct (panel A) were significant at a level of p<0.001. In K562 cells, Ef and Cf were significantly different at the level of p<0.001 ($P2Y_2R$-CFP/$G_{\alpha 16}$-YFP and CRFR1-CFP-YFP) and p<0.01 (β1AR-CFP/$P2Y_2R$-YFP).

method, we examined whether the $P2Y_2R$ was capable of forming such homo-oligomers. When co-expressed in HEK 293 cells, $P2Y_2R$-CFP and $P2Y_2R$-YFP gave rise to a FRET signal that was of similar magnitude as for receptor and G protein (Fig. 6A). Similar results were obtained in K562 cells (Fig. 6B): $P2Y_2R$-CFP and $P2Y_2R$-YFP gave a highly significant FRET signal, although of lesser magnitude than in the HEK cell expression system. This difference may be explained by lower levels of protein expression in K562 cells, because FRET signal intensities depend to some degree on the amount of plasmid used for transfection (not shown and Ref. [42]).

The FRET signal from $P2Y_2R$-CFP and $P2Y_2R$-YFP was also significantly higher (3.7-fold) than the one observed with β1AR-CFP as the donor, positively qualifying the FRET signal originating from the $P2Y_2R$ fusion proteins. It

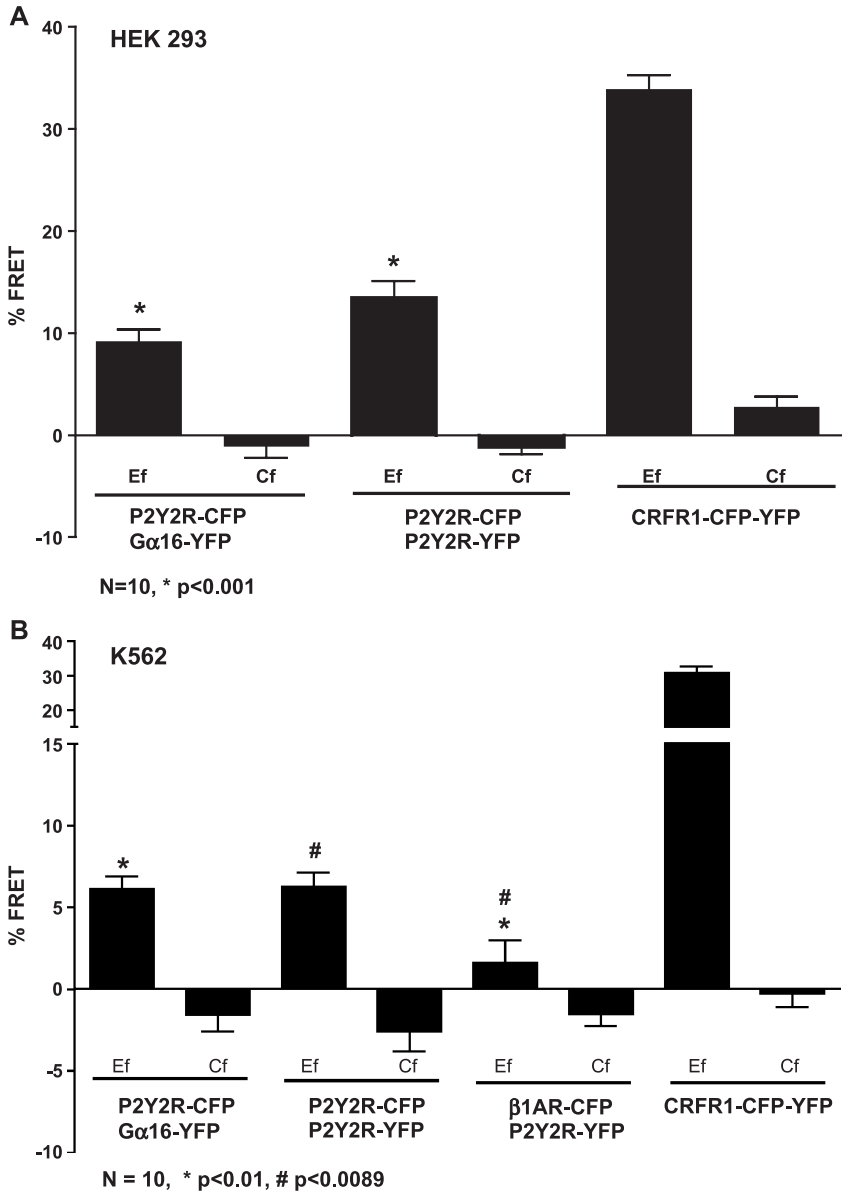

Fig. 6. Homo-oligomerization of the $P2Y_2$ receptor. HEK 293 or K562 cells were transiently transfected with $P2Y_2R$-CFP, $P2Y_2R$-YFP, CRFR1-CFP-YFP or β1AR-CFP as indicated in the figures. FRET signals were determined in regions of interest as described under Fig. 4. Per condition N=10 cells from a single experiment were analyzed. Representative experiments out of four (A) or six (B) independent experiments are shown in panels A and B. (A) HEK 293 cells, (B) K562 cells. All differences between the Ef and Cf values were significant at the level of $p<0.001$ or better, except for the difference observed in K562 cells co-expressing β1AR-CFP and P2Y2R-YFP, which was not significant. In panel A, asterisks indicate significant differences ($p < 0.001$) of the Ef values from the respective Cf values.

should be noted that, while co-expression of β1AR-CFP and $P2Y_2R$-YFP gave a significant FRET signal in some experiments (Fig. 5), it did not in others (Fig. 6). The level of the signal seems to be at the limit of detection for this, perhaps non-specific, interaction of the two non-related receptors. Thus, the observed FRET signal from the $P2Y_2R$ constructs is unlikely to be the result of random collisions, while this cannot be excluded for the heterologous interaction of β1AR-CFP with $P2Y_2R$-YFP.

The FRET signal in plasma membrane regions of cells that co-expressed $P2Y_2R$-CFP and $P2Y_2R$-YFP remained unchanged upon exposure to the agonist (UTP, 100 μM) for 10 min prior to fixation of the cells (not shown). This makes it unlikely that binding of agonist leads to the dissociation of the oligomeric complexes, although it cannot be excluded that agonist treatment resulted in dissociation of only a small fraction of receptors while a large portion of receptors remained in oligomeric assemblies. Such a small proportion of dissociation might go unnoticed by any FRET method since the small FRET decrease would have to be detected against a background of a strong FRET signal from all the non-responding oligomers.

Our experiments indicate that the $P2Y_2R$ forms homo-oligomeric assemblies and that the formation of $P2Y_2R$

oligomers does not depend on the presence of UTP as an agonist. Such constitutive complexes have previously been described for other receptors; a general assessment of the influence of agonists on receptor oligomerization suggests that FRET signals may be influenced by agonists in a majority of cases, but examples of no changes in FRET, or even a decrease of FRET have also been reported [38].

3.5. Conclusions

Our study demonstrates that FRET analysis may be used to screen for physical GPCR–G protein interactions. As the interactions appear to be constitutive in many cases, FRET analysis will be suitable to assess the possible interactions between a receptor and various G proteins in the absence of knowledge of the ligand, and thus obtain initial information about the coupling potential towards the main second messenger systems. This is of particular interest for studies on orphan receptors. It further offers the possibility to analyze GPCR–G protein interactions in non-adherent cell lines, or even primary hematopoietic cells, where transfection efficiencies and expression levels tend to be low, because the analysis can be done in single cells and makes use of spatially highly defined data from confocal microscopy images. The method makes it possible to study such protein–protein interactions in dependence of the intracellular localization of the interacting partners of the signalling axis.

The present study was focused on the analysis of constitutive interactions of fluorescent receptors and G proteins. Due to the duration of the photobleaching cycle, the method was not suitable for live cell FRET analysis, but rather required to fix cells in order to ensure focal stability and limit diffusional redistribution of fluorescent material. Further studies using direct observation of acceptor emission, fluorescence life time imaging or similar approaches will have to be conducted to obtain additional information on agonist induced changes in real time.

The main finding in our study—a constitutive, physical interaction between the $P2Y_2R$ and $G_{\alpha16}$ in the hematopoietic K562 cell—confirms a postulated link between the hematopoietic-cell specific G protein $G_{\alpha16}$ and the $P2Y_2$ receptor, as suggested from our earlier functional studies. Our data imply that G protein-dependent signal transduction may not only be regulated by agonist binding to the receptor but also be controlled by the extent of pre-existing receptor–G protein complexes. P2Y receptors, including the $P2Y_2$ receptor, have been implied in hematopoietic differentiation processes [43]. A recent publication [27] found that $CD34^+$ hematopoietic stem cells (HSCs) express $P2Y_2$ receptors, which, when activated by UTP, amplify cytokine-dependent cell growth. Based on its constitutive, physical interaction with the $P2Y_2$ receptor, a potentially important role of $G_{\alpha16}$ in transmitting this signal may be expected. Interestingly, high $G_{\alpha16}$ expression levels are associated with early differentiation stages, which are characterized by high proliferation [7,8], as well as with early hematopoietic cell proliferation and the progenitor marker $CD34^+$ [44,45]. Furthermore, our laboratory demonstrated that overexpression of activated $G_{\alpha16}$ in the factor-dependent (GM-CSF) leukemia cell line MB-02 initiated an erythroid differentiation program [9]. Further studies will have to address the involvement and the significance of the $P2Y_2R$-$G_{\alpha16}$ signalling axis in HSCs and early hematopoietic progenitor cells in proliferation and differentiation.

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