available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Involvement of basic amino acid residues in transmembrane regions 6 and 7 in agonist and antagonist recognition of the human platelet $P2Y_{12}$-receptor

Kristina Hoffmann, Uta Sixel, Francesca Di Pasquale [1], *Ivar von Kügelgen* *

Department of Pharmacology, University of Bonn, Reuterstrasse 2b, D-53113 Bonn, Germany

ARTICLE INFO

Article history:
Received 28 June 2008
Accepted 27 August 2008

Keywords:
Platelet $P2Y_{12}$-receptor
Ligand recognition
Site-directed mutagenesis
Cangrelor
Reactive blue-2

ABSTRACT

The $P2Y_{12}$-receptor plays a prominent role in ADP-induced platelet aggregation. In the present study, we searched for amino acid residues involved in ligand recognition of the human $P2Y_{12}$-receptor. Wild-type or mutated receptors were expressed in 1321N1 astrocytoma cells and Chinese hamster ovary (CHO) cells. There were no major differences in cellular expression of the constructs. Cellular cAMP production and cAMP response element (CRE)-dependent luciferase expression was increased by isoproterenol (astrocytoma cells) or forskolin (CHO cells). In cells expressing wild-type receptors, R256K or S101A mutant constructs, 2-methylthio-ADP inhibited the induced cAMP production with IC_{50} concentrations of about 0.3 nM. In cells expressing R256A constructs, the IC_{50} concentration amounted to 25 nM. In cells expressing H253A/R256A, Y259D and K280A constructs, 2-methylthio-ADP failed to affect the cellular cAMP production. Moreover, in cells expressing Y259D and K280A constructs, 2-methylthio-ADP did also not change the forskolin-induced CRE-dependent luciferase expression and caused only small increases in the serum response element-dependent luciferase expression. The antagonist cangrelor had similar potencies at wild-type receptors and R256A constructs (apparent pK_B-value at wild-type receptors: 9.2). In contrast, reactive blue-2 had a lower potency at the R256A construct (apparent pK_B-value at wild-type receptors: 7.6). In summary, the data indicate the involvement of Arg256, Tyr259 and, possibly, H253 (transmembrane region TM6) as well as Lys280 (TM7) in the function of the human $P2Y_{12}$-receptor. Arg256 appears to play a role in the recognition of nucleotide agonists and the non-nucleotide antagonist reactive blue-2, but no role in the recognition of the nucleotide antagonist cangrelor.

* *Corresponding author at*: Department of Pharmacology and Toxicology, University of Bonn, D-53113 Bonn, Germany. Tel.: +49 228 735445; fax: +49 228 735443.
E-mail address: kugelgen@uni-bonn.de (I. von Kügelgen).
[1] Present address: Department of Chemistry, University of Konstanz, D-78457 Konstanz, Germany.

Abbreviations: ANOVA, analysis of variance; CHO cells, Chinese hamster ovary cells; CRE, cAMP response element; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDTA, ethylendiaminetetraacetic acid; ERK, extracellular signal-regulated protein kinase; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; GPCR, G protein-coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hank's balanced salt solution; 2-methylthio-ADP, 2-methylthioadenosine 5′-diphosphate; SRE, serum response element; TM, transmembrane region.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.029

1. Introduction

The $P2Y_{12}$-receptor plays a crucial role in ADP-induced platelet aggregation [1–4]. The receptor is the site of action of potent inhibitors of platelet aggregation including active metabolites of thienopyridine compounds such as clopidogrel [5,6] and nucleotide $P2Y_{12}$-receptor antagonists such as cangrelor [7,8]. The $P2Y_{12}$-receptor is known to couple via Gi-proteins to the inhibition of adenylate cyclase activity. It belongs to the second subgroup ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) of the heterogenous family of P2Y receptors which are G-protein-coupled receptors (GPCRs) for extracellular nucleotides [9,10]. The molecular properties of the members of the first subgroup (consisting of the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors) have been analysed in several mutagenesis studies. These studies demonstrated the involvement of polar amino acid residues within the transmembrane regions TM3, TM6 and TM7 in ligand recognition. A conserved basic residue (lysine or arginine) in the predicted upper third of TM6 was shown to play a key role [11–16]. A basic residue in the predicted upper third of TM6 is also present in all receptor proteins of the second subgroup of P2Y receptors (e.g. Arg256 of the human $P2Y_{12}$-receptor).

Despite the fact that the $P2Y_{12}$-receptor plays a very important role in physiology and pharmacotherapy, direct experimental evidence for a contribution of residues in ligand recognition of the receptor protein is still limited. One interesting study demonstrated that a patient with a congenital bleeding disorder carries a polymorphism with a change of arginine in position 256 to glutamine. When expressed in cells, the R256Q mutation caused a defective receptor function, suggesting a role of Arg256 in receptor function [17]. Moreover, another patient with an impaired ADP-induced platelet aggregation was recently reported to carry a heterozygous P258T mutation within the $P2Y_{12}$-receptor protein [18]. There is evidence for the contribution of cysteine residues within the extracellular loops of the receptor protein in the interaction with active metabolites of clopidogrel [6]. A study with chimeric $P2Y_1$/$P2Y_{12}$ constructs indicated the involvement of the C-terminus of the $P2Y_{12}$-receptor in the activation of G-proteins [19]. The intracellular motif ''DRY'' has been reported to contribute to constitutive activity of the receptor [20]. And, finally, two potential N-linked glycosylation sites at the extracellular amino-terminus of the $P2Y_{12}$-receptor protein have been proposed to modulate the signal transduction of the receptor [21].

In the present study, we demonstrate the involvement of additional residues in receptor function of the $P2Y_{12}$-receptor by analysing the effects of receptor activation on, first, the receptor-mediated inhibition of cellular cAMP production and cAMP response element (CRE)-dependent luciferase expression and, second, on the receptor-mediated activation of the serum response element (SRE)-directed luciferase expression. The native $P2Y_{12}$-receptor mediates both an inhibition of the activity of the adenylate cyclase and an activation of the extracellular signal-regulated protein kinase ERK [22,23]. ERK activation is related to the activation of the SRE-pathway [24]. Other Gi-coupled receptors including the dopamine D2-receptor have previously also been shown to couple to the SRE-pathway [25]. Furthermore, we demonstrate that the residue Arg256 plays a role in the recognition of the non-nucleotide antagonist reactive blue-2 [26], but no role in the interaction with the nucleotide antagonist cangrelor. Some of the results have been presented in abstract form at meetings [27,28].

2. Methods and materials

2.1. Molecular biological experiments

The sequence encoding for the human $P2Y_{12}$-receptor was cloned from human brain cDNA (Stratagene, Amsterdam, Netherlands) into the expression vectors pcDNA3.1D/V5-His-TOPO (Invitrogen, Karlsruhe, Germany) and pcDNA5/FRT-V5-His-TOPO (Invitrogen, Karlsruhe, Germany). Site-directed mutations were introduced using mutagenic primers and standard molecular biology techniques. All polymerase chain reactions were performed using 2% dimethyl sulfoxide (DMSO, Sigma, Deisenhofen, Germany). The sequences encoding for wild-type and mutant $P2Y_{12}$-receptors were then verified by cycle sequencing (SequiTherm Exel II DNA sequencing kit; Epicentre Technologies, Madison, WI, USA) using a LICOR Gene READIR 4200 sequencer (MWG-Biotech, Ebersberg, Germany). The sequencing was repeated at GATC Biotech (Konstanz, Germany) with identical results.

2.2. Expression of wild-type and mutant receptors in mammalian cell lines

1321N1 astrocytoma cells (European cell culture collection, Salisbury, UK) were cultured at 5% CO_2 and 36.5 °C in Dulbecco's modified Eagle's medium (DMEM, 419660-029, Invitrogen, Karlsruhe, Germany) containing Glutamax I (35050-038, Invitrogen, Karlsruhe, Germany) or L-Glutamine (M11-004, PAA, Pasching, Austria) and 10% foetal bovine serum (FBS; 10108-165, Invitrogen, Karlsruhe, Germany, or A15-101, PAA, Pasching, Austria). The cells were split once a week by treating with trypsin–EDTA (trypsin 0.5 g/l, EDTA 0.54 mM; 25300-054, Invitrogen, Karlsruhe, Germany, or L11-660, PAA, Pasching, Austria). Chinese hamster ovary (CHO) Flp-In cells (Invitrogen, Karlsruhe, Germany) or CHO K1 cells were grown at 5% CO_2 and 36.5 °C in Ham's F12 medium (21765029, Invitrogen, Karlsruhe, Germany, or E15-817, PAA, Pasching, Austria) supplemented with Glutamax I, 10% FBS and (CHO Flp-In cells) Zeocin (100 μg/l: 4605059, Invitrogen, Karlsruhe, Germany) and were split every 3 days. 1321N1 astrocytoma cells and CHO K1 cells were transfected using the pcDNA3.1 expression vector and CHO Flp-In cells by the use of the pcDNA5-FRT expression vector combined with the pOG44 vector (1:10; Invitrogen, Karlsruhe, Germany). For transfection, LIPOFECTAMINE 2000™ (11668-027, Invitrogen, Karlsruhe, Germany) was used as described by the manufacturer. Cells stably expressing the receptor constructs were selected 2 days after transfection by culturing in the presence of 800 μg/ml GENETICINE™ (G418; 11811-064, Invitrogen, Karlsruhe, Germany; 1321N1 astrocytoma cells and CHO K1 cells) or 500 μg/ml hygromycin (10687-010, Invitrogen, Karlsruhe, Germany, CHO Flp-In cells). For each receptor construct at least 6–14 different clones were isolated from the transfected 1321N1

astrocytoma cells or CHO K1 cells and analysed for receptor expression and function (see below). Cells from passages 4 to 40 of the cloned 1321N1 astrocytoma cells or CHO K1 cells and cells from passages 3 to 40 of the isogenic CHO Flp-In cells were used for further experiments. In some experiments, cells were pretreated with pertussis toxin (Sigma, Deisenhofen, Germany) or its solvent (glycerol Tris–glycine buffer; Sigma, Deisenhofen, Germany) for 20 h.

2.3. Expression analysis

Cells were cultured on coverslips for 2 days (CHO cells) or 3 days (astocytoma cells). Expression levels of wild-type and mutant $P2Y_{12}$-receptors were assessed by direct immunofluorescence staining using a FITC-coupled monoclonal antibody against the V5-receptor epitope (anti-V5-FITC, 46-0308, Invitrogen, Karlsruhe, Germany; 1:500; incubation period 1 h; used according to the protocol of Invitrogen). After washing of the cells, expression levels were quantified by fluorescence microscopy on a Zeiss Axiovert 100 microscope equipped with an oil immersion 100× objective (Zeiss, Jena, Germany), a charge-coupled device camera and a Polychrome II monochromator (TILL photonics, Planegg, Germany; excitation wavelength 475 nm; emission wavelength 510 nm; exposure time 2 s, CHO cells; 1 s, astrocytoma cells). Membrane areas outside the nucleus were evaluated (arbitrary fluorescence pixels above background).

2.4. Analysis of cellular cyclic AMP accumulation

In most experiments, receptor function was assessed by analyzing changes in the cellular levels of cAMP as measure of the activity of cellular adenylate cyclase. For this purpose, non-transfected cells as well as 1321N1 astrocytoma cells or CHO cells stably expressing wild-type or mutant human $P2Y_{12}$-receptor constructs were cultured on 24-well plates for 48 h (1321N1 astrocytoma cells) or 24 h (CHO cells; culture media, see above). After removal of the culture medium, cells were washed with HBSS buffer (containing 20 mM HEPES, Sigma, Deisenhofen, Germany; pH 7.3) and then incubated with HBSS buffer for 2 h at 36.5 °C. Cellular cAMP production was then stimulated by addition of 10 nM isoproterenol (1321N1 astrocytoma cells) or 10 μM forskolin (CHO cells) at 36.5 °C. Solvent (control), ADP or 2-methylthio-ADP was added together with isoproterenol or forskolin. In some experiments, $P2Y_{12}$-receptor antagonists were given 5 min (cangrelor) or 10 min (reactive blue-2) before the agonist 2-methylthio-ADP. The reaction was stopped after 10 min by removal of the reaction buffer followed by the addition of a hot lysis solution (500 μl; 90 °C; Na_2EDTA 4 mM; Triton X100 0.1‰, Sigma, Deisenhofen, Germany, pH 7.5). The multi-well plates were shaken on ice for 1 h. cAMP levels in the supernatant were then quantified by incubation of an aliquot with cAMP binding protein and [^{3}H]-cAMP (cAMP assay, TRK 432, Amersham Biosciences, Freiburg, Germany), followed by liquid scintillation counting after removal of the unbound [^{3}H]-cAMP by charcoal. cAMP levels per well were calculated by linear regression from a standard curve determined for each experiment. The isoproterenol- or forskolin-induced cAMP production in the presence of ADP or 2-methylthio-ADP was expressed as percentage of the isoproterenol- or forskolin-induced cAMP production in the absence of $P2Y_{12}$-receptor agonists (% of respective control).

2.5. Analysis of CRE- or SRE-dependent luciferase activity

In some experiments, receptor function was assessed by analyzing changes in the cAMP response element (CRE)- or serum response element (SRE)-dependent expression of luciferase in CHO Flp-In cells expressing wild-type or mutant human $P2Y_{12}$-receptor constructs. For these experiments, the cells were transiently transfected with the pCRE-luc or pSRE-luc vector (Stratagene, Amsterdam, Netherlands) using LIPOFECTAMINE 2000™ (11668-027, Invitrogen, Karlsruhe, Germany). After a period of 18 h, the cells were cultured on 24-well plates for additional 20 h. The culture medium was then removed and the cells were incubated with HBSS buffer (without HEPES, pH 7.3) for 3.5 h (CRE) or 4 h (SRE) at 36.5 °C. Cellular cAMP production was stimulated by addition of 1 μM forskolin (pCRE-luc transfected cells). Solvent (control) or 2-methylthio-ADP was added together with forskolin, when used. In some experiments, the $P2Y_{12}$-receptor antagonist reactive blue-2 was given 10 min before the agonist 2-methylthio-ADP. The reaction was stopped after 3.5 h (CRE) or 4 h (SRE) by removal of the reaction buffer followed by the addition of 50 μl HBSS and 50 μl BRIGHT-GLO™ luciferase assay solution (Promega, Mannheim, Germany). The activity of luciferase in the supernatant was analyzed using a single photon luminometer (Berthold, Wildbad, Germany). The forskolin-induced (CRE) increase in luciferase activity in the presence of 2-methylthio-ADP was expressed as percentage of the response to forskolin in the absence of $P2Y_{12}$-receptor agonists (% of respective control). For experiments with cells transfected with the pSRE-luc vector, the 2-methylthio-ADP-induced increase in luciferase activity was expressed as percentage of the values obtained in the absence of 2-methylthio-ADP (% of basal activity).

2.6. Data analysis and statistics

Results are presented as means ± S.E. from n experiments. Differences between means were tested for significance by an analysis of variance followed by the Bonferroni or Dunnett post-test (Prism 4.03, Graph Pad, San Diego, CA). Concentration–response data were fitted by non-linear regression to estimate IC_{50} values (concentrations causing half-maximal inhibition) and EC_{50} values (concentrations causing half-maximal increases in SRE-dependent luciferase-expression) for 2-methylthio-ADP. Apparent pK_B-values were calculated according to: $pK_B = \log(\text{dose ratio} - 1) - \log[B]$. Differences between the estimated IC_{50} or EC_{50} values or pK_B-values were tested for significance by the F-test (Prism 4.03). $p < 0.05$ or lower was the significance criterion.

2.7. Reagents

The following drugs were used: adenosine 5′-diphosphate sodium (ADP; A8621, Sigma, Deisenhofen, Germany), forskolin (F-6886, Sigma, Deisenhofen, Germany), isoproterenol (I-5627, Sigma, Deisenhofen, Germany), 2-methylthio adenosine 5′-

diphosphate trisodium (2-methylthio-ADP; M-152, Sigma, Deisenhofen, Germany), pertussis toxin (P2980, Sigma, Deisenhofen, Germany), cangrelor (AR-C69931MX; N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene adenosine 5′-triphosphate, Astra, Hässle, Mölndal, Sweden), reactive blue-2 sodium (Ega Chemie, Mannheim, Germany). Stock solutions of drugs were prepared either with distilled water or with a mixture of ethanol and DMSO (forskolin only). The solvents were added to the buffer used for control experiments.

3. Results

The first series of experiments were performed in 1321N1 astrocytoma cells which are known to possess no or very few endogenous P2Y receptors. As reported previously, these cells are equipped with endogenous $beta_2$-adrenoceptors mediating an activation of adenylate cyclase [29]. Hence, isoproterenol was used to increase cellular cAMP levels in 1321N1 astrocytoma cells. In non-transfected astrocytoma cells, ADP (100 nM) and 2-methylthio-ADP (0.01 nM to 10 μM) failed to alter the isoproterenol (10 nM)-induced increases in cellular cAMP accumulation (not shown).

After transfection with the plasmids encoding for the human wild-type $P2Y_{12}$-receptor or mutant receptor constructs (tagged with the V5-epitope used for the analysis of receptor expression), at least 6–14 clonal cell lines were isolated for each construct. The cell line with the highest inhibition of cAMP production due to addition of 2-methylthio-ADP 100 nM was used for further experiments. In 1321N1 astrocytoma cells stably expressing human wild-type $P2Y_{12}$-receptors, isoproterenol increased cellular cAMP levels 28-fold (see Table 1). The $P2Y_{12}$-receptor agonist 2-methylthio-ADP caused a concentration-dependent decrease in the isoproterenol-stimulated cAMP levels (solid line in Fig. 1). 2-Methylthio-ADP acted in nanomolar concentrations with a half-maximal concentration (IC_{50}) of 0.3 nM and a maximal inhibition of about 60% (Fig. 1 and Table 1).

3.1. Analysis of the role of Arg256 in transmembrane region 6 in agonist recognition

First, the levels of expression of mutant receptor constructs were studied in astrocytoma cells by a quantitative microfluorometric analysis. There were no differences in expression levels at the cell membrane in cells stably expressing wild-type receptors or mutant receptor constructs. This was also true for constructs in which Arg256 was replaced by the neutral residue alanine (R256A), the basic residue lysine (R256K) or the acidic residue aspartate (R256D; Table 1). When cells expressing these constructs were stimulated by isoproterenol, the increases in intracellular cAMP levels were also similar (28–50-fold; Table 1). Then, the effect of 2-methylthio-ADP on the cellular cAMP production was tested. In cells expressing R256K mutant receptor constructs, 2-methylthio-ADP caused an inhibition (Fig. 1A) with an IC_{50}-value of 0.2 nM (Table 1; not significantly different from the IC_{50}-value determined for wild-type receptors; *F*-test). In contrast, in astrocytoma cells expressing R256A mutant receptors, 2-methylthio-ADP inhibited the cellular cAMP production only when tested at higher concentrations (Fig. 1A) with an IC_{50}-value of 25 nM (Table 1). The respective concentration–response curve of 2-methylthio-ADP was significantly shifted to the right (Fig. 1A, *F*-test). This shift corresponds to an 80-fold decrease in agonist potency (calculated from the ratio of IC_{50}-values). The maximal effect of 2-methylthio-ADP was also decreased in these experiments (Fig. 1A and Table 1). In agreement with these findings, a shift of the concentration–response curve of the physiological agonist ADP to the right

Table 1 – Basal and stimulated cAMP levels, inhibition by 2-methylthio-ADP (2-MeSADP), antagonistic potency (apparent pK_B-values) of cangrelor and expression levels of wild-type and mutant $P2Y_{12}$-receptor constructs expressed in 1321N1-astrocytoma cells

Construct	cAMP (pmol/well) solvent	cAMP (pmol/well) + isoproterenol (10 nM)	Max. inhibition by 2-MeSADP (% of control)	−(log IC_{50}) 2-MeSADP	Apparent pK_B-values of cangelor	Expression levels (% of wild-type)
Wild-type	1.5 ± 0.5 (9)	42.1 ± 2.9 (18)	60.6 ± 0.9 # (13)	9.5 ± 0.1	9.2	100.0 ± 4.9 (19)
R256K	1.4 ± 0.3 (8)	39.8 ± 3.9 (16)	40.4 ± 1.7 # ** (7)	9.7 ± 0.1	8.9	94.0 ± 4.9 (17)
R256A	1.0 ± 0.3 (11)	53.3 ± 2.5 (24)	25.7 ± 1.3 # ** (12)	7.6 ± 0.2 **	9.2	95.5 ± 7.0 (17)
R256D	1.8 ± 0.5 (12)	49.6 ± 2.3 (22)	16.3 ± 1.4 # ** (13)	6.8 ± 0.2 **		90.7 ± 4.1 (13)
H253A/R256A	2.0 ± 0.1 (6)	64.0 ± 3.8 ** (10)	3.6 ± 2.3 ** (6)			88.5 ± 4.0 (19)
S101A	2.8 ± 0.5 (14)	55.1 ± 5.5 (27)	31.0 ± 1.7 # ** (16)	8.9 ± 0.1	(9.1)[a]	95.5 ± 4.2 (17)
Y259D	1.8 ± 0.4 (4)	42.8 ± 5.5 (6)	5.0 ± 4.9 ** (6)			85.6 ± 3.0 (16)
K280A	0.8 ± 0.2 (4)	6.3 ± 0.8 ** (6)	8.4 ± 5.6 ** (6)			87.8 ± 5.6 (19)

Means ± S.E. of (*n*) experiments. #$p < 0.01$, significant differences vs. respective controls (ANOVA followed by the Bonferroni post-test). **$p < 0.01$, significant differences vs. values determined at wild-type $P2Y_{12}$-receptors (*F*-test). The table shows basal cAMP levels, cAMP levels stimulated by isoproterenol 10 nM, the maximal inhibition of the induced cAMP production by 2-methylthio-ADP and the respective (log of the half-maximal concentration IC_{50}). The table also summarizes the apparent pK_B-values determined for the antagonist cangrelor at cells expressing the respective constructs and cellular expression levels of these constructs. Expression levels were estimated by averaging fluorescence values from the membrane regions of cells after immunofluorescence staining with a monoclonal antibody against the V5 receptor epitope.

[a] Please note the non-surmountable antagonism of cangrelor at S101A mutant receptors.

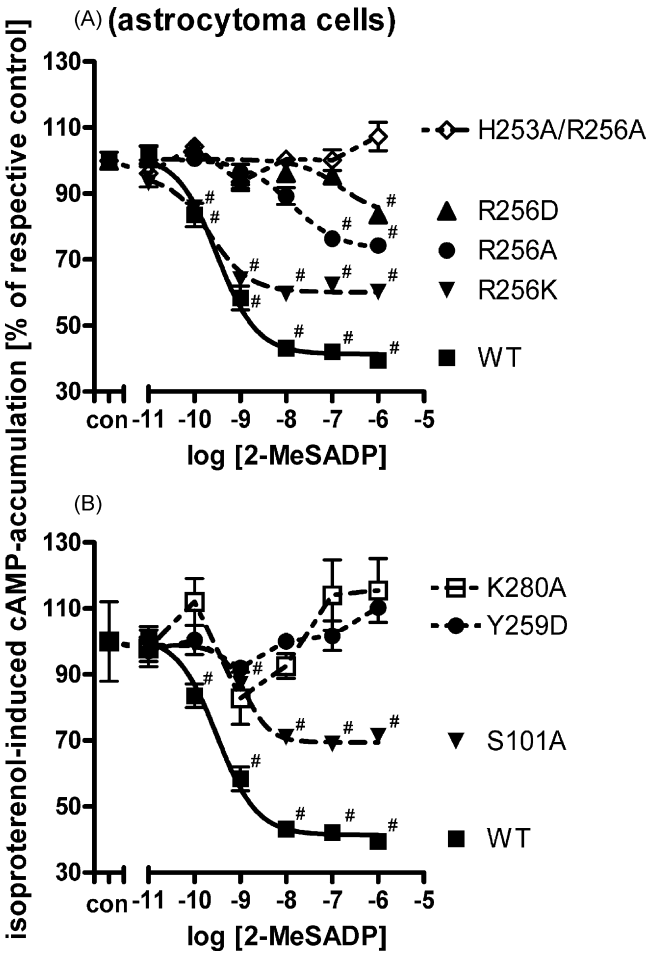

Fig. 1 – Involvement of polar amino acid residues in transmembrane regions 3, 6 and 7 in ligand recognition: 2-Methylthio-ADP-induced inhibition of cAMP accumulation in 1321N1 astrocytoma cells expressing wild-type or mutant receptor constructs (A, R256K, R256A, R256D, H253A/R256A; B, S101A, Y259D, K280A). Cellular cAMP production was increased by addition of isoproterenol 10 nM for 10 min at 36.5 °C. The figure shows the concentration-dependent inhibition of intracellular cAMP accumulation by the $P2Y_{12}$-receptor agonist 2-methylthio-ADP [2-MeSADP; concentrations indicated]. Cellular cAMP levels are expressed as percentage of those determined in respective experiments with isoproterenol, but without 2-methylthio-ADP (% of respective control, con). Log IC_{50} values are summarized in Table 1. Means ± S.E. of 6–40 experiments. $^{\#}p < 0.01$, significant differences vs. respective control (ANOVA followed by the Bonferroni post-test).

was observed in CHO K1 cells expressing R256A mutant receptors (when compared with the concentration–response curve of ADP obtained in CHO K1 cells expressing wild-type receptors; not shown). Moreover, in astrocytoma cells expressing a construct with the acidic residue aspartate at the position 256 (R256D), the inhibitory action of 2-methylthio-ADP was markedly reduced. Only at the highest concentration analyzed (1 μM), there was a significant inhibition in cAMP production in cells expressing R256D mutant constructs (Fig. 1A and Table 1). In astrocytoma cells expressing a double mutant construct (H253A/R256A), 2-methylthio-ADP even failed to cause any inhibition (Fig. 1A) despite a similar expression level of this receptor construct (Table 1) and a similar relative increase in cellular cAMP in response to isoproterenol in the absence of 2-methylthio-ADP (32-fold; Table 1).

3.2. *Role of other polar residues in TM3, TM6 and TM7 in receptor function*

Next, the possible roles of Ser101 in TM3 of the human $P2Y_{12}$-receptor (corresponding to His132 of the human $P2Y_1$-receptor [10]), of the polar residue Tyr259 in the upper portion of TM6 and of the basic residue Lys280 in TM7 (corresponding to Arg307 of the human $P2Y_1$-receptor [10] in agonist recognition were studied. When stably expressed in astrocytoma cells, the constructs S101A, Y259D and K280A showed similar expression levels as the wild-type receptor (Table 1). Isoproterenol (10 nM) also caused similar increases in cAMP levels in cells expressing S101A and Y259D constructs when compared with those observed in cells expressing wild-type receptors (Table 1), while some clonal cell lines expressing the K280A construct showed smaller increases (Table 1). In cells expressing the S101A constructs, 2-methylthio-ADP inhibited the isoproterenol-stimulated cAMP accumulation with a similar IC_{50} concentration as observed in cells expressing wild-type receptors (Fig. 1B and Table 1). In contrast, 2-methylthio-ADP failed to cause any inhibition in cAMP production in cells expressing Y259D and K280A constructs (Fig. 1B and Table 1; analyzed for 11 and 13 distinct clonal cell lines, respectively).

To confirm the lack of any agonistic effect of 2-methylthio-ADP at the Y259D and K280A mutant constructs, these constructs were stably expressed in CHO Flp-In cells. This cellular expression system generates isogenic cells with a single site of incorporation of the recombinant sequences in the cellular genome. Immunofluorescence staining revealed identical expression levels of wild-type receptors and mutant receptor constructs in these cells (100.1 ± 1.3% and 100.2 ± 1.2% of the values determined for wild-type receptors, $n = 12$ and 16, respectively). Forskolin (10 μM) was used to increase cellular cAMP production. In cells expressing the wild-type receptor, forskolin increased the cAMP accumulation about 23-fold (0.6 ± 0.2 pmol cAMP per well in the absence and 12.4 ± 0.9 pmol cAMP per well in presence of forskolin, $n = 16$ and 32, respectively). In these cells, 2-methylthio-ADP caused a concentration-dependent inhibition of cAMP production with an IC_{50} concentration of about 1.8 nM and a maximal effect by about 40% (solid line in Fig. 2A). In CHO Flp-In cells expressing the Y259D and K280A constructs, total forskolin-induced cAMP accumulation amounted to 8.8 ± 0.6 and 7.0 ± 0.7 pmol/well, respectively. In agreement with the results obtained at astrocytoma cells, 2-methylthio-ADP failed to alter cAMP levels in CHO Flp-In cells expressing the Y259D and K280A constructs (Fig. 2A). Moreover, the physiological agonist ADP also caused no inhibition of the cellular cAMP production in CHO Flp-In cells expressing K280A constructs (Table 2) and in CHO K1 cells expressing K280A constructs (not

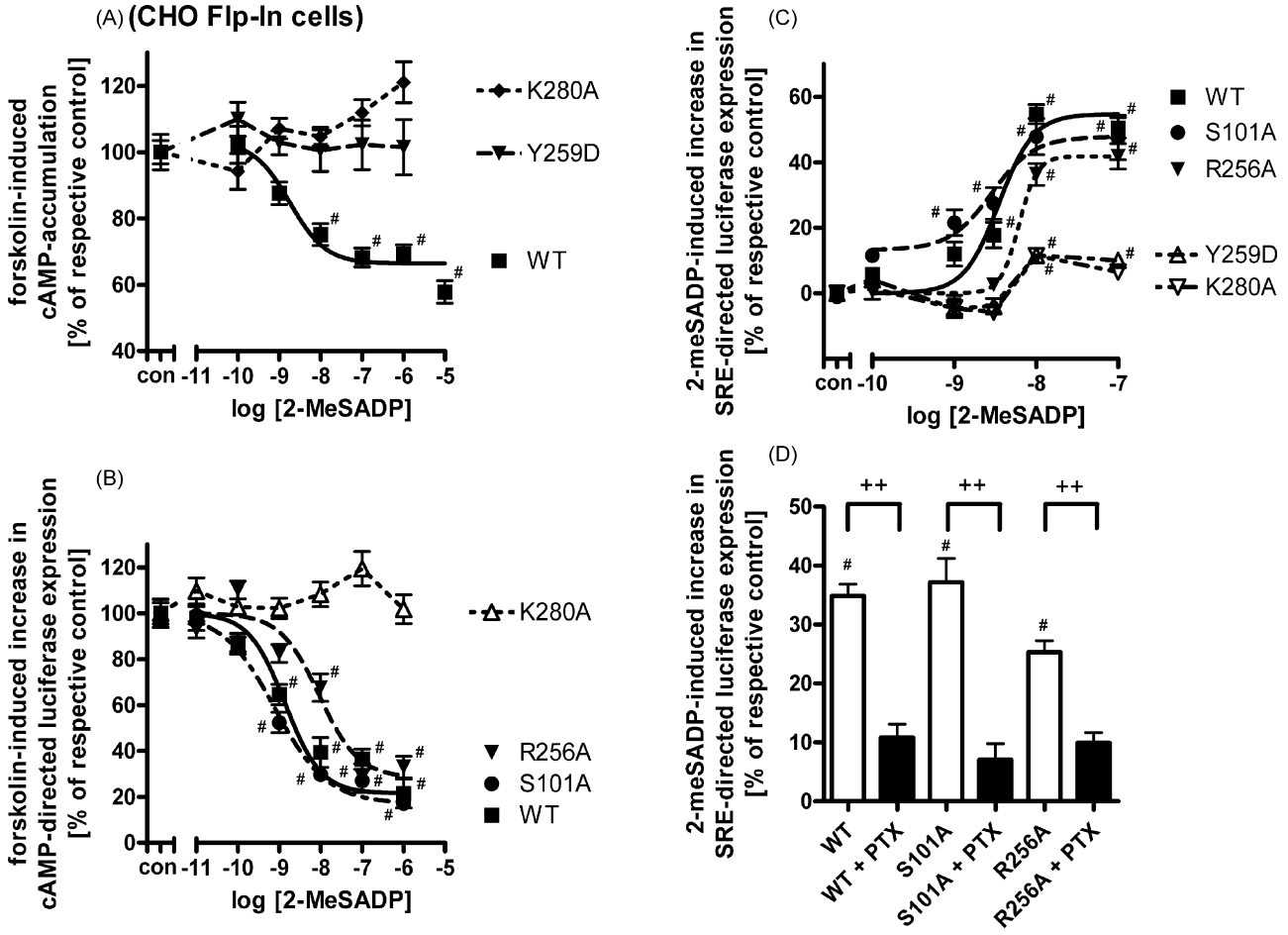

Fig. 2 – Inhibition of cAMP accumulation (A) and cAMP response element (CRE, B) or serum response element (SRE, C and D)-induced changes in luciferase activity in CHO Flp-In cells expressing wild-type (WT) or mutant $P2Y_{12}$-receptor constructs. (A) Cellular cAMP production was increased by addition of forskolin 10 μM for 10 min at 36.5 °C. The figure shows the concentration-dependent inhibition of intracellular cAMP accumulation by 2-methylthio-ADP. Cellular cAMP levels are expressed as percentage of those determined in respective experiments with forskolin, but without 2-methylthio-ADP (% of respective control, con). (B) CRE-dependent luciferase expression was increased by addition of forskolin 1 μM for 3.5 h; (% of respective control, con). (C) Increases in SRE-dependent luciferase expression; (% of basal activity). (D) Cells were pretreated with solvent or pertussis toxin (PTX, 400 ng/ml) for 20 h. Means ± S.E. of 6–32 experiments. $^{\#}p < 0.05$, significant differences vs. respective control; $^{++}p < 0.01$ significant differences vs. respective values in the absence of PTX (ANOVA followed by the Bonferroni post-test).

shown). The lack of any agonistic activity of 2-methylthio-ADP at the Y259D and K280A constructs was further confirmed in experiments analyzing the effect of the agonist on the forskolin-induced increase of CRE-dependent luciferase activity. For this purpose, CHO Flp-In cells expressing wild-type or mutant receptor constructs were transiently transfected with the pCRE-luc vector. The cellular adenylate cyclase activity was increased by addition of forskolin (1 μM) for 3.5 h. This treatment by forskolin increased the activity of luciferase about 2-fold above basal (not shown). In cells expressing wild-type receptors, S101A constructs or R256A constructs, 2-methylthio-ADP (1 nM to 1 μM) caused the expected inhibition of the response to forskolin with IC_{50}-values of 1.4 nM, 0.8 nM and 9 nM, respectively (Fig. 2B). In agreement with the results analyzing the cellular cAMP content, 2-methylthio-ADP had no effect on the forskolin-induced luciferase expression in cells expressing the Y259D- and K280A-constructs (Fig. 2B).

Subsequently, we studied the question whether mutating the receptor alters receptor signalling to another intracellular pathway. For this purpose, CHO Flp-In cells expressing wild-type or mutant receptor constructs were transiently transfected with the pSRE-luc vector. In cells transfected with the empty pcDNA5 vector and the pSRE-luc vector, 2-methylthio-ADP (0.1 nM to 0.1 μM) caused no response (not shown). In contrast, 2-methylthio-ADP (0.1 nM to 0.1 μM) increased the SRE-dependent luciferase expression by about 50% with an EC_{50} concentration of 3.5 nM in cells expressing wild-type $P2Y_{12}$-receptors (Fig. 2C). Similar increases were observed in cells expressing S101A and R256A constructs with EC_{50} concentrations of 2.9 and 6.2 nM, respectively (Fig. 2C). These

Table 2 – Effects of ADP on cAMP accumulation in CHO Flp-In cells expressing wild-type- and K280A mutant $P2Y_{12}$-receptors

	cAMP-accumulation (% of control)	
	Wild-type	K280A
Control	100.0 ± 4.0 (4)	100.0 ± 3.0 (4)
ADP 1 μM	70.4 ± 2.7 $^{\#\#}$ (6)	113.7 ± 2.9 (6)
ADP 10 μM	82.6 ± 4.9 $^{\#}$ (6)	155.4 ± 3.6 $^{\#\#}$ (6)

Means ± S.E. of (*n*) experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant differences vs. respective control (ANOVA followed by the Bonferroni post-test). Cellular cAMP-accumulation was increased by forskolin (10 μM). The table shows the changes in cAMP-accumulation by the agonist ADP added for 10 min.

responses to 2-methylthio-ADP were markedly reduced in cells pretreated with pertussis toxin (Fig. 2D). In non-pretreated cells expressing the Y259D and K280A constructs, 2-methylthio-ADP caused very small, but statistically significant increases in SRE-dependent luciferase expression (Fig. 2C).

3.3. *Recognition of a nucleotide antagonist*

Next, we performed experiments in order to identify residues involved in the binding of a nucleotide antagonist. For this purpose, the interaction of 2-methylthio-ADP with the nucleotide antagonist cangrelor was first studied by analyzing changes in cAMP production in 1321N1 astrocytoma cells expressing wild-type or mutant receptors. In cells expressing the wild-type human $P2Y_{12}$-receptor, cangrelor (30 nM) shifted the concentration–response curve of 2-methylthio-ADP to the right (Fig. 3A). The effects of the antagonist were clearly surmountable (Fig. 3A). The respective apparent pK_B-value amounted to 9.2 (Table 1). In cells expressing the R256K construct, cangrelor (30 nM) again shifted the concentration–response curve of 2-methylthio-ADP to the right (Fig. 3B). The shift corresponded to an apparent pK_B-value of 8.9 and was not statistically different from the shift observed at wild-type receptors (Table 1). An almost identical shift to the right of the concentration–response curve of 2-methylthio-ADP by cangrelor was also observed in cells expressing the R256A construct (Fig. 3C and Table 1) despite the fact that the

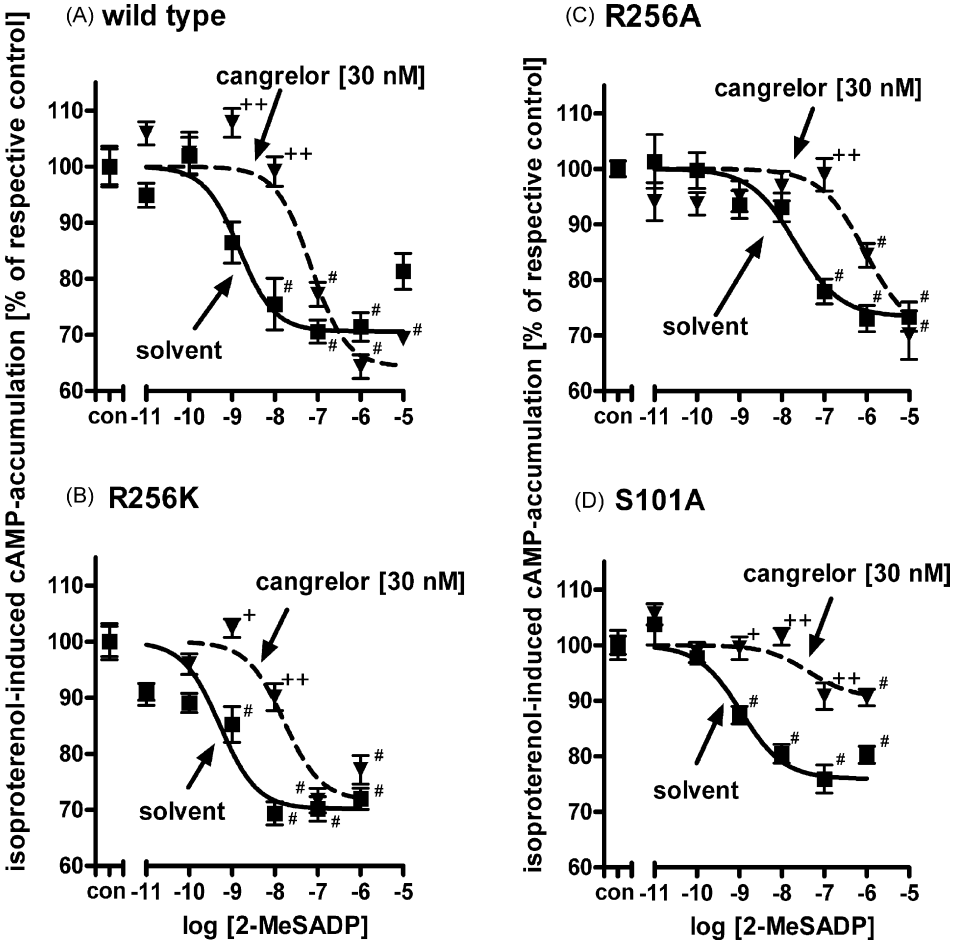

Fig. 3 – Inhibition of cAMP accumulation: interaction with the nucleotide antagonist cangrelor in 1321N1 astrocytoma cells expressing wild-type (A, WT) or mutant $P2Y_{12}$-receptor constructs (B, R256K; C, R256A; D, S101A). The figures show concentration–response curves for 2-metylthio-ADP (2-MeSADP) in the absence (solid lines) or presence of cangrelor (AR-C69931MX, interrupted lines). Cangrelor was added 5 min before the agonist at the concentrations indicated. The apparent pK_B-values of cangrelor are summarized in Table 1. Means ± S.E. of 4–24 experiments. $^{\#}p < 0.01$, significant differences vs. respective control. $^{+}p < 0.05$, $^{++}p < 0.01$, significant differences vs. respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post-test). For further details see legend to Fig. 1.

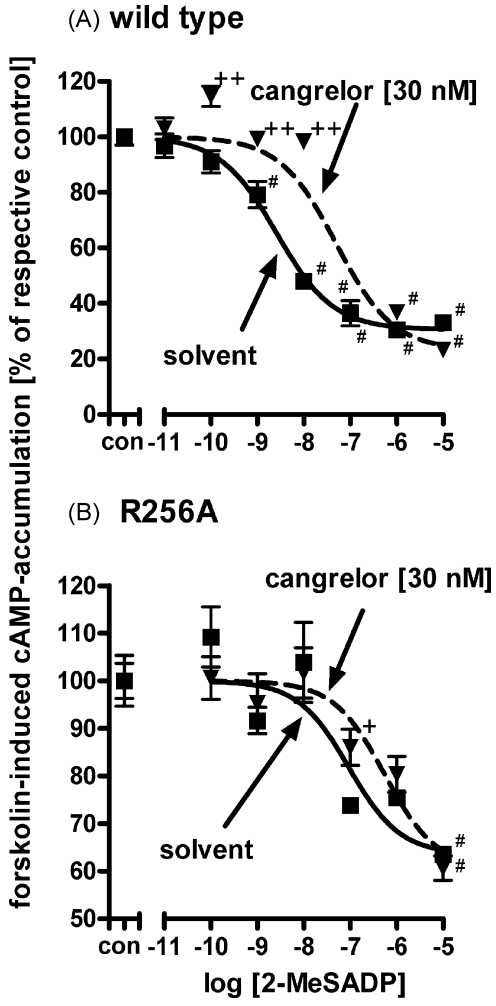

Fig. 4 – Inhibition of cAMP accumulation: interaction with the nucleotide antagonist cangrelor in CHO Flp-In cells expressing wild-type (A, WT) or R256A mutant $P2Y_{12}$-receptor constructs (B). Cangrelor (interrupted lines) was added 5 min before the agonist at the concentrations indicated. The apparent pK_B-values of cangrelor are summarized in Table 4. Means ± S.E. of 4–20 experiments. $^{\#}p < 0.05$, significant differences vs. respective control. $^{+}p < 0.05$, $^{++}p < 0.01$, significant differences vs. respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post-test). For further details see legend to Figs. 2 and 3.

potency of the agonist 2-methylthio-ADP was clearly decreased in cells expressing this mutant receptor construct (compare solid lines of Fig. 3A and C; see also Fig. 1 and Table 1). Finally, in astrocytoma cells expressing S101A constructs, cangrelor (30 nM) inhibited the effects of 2-methylthio-ADP in a non-surmountable manner. Cangrelor itself did not change basal cAMP levels or the isoproterenol-induced cAMP production in 1321N1 astrocytoma cells expressing wild-type or mutant receptors (not shown).

The determination of the potency of cangrelor at the wild-type receptor and the R256A mutant construct was confirmed in experiments performed on CHO cells. In CHO Flp-In cells expressing wild-type receptors or the R256A construct, cangrelor shifted the concentration–response curves of 2-methylthio-ADP to the right with pK_B values of 8.63 and 8.50, respectively (no statistically significant difference; Fig. 4 and Table 3). Cangrelor did not change basal or forskolin-stimulated cAMP levels in CHO cells (not shown).

3.4. *Recognition of a non-nucleotide antagonist*

CHO Flp-In cells were then used in order to study the interaction with the non-nucleotide antagonist reactive blue-2 which is known to block $P2Y_{12}$-receptors [10,26]. Reactive blue-2 (3 μM) clearly acted as a potent and surmountable antagonist in cells expressing the wild-type $P2Y_{12}$-receptor. It shifted the concentration–response curve of the agonist to the right with an apparent pK_B-value of 7.6 (Fig. 5A and Table 3). In contrast, in cells expressing the R256A construct, reactive blue-2 used at the same concentration of 3 μM caused only a modest shift of the agonist concentration–response curve to the right (Fig. 5B). This shift corresponded to an apparent pK_B-value of reactive blue-2 of 6.7 at the R256A construct (statistically significant difference; Table 3). Reactive blue-2 itself markedly increased cellular cAMP levels in the presence of forskolin (10 μM; Table 4). Therefore, we used lower concentrations of forskolin of 5 (wild-type) and 6 (R256A) μM for the experiments in the presence of reactive blue-2 shown in dashed lines in Fig. 5 in order to compensate for the reactive blue-2-induced increase in cAMP levels (Table 4). Shifts of the concentration–response curves by reactive blue-2 were also observed in experiments without a compensation for the increase in total cAMP concentration (not shown).

To confirm the differences in pK_B-values of reactive blue-2 determined at the wild-type receptor and the mutant R256A construct, the interaction of reactive blue-2 was then studied

Table 3 – Inhibition of cAMP accumulation by 2-methylthio-ADP and antagonist potency (apparent pK_B-values) of cangrelor and reactive blue-2 (RB 2) at wild-type and R256A-mutant $P2Y_{12}$-receptor constructs expressed in CHO Flp-In cells

Construct	Max. inhibition (% of control) (cAMP assay)	$-\log IC_{50}$ 2-MeSADP (cAMP assay)	Apparent pK_B-values of cangrelor (cAMP assay)	Apparent pK_B-values of RB 2 (cAMP assay)	Apparent pK_B-values of RB 2 (cAMP assay) (luciferase-assay)
Wild-type	66.6 ± 4.9 $^{\#}$ (5)	8.8 ± 0.1	8.63	7.63	7.37
R256A	42.2 ± 4.0 $^{\#}$ ** (9)	7.8 ± 0.2 **	8.50	6.75 *	5.91 *

Means ± S.E. of (n) experiments. $^{\#}p < 0.01$, significant differences vs. respective controls (ANOVA followed by the Bonferroni post-test). $^{*}p < 0.05$, $^{**}p < 0.01$, significant differences vs. values determined at wild-type $P2Y_{12}$-receptors (F-test). For further details see legends to Figs. 5 and 6 and Table 1.

Table 4 – Effects of reactive blue-2 (RB 2) on cellular cAMP accumulation in CHO Flp-In cells expressing wild-type and R256A mutant $P2Y_{12}$-receptors in the absence and presence of forskolin

	cAMP-level (pmol/well)					
	Solvent –	Solvent +RB 2 (3 μM)	Forskolin (10 μM) –	Forskolin (10 μM) +RB 2 (3 μM)	Forskolin (5 μM) +RB 2 (3 μM)	Forskolin (6 μM) +RB 2 (3 μM)
Wild-type	0.3 ± 0.3 (11)	0.2 ± 0.2 (12)	8.7 ± 0.5 (10)	17.7 ± 0.9^{++} (15)	5.6 ± 0.4^{+} (6)	
R256A	0.3 ± 0.3 (10)	0.6 ± 0.3 (9)	6.3 ± 0.4 (20)	11.7 ± 0.3^{++} (11)		6.0 ± 0.2 (7)

Means ± S.E. of (*n*) experiments. $^{+}p < 0.05$, $^{++}p < 0.01$ significant differences vs. respective values in the absence of antagonist (ANOVA followed by the Bonferroni post-test). Forskolin caused significant increases in cAMP levels ($p < 0.01$ vs. respective values obtained in the presence of solvent used for forskolin). Reactive blue-2 (RB 2) (3 μM) was added 10 min before forskolin. The table shows values determined in the absence and presence of reactive blue-2. In some experiments, the concentration of forskolin was reduced in order to compensate the increase in cAMP levels induced by reactive blue-2 itself (see legend to Fig. 5).

by analyzing the 2-methylthio-ADP mediated inhibition of the CRE-dependent luciferase activity in CHO Flp-In cells transiently transfected with the pCRE-luc vector. In this assay system with forskolin used at a concentration of 1 μM, reactive blue-2 itself had no significant effect of the response to forskolin alone (not shown). In cells expressing wild-type receptors, reactive blue-2 caused a marked shift of the concentration–response curve of 2-methylthio-ADP (apparent pK_B-value of 7.4; Fig. 6A and Table 3). Moreover, the shift caused by reactive blue-2 was smaller in cells expressing the R256A construct (apparent pK_B-value of 5.9; Fig. 6B and Table 3). In cells expressing the S101A construct, the apparent pK_B-value of reactive blue-2 amounted to 7.6 (Fig. 6C).

4. Discussion

The first series of experiments of this study have been performed on 1321N1 astrocytoma cells which are known to possess no or very few endogenous P2Y receptors. In non-transfected astrocytoma cells, the P2Y receptor agonist 2-methylthio-ADP (up to 10 μM) caused no change in cAMP levels in agreement with the absence of endogenous receptors for 2-methylthio-ADP in these cells. We performed additional experiments on CHO cells in order to confirm our conclusions. CHO cells are known to express endogenous P2Y receptors including the Gq-coupled $P2Y_1$-receptor. However, in non-transfected and mock-transfected CHO Flp-In cells, 2-methylthio-ADP did not change cAMP levels, the CRE-dependent luciferase activity or the SRE-dependent luciferase activity indicating that CHO Flp-In cells are also a suitable expression cell line for the analysis of the pharmacological properties of the human $P2Y_{12}$-receptor. Isoproterenol and forskolin were used to accelerate the cAMP production in astrocytoma cells and CHO cells, respectively.

In 1321N1 astrocytoma cells expressing recombinant wild-type human $P2Y_{12}$-receptors, there was in fact a clear inhibitory action of the prototype $P2Y_{12}$-receptor agonist 2-methylthio-ADP on the isoproterenol-stimulated cAMP production with a half-maximal concentration (IC_{50}) of about 0.3 nM. This value is similar to published data [1]. Similar IC_{50} values for 2-methylthio-ADP were also determined in our experiments performed on CHO Flp-In cells expressing the recombinant human wild-type $P2Y_{12}$-receptors using two different assay systems: the determination of cellular cAMP levels by the radioligand competition assay (which was also used for the experiments with astrocytoma cells) and the analysis of the CRE-dependent luciferase activity (CHO cells only). When analyzing effects on the SRE-dependent luciferase expression, 2-methylthio-ADP showed an EC_{50} value of 3.5 nM in cells expressing the wild-type $P2Y_{12}$-receptor. Gi-coupled receptors including the dopamine D2-receptor have previously been demonstrated to couple via pertussis toxin sensitive G-proteins to the stimulation of SRE-dependent gene expression related to the extracellular signal-regulated kinase (ERK) pathway [24,25]. The native $P2Y_{12}$-receptor is known to mediate an activation of ERK [22,23]. To our knowledge, we demonstrate here for the first time the pertussis toxin-sensitive coupling of the recombinant human $P2Y_{12}$-receptor to a change in the SRE-dependent gene expression. The cellular expression levels of wild-type receptors and mutant receptor constructs were almost identical within one cell line (astrocytoma cells or CHO cells) excluding that differences in functional responses are due to differences in receptor expression at the cell membrane.

4.1. *Role of Arg256 in recognition of a negatively charged domain of an agonist*

Our data now provide direct experimental evidence for an involvement of Arg256 in TM6 of the human $P2Y_{12}$-receptor in the recognition of a negatively charged domain of the agonist molecule, most likely a portion of the diphosphate chain. This is shown by the observation that the replacement of arginine by the basic residue lysine is well tolerated for receptor function, while the replacement by the neutral residue alanine caused an intermediate reduction in agonist potency (as shown by the increase in IC_{50} concentration) and the replacement by the acidic residue aspartate induced a very marked decrease in agonist activity of 2-methylthio-ADP. A recently published molecular model of the $P2Y_{12}$-receptor protein based on homology calculations suggests that Arg256 of the $P2Y_{12}$-receptor is located in the proximity of the alpha-phosphate group of the nucleotide agonist [30]. An important role of Arg256 in the function of the $P2Y_{12}$-receptor has previously been shown by Cattaneo et al., who described the effect of the mutation R256Q within the $P2Y_{12}$-receptor in a patient with a bleeding disorder [17].

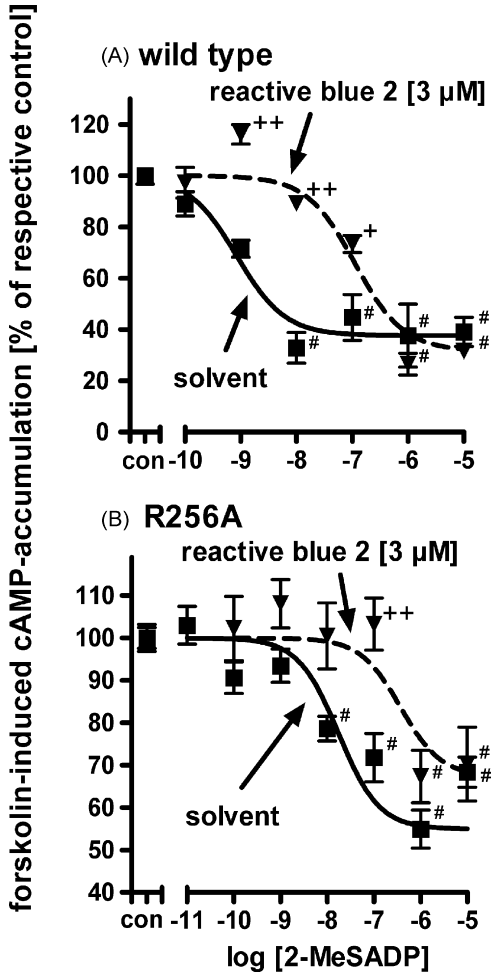

Fig. 5 – Inhibition of cAMP accumulation: interaction of the non-nucleotide antagonist reactive blue-2 (RB 2) in CHO cells expressing wild-type (A, WT) or mutant $P2Y_{12}$-receptor constructs (B, R256A). Reactive blue-2 (RB 2, interrupted lines) was added 10 min before the agonist at the concentration of 3 μM. The apparent pK_B-values of reactive blue-2 are summarized in Table 4. Means ± S.E. of 4–20 experiments. $^{\#}p < 0.01$, significant differences vs. respective control. $^{+}p < 0.05$, $^{++}p < 0.01$, significant differences vs. respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post-test). For further details see legend to Figs. 2 and 3.

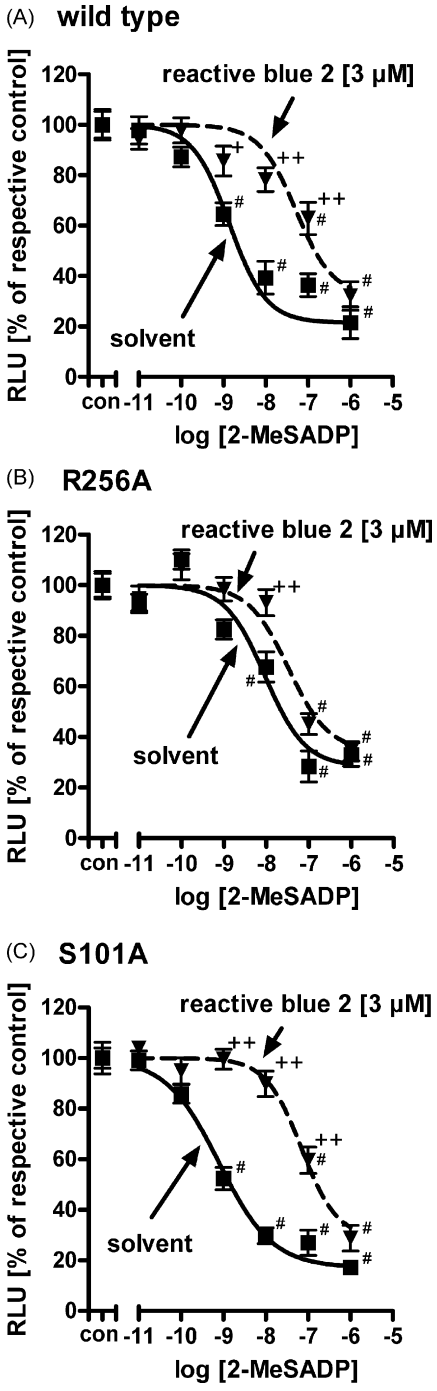

Fig. 6 – Inhibition of CRE-dependent luciferase activity: interaction of the non-nucleotide antagonist reactive blue-2 (RB 2) in CHO cells expressing wild-type (A, WT) or mutant $P2Y_{12}$-receptor constructs (B, R256A; C, S101A). Reactive blue-2 (RB 2, interrupted lines) was added 10 min before the agonist at the concentration of 3 μM. The apparent pK_B-values of reactive blue-2 are summarized in Table 4. Means ± S.E. of 10–23 experiments. $^{\#}p < 0.05$, significant differences vs. respective control. $^{+}p < 0.05$, $^{++}p < 0.01$, significant differences vs. respective values in the absence of the antagonist (ANOVA followed by the Bonferroni post-test). For further details see legends to Figs. 2 and 3.

4.2. *Roles of His253, Tyr259 and Lys280*

In the present study, we identified the involvement of additional residues of the human $P2Y_{12}$-receptor in ligand recognition. Tyr259 as well as Lys280 appear to be crucial for the activation of the receptor by agonists as shown by the absence of any receptor-mediated inhibition of cAMP production or cAMP-dependent gene expression in 1321N1 cells and CHO Flp-In cells expressing receptor constructs in which the residues were replaced by alanine (K280A) or aspartate (Y259D). The loss of any responses in cells expressing the double mutant H253A/R256A suggests a similar role for His253. One should note that the single mutation R256A only caused

an intermediate change in receptor function. The residues His253 and Tyr259 are located in the upper portion of the predicted TM6, His253 one predicted alpha helix turn below and Tyr259 one predicted alpha helix turn above the residue Arg256. These data underline the important role of TM6 in ligand recognition of the human $P2Y_{12}$-receptor. Lys280 is located at the upper third of TM7 of the human $P2Y_{12}$-receptor [10]. Lys280 appears to be very important for agonist recognition of the human $P2Y_{12}$-receptor as shown by the absence of any responses to 2-methylthio-ADP or ADP in experiments analyzing the cAMP pathway (present study). The corresponding residue Gln307 of the human $P2Y_1$-receptor also plays a dominant role as shown by a 360-fold increase in the EC_{50} value of 2-methylthio-ADP at the Q307A mutant construct of the $P2Y_1$-receptor [12].

Interestingly, some differences were observed in experiments analyzing the signalling of mutant receptors to a distinct intracellular pathway. When analyzing effects on the SRE-dependent luciferase expression, there were no significant differences in the responses to the agonist 2-methythio-ADP in cells expressing wild-type and R256A mutant receptors (EC_{50} concentrations of 3.5 nM vs. 6.2 nM, respectively). Differential coupling of mutant receptors to the cAMP- and the SRE-dependent pathways has previously been reported for the D2-receptor [24]. In agreement with very important roles of Tyr259 and Lys280 in receptor function, 2-methylthio-ADP caused only small increases in SRE-dependent luciferase expression in cells expressing Y259D and K280A constructs (Fig. 2C).

4.3. *Recognition of antagonists*

In astrocytoma cells expressing recombinant human $P2Y_{12}$-receptors, the nucleotide antagonist cangrelor acted in a manner compatible with a competitive antagonism as suggested for the action of cangrelor at the human $P2Y_{12}$-receptor [31]. The apparent pK_B-value of 9.2 of cangrelor determined in our study at the recombinant $P2Y_{12}$-receptor expressed in astrocytoma cells fits well to the affinity value of 0.4 nM determined for cangrelor at the native $P2Y_{12}$-receptor expressed in human platelets [7]. The anthraquinone derivative reactive blue-2 blocks a number of P2 receptors when used at micromolar concentrations [10]. It has, however, been shown to act in nanomolar concentrations as a competitive antagonist at the native rat $P2Y_{12}$-receptor expressed in C6 glioma cells [26]. The pK_B-value determined by Boyer and co-workers [26] for reactive blue-2 at the native rat $P2Y_{12}$-receptor (pK_B 7.6) is identical with or very close to the apparent pK_B-value determined for reactive blue-2 in the present study at the recombinant human $P2Y_{12}$-receptor (apparent pK_B 7.6 or 7.4; Table 4).

The analysis of antagonistic properties on cells expressing the R256A mutant construct now provides a more detailed view of the role of Arg256 in ligand recognition of the human $P2Y_{12}$-receptor. While the replacement of Arg256 by alanine had a marked effect on the agonist potency (IC_{50} concentration), there was almost no change in the antagonistic potency (apparent pK_B) of the triphosphate nucleotide antagonist cangrelor in both 1321N1 astrocytoma cells and CHO Flp-In cells expressing the R256A mutant receptor construct. This result indicates that Arg256 contributes to nucleotide agonist recognition but not to the interaction with the nucleotide antagonist (at least in the case of cangrelor). One might speculate that Arg256 is involved in the stabilization of the active conformation of the receptor protein possibly by interaction with the phosphate chain of the nucleotide agonist molecule. The important role of the beta-phosphate group of the agonist in the activation process of the receptor is known from a number of functional studies. 2-Methylthio-AMP acts as an antagonist at the $P2Y_{12}$-receptor while 2-methylthio-ADP is a very potent $P2Y_{12}$-receptor agonist [1,10].

A key role of Arg256 within the ligand binding site of the human $P2Y_{12}$-receptor is further confirmed by the experiments analyzing the interaction with the non-nucleotide antagonist reactive blue-2. Interestingly, reactive blue-2 had a markedly lower antagonistic potency (apparent pK_B) in cells expressing R256A constructs indicating that Arg256 belongs to the overlapping portion of the binding sites of reactive blue-2 and the agonist 2-methylthio-ADP. Hence, our data support the view that reactive blue-2 acts as a competitive antagonist at the $P2Y_{12}$-receptor. It may serve as a chemical lead for the development of potent and competitive non-nucleotide antagonists. The corresponding residue Lys280 in TM6 of the human $P2Y_1$-receptor has also been shown to be involved in binding of the non-nucleotide antagonist reactive blue-2 [32].

Ser101 of the human $P2Y_{12}$-receptor corresponds to His132 of the human $P2Y_1$-receptor. While His132 of the $P2Y_1$-receptor is involved in agonist recognition [12,13], Ser101 of the $P2Y_{12}$-receptor does not seem to play the same role in the $P2Y_{12}$-receptor protein. Nevertheless, this residue is likely to be located close to the ligand binding site as shown by the marked change in antagonistic properties of cangrelor in cells expressing the S101A mutant receptor construct. While in cells expressing the wild-type $P2Y_{12}$-receptor the mode of action of cangrelor is consistent with competitive antagonism, cangrelor acted in a non-surmountable manner in cells expressing S101A mutant receptor constructs. Possibly, the mutated construct favours an allosteric mode of interaction of cangrelor with the receptor.

In summary, our data indicate that Arg256 in the upper third of TM6 of the human $P2Y_{12}$-receptor interacts with a negatively charged domain of the agonist, most likely the phosphate chain. Interestingly, the exchange of the residue did not modify the potency of the triphosphate nucleotide antagonist cangrelor, suggesting an involvement of the residue Arg256 in the stabilization of the active state of the receptor protein. Furthermore, we provide evidence for the contribution of His253 (TM6), Tyr259 (TM6) and Lys280 (TM7) in ligand recognition of the human $P2Y_{12}$-receptor. And finally, we show for the Y259D and K280A mutant constructs that effects of mutations on the receptor function are not restricted to the coupling to the cAMP pathway, but also affect the coupling to the SRE-dependent luciferase expression.

Acknowledgements

We thank Kenneth A. Jacobson PhD (National Institutes of Health, NIDDK, Bethesda MD) for helpful discussion, Prof. Dr.

P. Illes (Rudolph-Boehm-Institut für Pharmakologie und Toxikologie, University of Leipzig, Leipzig, Germany) for the supply of drugs and Petra Spitzlei (Department of Pharmacology and Toxicology, University of Bonn, Bonn, Germany) for expert technical assistance. The study was supported by grants of the University of Bonn (Germany) and the Doktor Robert Pfleger Stiftung (Bamberg, Germany).

REFERENCES

[1] Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001;409:202–7.
[2] Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, et al. Molecular cloning of the platelet $P2T_{AC}$ ADP receptor: pharmacological comparison with another ADP receptor, the $P2Y_1$ receptor. Mol Pharmacol 2001;60:432–9.
[3] Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, et al. ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J Biol Chem 2001;276:8608–15.
[4] Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. J Clin Invest 2004;113: 340–5.
[5] Savi P, Labouret C, Delesque N, Guette F, Lupker J, Herbert JM. $P2y_{12}$, a new platelet ADP receptor, target of clopidogrel. Biochem Biophys Res Commun 2001;283: 379–83.
[6] Savi P, Zachayus JL, Delesque-Touchard N, Labouret C, Hervé C, Uzabiaga MF, et al. The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci USA 2006;103:11069–74.
[7] Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 1999;42:213–20.
[8] Cattaneo M. Platelet P2 receptors: old and new targets for antithrombotic drugs. Expert Rev Cardiovasc Ther 2007;5:45–55.
[9] Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 2006;58:281–341.
[10] von Kügelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacol Ther 2006;110:415–32.
[11] Erb L, Garrad R, Wang Y, Quinn T, Turner JT, Weisman GA. Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. J Biol Chem 1995;270:4185–8.
[12] Jiang Q, Guo D, Lee BX, Van Rhee AM, Kim YC, Nicholas RA, et al. A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. Mol Pharmacol 1997;52:499–507.
[13] Hoffmann C, Moro S, Nicholas RA, Harden TK, Jacobson KA. The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. J Biol Chem 1999;274: 14639–47.
[14] Qi AD, Zambon AC, Insel PA, Nicholas RA. An arginine/glutamine difference at the juxtaposition of transmembrane domain 6 and the third extracellular loop contributes to the markedly different nucleotide selectivities of human and canine P2Y11 receptors. Mol Pharmacol 2001;60:1375–82.
[15] Ivanov AA, Costanzi S, Jacobson KA. Defining the nucleotide binding sites of P2Y receptors using rhodopsin-based homology modeling. J Comput Aided Mol Des 2006;20:417–26.
[16] Zylberg J, Ecke D, Fischer B, Reiser G. Structure and ligand-binding site characteristics of the human P2Y11 nucleotide receptor deduced from computational modelling and mutational analysis. Biochem J 2007;405:277–86.
[17] Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, Conley PB, et al. Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. Proc Natl Acad Sci USA 2003;100:1978–83.
[18] Remijn JA, IJsseldijk MJ, Strunk AL, Abbes AP, Engel H, Dikkeschei B, et al. Novel molecular defect in the platelet ADP receptor P2Y12 of a patient with haemorrhagic diathesis. Clin Chem Lab Med 2007;45:187–9.
[19] Ding Z, Kim S, Kunapuli SP. Identification of a potent inverse agonist at a constitutively active mutant of human P2Y12 receptor. Mol Pharmacol 2006;69:338–45.
[20] Schöneberg T, Hermsdorf T, Engemaier E, Engel K, Liebscher I, Thor D, et al. Structural and functional evolution of the P2Y12-like receptor group. Purinergic Signal 2007;3:255–68.
[21] Zhong X, Kriz R, Seehra J, Kumar R. N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression. FEBS Lett 2004;562:111–7.
[22] Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. J Thromb Haemost 2006;4:638–47.
[23] Garcia A, Shankar H, Murugappan S, Kim S, Kunapuli SP. Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. Biochem J 2007;404:299–308.
[24] Takeuchi Y, Fukunaga K. Dopamine D2 receptor activates extracellular signal-regulated kinase through the specific region in the third cytoplasmic loop. J Neurochem 2004;89:1498–507.
[25] Wang Y, Ho G, Zhang JJ, Nieuwenhuijsen B, Edris W, Chanda PK, et al. Regulator of G protein signaling Z1 (RGSZ1) interacts with Galpha i subunits and regulates Galpha i-mediated cell signaling. J Biol Chem 2002;277:48325–32.
[26] Boyer JL, Zohne IE, Jacobson KA, Harden TK. Differential effects of P2-purinoceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P2Y-purinoceptors. Br J Pharmacol 1994;113:614–20.
[27] Hoffmann K, Algaier I, von Kügelgen I. Evidence for the involvement of basic amino acid residues in transmembrane regions 6 and 7 of the human platelet P2Y12-receptor in ligand recognition. Purinergic Signal 2006;2:199–200.
[28] Hoffmann K, von Kügelgen I. Analysis of the potencies of cangrelor and reactive blue 2 at wild type and mutant human P2Y12-receptors. Naunyn Schmiedebergs Arch Pharmacol 2007;375(Suppl 1):26–7.
[29] Li Q, Schachter JB, Harden TK, Nicholas RA. The 6H1 orphan receptor, claimed to be the p2y5 receptor, does not mediate nucleotide-promoted second messenger responses. Biochem Biophys Res Commun 1997;236:455–60.

[30] Jacobson KA, Mamedova L, Joshi BV, Besada P, Costanzi S. Molecular recognition at adenine nucleotide (P2) receptors in platelets. Semin Thromb Hemost 2005;31: 205–16.
[31] Communi D, Gonzalez NS, Detheux M, Brézillon S, Lannoy V, Parmentier M, et al. Identification of a novel human ADP receptor coupled to G(i). J Biol Chem 2001;276:41479–85.
[32] Guo DP, von Kügelgen I, Moro S, Kim YC, Jacobson KA. Evidence for the recognition of non-nucleotide antagonists within the transmembrane domains of the human P2Y1 receptor. Drug Devel Res 2003;57:173–81.