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

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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.

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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.

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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