Molecular bases of defective signal transduction in the platelet P2Y₁₂ receptor of a patient with congenital bleeding

Marco Cattaneo^{*†‡}, Maddalena L. Zighetti^{*}, Rossana Lombardi^{*}, Constantino Martinez[†], Anna Lecchi^{*}, Pamela B. Conley[§], Jerry Ware[†], and Zaverio M. Ruggeri^{†¶}

^{*}A. Bianchi Bonomi Hemophilia and Thrombosis Center, Department of Internal Medicine, Istituto di Ricovero e Cura a Carattere Scientifico–Ospedale Maggiore, University of Milan, 20122 Milan, Italy; [†]Roon Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Thrombosis and Hemostasis, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037; and [§]Millennium Pharmaceuticals, South San Francisco, CA 94080

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We have identified structural attributes required for signal transduction through a seven-transmembrane-domain receptor. Platelets from a patient (AC) with a congenital bleeding disorder had normal shape change but reduced and reversible aggregation in response to 4 μ M ADP, similar to normal platelets with blocked P2Y₁₂ receptor. The response to 20 μ M ADP, albeit still decreased, was more pronounced and was reduced by a P2Y₁₂ antagonist, indicating some residual receptor function. ADP failed to lower the adenylyl cyclase activity stimulated by prostaglandin E₁ in the patient's platelets, even though the number and affinity of 2-methylthioadenosine 5'-[³³P]diphosphate-binding sites was normal. Analysis of the patient's P2Y₁₂ gene revealed a G-to-A transition in one allele, changing the codon for Arg-256 in the sixth transmembrane domain to Gln, and a C-to-T transition in the other allele, changing the codon for Arg-265 in the third extracellular loop to Trp. Neither mutation interfered with receptor surface expression but both altered function, since ADP inhibited the forskolin-induced increase of cAMP markedly less in cells transfected with either mutant P2Y₁₂ as compared with wild-type receptor. These studies delineate a region of P2Y₁₂ required for normal function after ADP binding.

ADP | platelet function disorder | G-protein coupled receptors | platelet aggregation

Platelets possess at least two P2 receptors whose combined action is required for full activation and aggregation in response to stimulation by ADP (1, 2). One receptor, P2Y₁, is coupled to the heterotrimeric GTP-binding protein G_q and to phospholipase C- β ; it induces mobilization of cytoplasmic calcium and mediates shape change followed by an initial wave of rapidly reversible aggregation (3, 4). The other, P2Y₁₂, is negatively coupled to adenylyl cyclase through G_i; it mediates progressive and sustained platelet aggregation in the absence of shape change (2, 3, 5) and plays an important role in the potentiation of secretion induced by several agonists (6, 7). P2Y₁₂ is the therapeutic target of ticlopidine and clopidogrel (8), two platelet aggregation inhibitors used for the prevention and treatment of arterial thrombosis (9), and its congenital deficiency results in a bleeding disorder (7, 10, 11). Platelets deficient in P2Y₁₂ exhibit normal ADP-induced shape change but only slight and rapidly reversible aggregation, as well as a failure of ADP to inhibit the rise of cAMP levels after stimulation with prostaglandin E₁ (PGE₁) (1). The P2Y₁₂ defect is inherited as an autosomal recessive trait (1), and heterozygous patients display a mild abnormality of platelet function similar to that seen in the relatively common primary secretion defects. These are a heterogeneous group of congenital platelet alterations characterized by reduced platelet secretion despite normal thromboxane production and storage granule content (12). In some of these patients, platelets that produce normal amounts of thromboxane

A₂ fail to respond to the agonist because of defects in the corresponding receptor, but in many others the pathogenetic mechanism is poorly defined (12). Thus, congenital P2Y₁₂ defects may be more common than presently recognized, but they may be mistakenly identified with other disorders of platelet function (13).

The P2Y₁₂ locus has been analyzed in some patients in whom the platelet binding sites for ADP and the nonhydrolyzable ADP analogue 2-methylthioadenosine 5'-diphosphate (2MeS-ADP) were reduced by 70–80% (7, 11, 14). In two instances, different homozygous frameshift mutations were found to cause premature termination of translation.^{||} In another case, one allele presented a reading frame shift caused by the deletion of two nucleotides, whereas the other had a normal coding sequence but reduced expression, possibly resulting from another mutation in a regulatory region of the gene (15). In this report we describe a patient with a congenital bleeding disorder who was a compound heterozygote for two distinct amino acid substitutions that independently impair all of the responses mediated by P2Y₁₂ without affecting ADP binding. These two closely spaced residues define a structural motif that appears to be crucial for postoccupancy signaling in a seven-transmembrane-domain receptor.

Materials and Methods

Patients. The proband (AC) is a 60-year-old white male with a lifelong history of easy bruising and excessive posttraumatic and postsurgical blood loss. His 28-year-old daughter (MC) and 30-year-old son (FC), who had never suffered abnormal bleeding episodes, were also investigated. The proband, his family members, and 63 healthy controls (25 men and 30 women; median age 38 years, range 25–52 years) were informed of the investigational purpose of these studies and all gave their consent according to the Declaration of Helsinki. All subjects entered in the study had normal platelet counts, coagulation tests (prothrombin time and activated partial thromboplastin time), and plasma levels of von Willebrand factor antigen and ristocetin cofactor activity. The

Abbreviations: PGE₁, prostaglandin E₁; 2MeS-ADP, 2-methylthioadenosine 5'-diphosphate; A2P5P, adenosine 2'-phosphate 5'-phosphate; U46619, 9,11-dideoxy-11 α ,9 α -epoxy-methanoprostaglandin F_{2 α} ; PAF-acether, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); PGI₂, prostaglandin I₂; IBMX, 3-isobutyl-1-methylxanthine; PRP, platelet-rich plasma; [Ca²⁺]_i, cytoplasmic concentration of calcium ions; CHO cells, Chinese hamster ovary cells; TM6, sixth transmembrane domain; EL3, third extracellular loop; GPCR, G-protein-coupled receptor.

^{*}To whom correspondence may be sent at present address: Unit of Hematology and Thrombosis, Department of Medicine, Surgery and Dentistry, Ospedale San Paolo, University of Milan, Via di Rudini, 8, 20142 Milan, Italy. E-mail: marco.cattaneo@unimi.it.

[†]To whom correspondence may be addressed at: MEM 175, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: ruggeri@scripps.edu.

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template bleeding time (Symplate II; Organon Teknika, Durham, NC), was 19 and 30 min in two different determinations in AC (normal range: 2–8 min); it was 7.0 min in MC and 6.5 min in FC. Ristocetin-induced platelet agglutination, serum thromboxane B₂ levels, and the platelet contents of serotonin, ADP, ATP, and fibrinogen were normal in all subjects.

Materials. 2MeS-ADP was from Boehringer Mannheim. [³³P]2MeS-ADP was from DuPont/NEN and [³H]2MeS-ADP was from Amersham Pharmacia. ADP, adenosine 2'-phosphate 5'-phosphate (A2P5P), epinephrine, arachidonic acid, collagen, the thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F_{2 α} (U46619), platelet-activating factor (PAF-acether), prostaglandin I₂ (PGI₂), PGE₁, acetylsalicylic acid (ASA), forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma. Arachidonate was prepared under a stream of nitrogen to prevent oxidative changes. AR-C69931MX, a P2Y₁₂ antagonist (16), was a gift from AstraZeneca R&D (Charnwood, U.K.). Fura-2/AM was from Calbiochem. Apyrase was a gift of R. L. Kinlough-Rathbone (McMaster University, Hamilton, ON, Canada). Commercial preparations of luciferin/luciferase (ATP Assay Kit, BioOrbit Oy, Turku, Finland) were used to measure platelet ATP/ADP content and secretion concurrently with aggregation (Chronolume, Chrono-log, Havertown, PA). The RIA kit for measurement of thromboxane B₂ was from New England Nuclear.

Preparation of Platelet-Rich Plasma (PRP) and Washed Platelet Suspensions. For the preparation of PRP, 9 vol of blood was drawn into 1 vol of 129 mM trisodium citrate; for the preparation of washed platelets, 6 vol of blood was drawn into 1 vol of acid-citrate-dextrose anticoagulant. Twice-washed platelets were prepared according to the method of Mustard *et al.* (17), with the exception that 500 nM PGI₂ was added during the first and second washes. After the final resuspension in the absence of PGI₂ these washed platelets had normal cAMP levels, indicating that the PGI₂ effect was reversible in time. Platelet counts were adjusted to 3×10^{11} per liter in PRP and to 4×10^{11} per liter in washed platelet suspensions. For the measurement of cytoplasmic concentrations of calcium ions ([Ca²⁺]_i), platelets were loaded with 2 μ M fura-2/AM for 45 min at 37°C as previously described (7).

Studies of Platelet Aggregation and Secretion. Samples of PRP (0.45 ml) were incubated with 50 μ l of luciferin/luciferase reagent at 37°C for 30 sec and stirred at 1,000 rpm in a Chronolume lumiaggregometer. Ten microliters of an aggregating agent was then added and the aggregation and ATP secretion tracings were recorded for 3 min.

Measurement of Platelet Shape Change. Platelet shape change induced by 1 μ M ADP was measured in the aggregometer in citrated PRP to which 2 mM EDTA had been added to prevent platelet aggregation. Decrease in oscillations of the basal tracings and increase in optical density were interpreted as being caused by a platelet shape change.

Measurement of [Ca²⁺]_i. Aliquots of fura-2/AM-loaded platelets were transferred to quartz cuvettes at 37°C. Fluorescence was monitored continuously before and after ADP stimulation by using a spectrofluorimeter (LS50B, Perkin-Elmer). The excitation wavelength was alternately fixed at 340 and 380 nm, and emitted light was measured at 510 nm.

Binding of [³³P]2MeS-ADP to Washed Platelets. [³³P]2MeS-ADP (0.1 nM; 872–1044 Ci/mmol; 1 Ci = 37 GBq) was mixed with increasing concentrations of unlabeled ligand (0–49.9 nM) and

incubated with washed platelet suspensions (1-ml final volume) at 37°C for 5 min. After bound and free ligand had been separated by filtration through Whatman GF/C glass filters under vacuum, platelet bound radioactivity was measured by scintillation counting (Minibeta 1211; LKB). Nonspecific binding, determined by incubation in the presence of 1 μ M 2MeS-ADP, was 1–3% of total binding. Data were analyzed by a computer program (LIGAND) (18).

Measurement of Platelet cAMP. Platelet cAMP was measured by a radioisotopic assay, using a commercially available kit (Amersham Pharmacia). Duplicate samples of 1 ml of citrated PRP containing 1 mM theophylline were incubated with Tyrode's buffer and PGE₁ (1 μ M), Tyrode's buffer, PGE₁, and ADP or epinephrine (0.01, 0.1, and 1.0 μ M), or Tyrode's buffer alone in a control mixture. After incubation at 37°C (2 min), 1 ml of 5% trichloroacetic acid was added, and the samples were snap-frozen in dry ice and methanol, thawed at room temperature (22–25°C), and then shaken at 4°C for 45 min. After centrifugation at 4°C for 30 min, the supernatant was extracted three times with 5 ml of water-saturated ether, dried under a stream of nitrogen at 60°C, and stored at –20°C. Before assay, the samples were reconstituted with 0.05 M Tris buffer, pH 7.4, containing 4 mM EDTA.

Genetic Studies. Genomic DNA was isolated from blood lymphocytes by using standard procedures (19). The entire coding sequence of the P2Y₁₂ gene was amplified by a single PCR. The amplified fragments were subcloned into pCR2.1 (Invitrogen) and subjected to DNA sequence analysis. PCR was performed with the following primers: forward, 5'-CCTTAGGCTGAAAATAACCATCCTC-3' and reverse, 5'-GCGCTTTGCTTTAACGAGTTCTGAAC-3'. These primers can be found in GenBank accession no. AC024886.20 and correspond to nucleotides 127532–127556 (forward) and 128650–128675 (reverse). Restriction digestion fragments of the PCR products were analyzed with enzymes from New England Biolabs according to the supplier's instructions.

Heterologous Cell Expression of Recombinant P2Y₁₂. Mammalian cell expression vectors were constructed by moving the PCR-generated inserts present in pCR2.1 into the expression vector pcDNA3.1(–) (Invitrogen). Three different plasmids were generated with the published P2Y₁₂ coding sequence (wild-type) or the mutants described here, P2Y₁₂(R256Q) and P2Y₁₂(R265W). Each recombinant plasmid was purified through CsCl density gradients before transfection into Chinese hamster ovary (CHO)-K1 cells. Plasmid DNA was transfected into CHO-K1 cells by using the Transfast reagent (Promega) according to the supplier's recommended protocol. CHO-K1 cells were maintained in a 5% CO₂/95% atmosphere and grown in Dulbecco's MEM (DMEM) supplemented with 10% FCS, 0.5 mM each nonessential amino acid, 2 mM L-glutamine, 100 units/ml penicillin, and 0.25 μ g/ml Fungizone (BioWhittaker). Three weeks after transfection, antibiotic-resistant clones were isolated, expanded, and tested for the surface expression of P2Y₁₂, which was determined by measuring the binding of [³H]2MeS-ADP to individual CHO-K1 colonies. Briefly, transfected cells (5×10^5 per ml) were incubated with 11.9 nM [³H]2MeS-ADP for 10 min at 22°C. The reactions were stopped by addition of ice-cold phosphate-buffered saline (PBS), and the cells were immediately filtered through Whatman GF/B glass fiber filters. The filters were washed three times with PBS, and the remaining radioactivity was measured by liquid scintillation counting. Nonspecific binding was measured by adding 9.5 μ M 2MeS-ADP to the mixture; specific binding corresponded to the difference between total binding and nonspecific binding. The cell lines selected for subsequent studies, whether expressing normal

Fig. 1. Platelet aggregation induced by the indicated concentrations of ADP in citrated PRP from patient AC and a normal volunteer (traces labeled A). AR-C69931MX, a specific P2Y₁₂ antagonist, or PBS was added to PRP samples 1 min before the addition of ADP (traces labeled B). The tracings are representative of three experiments that gave similar results.

P2Y₁₂ sequence or each of the two mutants, had similar binding capacities for [³H]2MeS-ADP. These clones were further expanded and rechecked for their ability to bind similar levels of [³H]2MeS-ADP before cAMP analysis.

Measurement of cAMP in Transfected Heterologous Cells. CHO-K1 cells expressing recombinant P2Y₁₂ were seeded into 12-well plates (2×10^5 per well) containing complete DMEM supplemented with 1 unit/ml apyrase. After 48 h cells were washed twice with DMEM and returned to the incubator with 1 ml of DMEM. After 1 h cells were incubated at 37°C (10 min) with DMEM containing 0.5 mM IBMX. Cells were incubated for an additional 10 min with 10 μM forskolin, with or without increasing concentrations of ADP (0.1, 1, and 10 μM). The reactions were stopped by removing the medium and adding 1 ml of boiling water. The plates were kept at 22°C for 5 min, and then placed on ice. Cells were removed from the plate by scraping, and then pelleted (10,000 × g, 1 min). Levels of cAMP were determined from the supernatants in an RIA (Amersham Pharmacia Biotech cAMP [¹²⁵I] assay system) following the supplier's instructions.

Results

Platelet Shape Change, Aggregation, and Secretion. The platelets from patient AC changed shape normally after stimulation with 1 μM ADP, an effect inhibited by the P2Y₁ antagonist A2P5P (1 mM) (not shown); they also exhibited aggregation in response to 4 μM ADP that was markedly lower than normal and rapidly reversible (Fig. 1). The specific P2Y₁₂ antagonist AR-C69931MX rendered the response of normal platelets similar to that of patient platelets, but had no effect on the latter (Fig. 1). Higher concentrations of ADP (20 μM) induced a more pronounced, but still abnormally low, aggregation of patient platelets that was reduced by the P2Y₁₂ antagonist, suggesting the presence of residual receptor function (Fig. 1). The aggregation of these platelets in response to different agonists was also abnormal, as was that induced by 0.01–1.0 μM 2MeS-ADP (not shown). Little or no ATP secretion occurred from patient platelets stimulated with ADP and other agonists (Table 1).

ADP-Induced Increase in [Ca²⁺]_i. Stimulation of washed platelet suspensions with 5 μM ADP caused a rapid increase in [Ca²⁺]_i that was similar in the patient's platelets and in seven normal controls, both in the presence of 2 mM external CaCl₂ and in the absence of external Ca²⁺ (1 mM EDTA) (Table 2). The ADP-induced increase in [Ca²⁺]_i was abolished by 0.5 mM A2P5P, which blocks P2Y₁ function, in both the patient's and normal platelets (Table 2).

Table 1. Platelet ATP secretion induced by various agonists

Agonist	ATP, nmol/10 ⁹ platelets	
	AC*	Normal range†
ADP (2 μM)	ND	1.1–5.2
PAF-acether (0.2 μM)	ND	1.1–6.1
Collagen (2 μg/ml)	1.5	1.6–7.8
Arachidonate (0.7 mM)	ND	1.7–6.0
U46619 (1 μM)	0.6	1.3–5.6
Epinephrine (5 μM)	ND	1.4–5.1

PRP containing trisodium citrate as anticoagulant was stimulated by various agonists at the indicated concentrations.

*Mean of two experiments. ND, not detectable.

†The normal range is defined by the 2.5th and 97.5th percentile of the distribution of values obtained in 39 healthy subjects.

Inhibition of PGE₁-Induced Increase in Platelet cAMP. The basal level of cAMP in the platelets from patient AC was normal (12 ± 2.2 pmol per 10⁹ platelets, mean $\pm$ SD of four experiments; normal range: 6–17.9; $n = 17$) and increased normally after stimulation with 1 μM PGE₁ (68.3 ± 10.8 pmol per 10⁹ platelets; normal range: 22.5–71.4). ADP, in a concentration-dependent manner, inhibited the increase in production of cAMP by normal platelets exposed to PGE₁, but this response was greatly impaired in the patient platelets, and only a marginal inhibitory effect was detectable with the highest concentration of ADP tested (1 μM) (Fig. 2A). In contrast to ADP, epinephrine normally inhibited the production of cAMP in patient platelets exposed to PGE₁ (Fig. 2B), suggesting that the defect could be attributed to an abnormality in the platelet P2Y₁₂ receptor responsible for coupling specifically ADP stimulation to adenylyl cyclase inhibition.

Binding of [³³P]2MeS-ADP to Washed Platelet Suspensions. The binding of [³³P]2MeS-ADP to patient platelets was measured in two separate experiments and compared with that observed in 34 normal volunteers. The specific binding was saturable in both cases, and Scatchard plot analysis yielded a linear fit. The numbers of binding sites on normal and patient platelets were similar, as were the K_d values (Table 3).

Characterization of the P2Y₁₂ Gene. The observation that the patient's platelets had a normal number of binding sites for 2MeS-ADP despite a severely impaired function of P2Y₁₂ suggested that a dysfunctional receptor was being synthesized in normal amounts. To identify the underlying structural changes

Table 2. ADP-induced increase of platelet cytoplasmic Ca²⁺

Sample	[Ca ²⁺] _i , nM	
	Patient AC	Controls*
With CaCl ₂ (2 mM)		
Saline	74	79 (46–130)
ADP	294	465 (312–551)
A2P5P + saline	80	70 (59–109)
A2P5P + ADP	87	70 (61–109)
With EDTA (1 mM)		
Saline	103	75 (49–105)
ADP	327	480 (236–577)
A2P5P + saline	64	71 (41–119)
A2P5P + ADP	64	71 (58–109)

*The results of normal controls ($n = 7$) are expressed as median values with the observed range in parentheses. Washed platelets loaded with fura-2/AM were suspended in Tyrode's buffer without added CaCl₂. ADP was used at the final concentration of 5 μM; A2P5P at 0.5 mM.

Fig. 2. Effect of increasing concentrations of ADP (A) or epinephrine (B) on the inhibition of PGE₁-induced cAMP accumulation in platelets. One micromolar PGE₁ was added to platelets from patient AC and 17 normal volunteers. The results in AC are expressed as the mean of four experiments for stimulation with ADP, and two experiments for stimulation with epinephrine. Vertical bars indicate the SEM.

we analyzed the entire coding sequence for the P2Y₁₂ polypeptide (Fig. 3). DNA from the patient showed two missense mutations, a G-to-A transition at nucleotide 1011 (numbering according to GenBank sequence with accession no. BC017898) that changed the codon for Arg-256 (CGA) to Gln (CAA) and abolished an *Eco*RI restriction site, and a C-to-T transition at nucleotide 1037 that changed the codon for Arg-265 (CGG) to Trp (TGG) and abolished an *Hpa*II restriction site. Because the coding sequence in the P2Y₁₂ gene is contained in a single exon and was amplified by using one set of primers and a single PCR, we can conclude that the two mutations identified in the patient are on different alleles.

Heterologous Expression of Mutant and Wild-Type P2Y₁₂ Receptors.

To confirm that the two mutations identified in the patient were the cause for the functional defect of P2Y₁₂, protein with normal or mutant sequence was expressed in CHO-K1 cells and evaluated for the effect of ADP stimulation on adenylyl cyclase activity. At all tested concentrations (0.1–10 μM), ADP inhibited the forskolin-induced increase of cAMP in cells transfected with wild-type P2Y₁₂; in contrast, it had no effect in cells transfected with either P2Y₁₂(R256Q) or P2Y₁₂(R265W) when used at concentrations between 0.1 and 1.0 μM, and caused only marginal inhibition at 10 μM (Fig. 4).

Table 3. Parameters of [³³P]2MeS-ADP binding to platelets

Parameter	Patient AC		Normal range
	Exp. 1	Exp. 2	
Sites/platelet	754	629	454–1142
K _d , mM	6.7	8.3	2.3–7.7

The normal range is defined by the 2.5th and 97.5th percentile of the distribution of values obtained in 34 healthy subjects.

Fig. 3. Restriction enzyme analysis of the P2Y₁₂ gene in patient AC and a normal control. (A) A PCR was performed to generate a 1,144-bp fragment encompassing the entire coding sequence of the P2Y₁₂ polypeptide. In AC's DNA, a G-to-A transition at nucleotide 1011 abrogates an *Eco*RI restriction site, and a C-to-T transition at nucleotide 1037 abrogates a *Hpa*II restriction site; both restriction sites are present in the normal DNA. Presence (+) or absence (–) of the restriction enzyme is designated at the bottom of each lane. Lengths in bp are given on the left. (B) Diagrammatic representation of the PCR product generated from the P2Y₁₂ gene. The position and length of the fragments produced after digestion with *Eco*RI or *Hpa*II are shown. Asterisks indicate the restriction enzyme sites abrogated in the patient. The results demonstrate that AC is a compound heterozygote.

Studies of Heterozygous Carriers of a Dysfunctional P2Y₁₂ Receptor.

The two children of the proband (MC and FC) were found to have one allele coding for normal P2Y₁₂ and one coding for the R265W mutant. This mode of genetic transmission confirmed that the two mutations identified in the father were on separate alleles. Platelets from either MC or FC exhibited reversible aggregation when stimulated by 2 or 4 μM ADP, a response that was similar to that of normal platelets incubated *in vitro* with 0.5 mM acetylsalicylic acid for 30 min. Higher concentrations of ADP (up to 20 μM) induced normal platelet aggregation in both cases. The aggregation induced by 0.2 μM PAF-acether was reversible, whereas that induced by epinephrine was monophasic; collagen (2 μg/ml) and the thromboxane A₂ analogue U46619 (1 μM) induced normal aggregation in both MC and FC.

Fig. 4. Effects of increasing concentrations of ADP on the increase in cAMP levels induced by 10 μM forskolin in CHO cells transfected with wild-type P2Y₁₂ (WT), P2Y₁₂(R256Q), or P2Y₁₂(R265W). The results are means of four experiments. For better clarity, SEM bars are not shown; they ranged between 9.1% and 18.7% in WT cells, and between 5.6% and 12.4% in R256Q and R265W cells. The three lines of transfected CHO cells bound similar amounts of [³³H]2MeS-ADP (mean ± SD; n = 4): WT, 61 ± 20 cpm per 10³ cells; R256Q, 50 ± 8; and R265W, 76 ± 14; P > 0.05.

Table 4. Parameters of platelet function in the daughter (MC) and son (FC) of patient AC

	MC	FC	Normal range
ATP secretion, nmol/10 ⁹ platelets			
ADP (2 μM)	ND	ND	1.1–5.2
PAF-acether (0.2 μM)	ND	ND	1.1–6.1
Collagen (2 μg/ml)	2.1	4.8	1.6–7.8
Arachidonate (0.7 mM)	2.4	2.4	1.7–6.0
U46619 (1 μM)	1.3	2.2	1.3–5.6
Epinephrine (5 μM)	ND	ND	1.4–5.1
Inhibition of PGE ₁ -induced increase in cAMP, %			
ADP			
0.01 μM	13	6	0–23
0.1 μM	12	11	20–46
1.0 μM	57	52	39–80
Epinephrine			
0.01 μM	17	26	7–28
0.1 μM	52	60	25–63
1.0 μM	70	85	49–85
Parameters of [³³ P]2MeS-ADP binding to washed platelets			
Sites/platelet	852	1152	454–1142
K _d , nM	6.4	7.6	2.3–7.7

Results of patients MC and FC are expressed as mean values of two separate experiments. The normal range is defined by the 2.5th and 97.5th percentile of the distribution of values obtained in healthy subjects ($n = 39$ for ATP secretion; $n = 17$ for cAMP inhibition; $n = 34$ for [³³P]2MeS-ADP binding). ND, not detectable.

ATP secretion was absent or severely reduced when platelets were stimulated by ADP (2–4 μM), epinephrine (5 μM), or low concentrations of PAF-acether (0.2 μM), was borderline-low when platelets were stimulated by a higher concentration of PAF-acether (2 μM), and was normal when platelets were stimulated by collagen (2 μg/ml) or U46619 (1 μM) (Table 4). The basal cAMP levels in the platelets of MC and FC were normal and increased normally after stimulation with PGE₁ (from 15 to 50 and from 14 to 55 pmol per 10⁹ platelets in MC and FC, respectively). At 0.01 μM, both ADP and epinephrine caused little inhibition of the PGE₁-induced increase of cAMP levels in platelets from normal controls as well as from MC and FC (Table 4). At the intermediate concentration used (0.1 μM), epinephrine caused a similar inhibition in MC, FC, and normal platelets, whereas the inhibitory effect of ADP was below normal in the platelets from MC and FC (Table 4). At the higher concentration of 1.0 μM, both ADP and epinephrine were as effective in the platelets from MC and FC as they were in normal platelets (Table 4). Finally, as seen in the propositus, the number of sites and K_d for binding of 2MeS-ADP to platelets were normal in both MC and FC (Table 4).

Discussion

The P2Y₁₂ receptor for ADP is important for platelet function, as shown by the fact that its deficiency causes a bleeding diathesis (7) and its pharmacological inhibition reduces the risk of clinical events associated with arterial thrombosis (9–11). The defects of P2Y₁₂ described to date were characterized by decreased ADP binding to platelets caused by mutations that disrupt receptor synthesis (11, 15). In contrast, the patient described here presents abnormal P2Y₁₂-dependent platelet activation despite normal ADP binding to the membrane surface, consistent with the concept that agonist ligation is only the first in a sequence of events that include signal transduction through the membrane and transfer to the cytoplasm to induce cell activation. The platelets of patient AC, like those of previously reported patients with complete absence of P2Y₁₂ (7, 10, 11), changed shape normally when exposed to ADP, but underwent only a slight,

Fig. 5. Schematic representation of P2Y₁₂. Numbers in the open circles indicate the amino (1) and carboxyl (342) termini of the molecule; numbers in filled circles indicate the position of the two mutations identified in the propositus. TM, transmembrane domain; EL, extracellular loop; IL, intracytoplasmic loop. TM3, -5, -6, and -7, which contain residues necessary for nucleotide binding, are shaded.

rapidly reversible aggregation response and did not exhibit the normal inhibition of PGE₁-stimulated adenylyl cyclase. In addition, they did not undergo normal secretion when exposed to release-inducing platelet agonists. These findings are consistent with the presence on platelets of a dysfunctional P2Y₁₂ still able to bind ADP but unable to respond to the stimulus.

The defect observed in patient AC was milder than in previously described patients with reduced agonist binding, since the highest concentration of ADP tested evoked some inhibition of PGE₁-stimulated adenylyl cyclase and induced a slightly higher aggregation response than lower concentrations. In contrast, P2Y₁₂-deficient platelets typically failed to respond even to high concentrations of ADP. These findings suggest that the variant P2Y₁₂ of patient AC retains some activity, possibly indicating a finer regulation of receptor response to agonist ligation than allowed for by a simple on/off model. Of note, the observation that the heterozygous children of our patient exhibited parameters of platelet activation and aggregation considered typical of a primary secretion defect suggests that the underlying cause of at least some of these heterogeneous conditions (12) may be a specific defect in a P2Y₁₂-coupled signaling pathway.

Analysis of the P2Y₁₂ gene revealed that AC was a compound heterozygote for two missense mutations that caused the replacement of Arg-256 by Gln and Arg-265 by Trp. Surface expression of either P2Y₁₂ mutant receptor on CHO-K1 cells resulted in normal 2MeS-ADP binding but defective ADP-dependent inhibition of forskolin-stimulated adenylyl cyclase activity. The loss of function in each case was of similar severity, suggesting that the two Arg residues at positions 256 and 265 contribute independently to the generation of a signal through P2Y₁₂. The two mutations encompass a short sequence located between the extracellular end of the sixth transmembrane domain (TM6) and the beginning of the third extracellular loop (EL3; Fig. 5), a region of G-protein-coupled receptors (GPCRs) known to be important for function. For example, activation of rhodopsin (20) or β₂ adrenergic receptor (21, 22) appears to involve a rotation and/or tilting of TM6, which may be important for G-protein coupling. In addition, studies of the human A3 adenosine receptor (23) and muscarinic acetylcholine receptors (24) have identified regions that are critical for specific G-protein activation, and have suggested that TM6 may act as a switch defining the activation state of the receptor (25, 26). In various GPCRs, EL3 residues have been shown to influence receptor function or subtype specificity (24, 27–29). This region of the P2Y₁₂ molecule includes Arg-265, a residue homologous to Arg-287, which is important for the function of P2Y₁, the other

platelet ADP receptor (29, 30). Both our present and previous results, therefore, identify corresponding regions in two GPCRs activated by adenine nucleotides whose structural integrity is necessary for normal function.

The important role of Arg-287 in P2Y₁ function was demonstrated earlier by measuring agonist-induced [³H]inositol phosphate formation in transfected COS-7 cells (29, 30). Because a high-affinity radioligand specific for P2Y₁ was not easily available at the time, those studies could not distinguish between effects on agonist binding and G-protein coupling efficiency. Nonetheless, integration of the experimental findings with molecular modeling (30) led to the concept that accessory interaction sites for adenine nucleotides may exist in purinergic receptors with the function of orienting ligands properly and facilitating access to the main binding site formed by the transmembrane domains, principally TM3, -5, -6, and -7. One such accessory site in P2Y₁ was thought to include Arg-287 in EL3 (30). In this model, the contribution of accessory sites to receptor activity should precede agonist ligation at the main transmembrane cleft, and a defect of their function should reduce the amount of ligand bound or the affinity of interaction. Such was not the case in the patient we studied. The positive charge of Arg-265 in P2Y₁₂, which may interact with the phosphate groups of adenine nucleotides as proposed for Arg-287 in

P2Y₁ (30), was lost with the mutation to Trp but with no consequence for the binding of an ADP analogue to platelets or transfected CHO cells and no decrease in binding affinity. Rather, the mutation resulted in a marked reduction of cell activation apparently caused by defective signaling. EL3, where Arg-265 is located in P2Y₁₂, is in close proximity to TM6, whose movement appears to be important in the activation of many class I GPCRs. Our results suggest the possibility that changes in the conformation of EL3, possibly influenced by the interaction with ligand, may be propagated to TM6 and affect its role in receptor activation. The mutation of Arg-256, located at the end of TM6 immediately preceding EL3 on the cell surface (Fig. 5), may have similar effects. In conclusion, these studies have defined the phenotypic characteristics of defective P2Y₁₂ activation in platelets with normal ADP binding and have provided clues that clarify the structural requirements of the signaling mechanism through a P2 receptor.

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