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Article

ADP is a key agonist in hemostasis and thrombosis. ADP-induced platelet activation involves the purinergic $P2Y_1$ receptor, which is responsible for shape change through intracellular calcium mobilization. This process also depends on an unidentified P2 receptor (P2cyc) that leads to adenylyl cyclase inhibition and promotes the completion and amplification of the platelet response. $P2Y_1$-null mice were generated to define the role of the $P2Y_1$ receptor and to determine whether the unidentified P2cyc receptor is distinct from $P2Y_1$. These mice are viable with no apparent abnormalities affecting their development, survival, reproduction, or the morphology of their platelets, and the platelet count in these animals is identical to that of wild-type mice. However, platelets from $P2Y_1$-deficient mice are unable to aggregate in response to usual concentrations of ADP and display impaired aggregation to other agonists, while high concentrations of ADP induce platelet aggregation without shape change. In addition, ADP-induced inhibition of adenylyl cyclase still occurs, demonstrating the existence of an ADP receptor distinct from $P2Y_1$. $P2Y_1$-null mice have no spontaneous bleeding tendency but are resistant to thromboembolism induced by intravenous injection of ADP or collagen and adrenaline. Hence, the $P2Y_1$ receptor plays an essential role in thrombotic states and represents a potential target for antithrombotic drugs.

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Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor–null mice

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ADP is a key agonist in hemostasis and thrombosis. ADP-induced platelet activation involves the purinergic $P2Y_1$ receptor, which is responsible for shape change through intracellular calcium mobilization. This process also depends on an unidentified P2 receptor (P2cyc) that leads to adenylyl cyclase inhibition and promotes the completion and amplification of the platelet response. $P2Y_1$-null mice were generated to define the role of the $P2Y_1$ receptor and to determine whether the unidentified P2cyc receptor is distinct from $P2Y_1$. These mice are viable with no apparent abnormalities affecting their development, survival, reproduction, or the morphology of their platelets, and the platelet count in these animals is identical to that of wild-type mice. However, platelets from $P2Y_1$-deficient mice are unable to aggregate in response to usual concentrations of ADP and display impaired aggregation to other agonists, while high concentrations of ADP induce platelet aggregation without shape change. In addition, ADP-induced inhibition of adenylyl cyclase still occurs, demonstrating the existence of an ADP receptor distinct from $P2Y_1$. $P2Y_1$-null mice have no spontaneous bleeding tendency but are resistant to thromboembolism induced by intravenous injection of ADP or collagen and adrenaline. Hence, the $P2Y_1$ receptor plays an essential role in thrombotic states and represents a potential target for antithrombotic drugs.

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Introduction

ADP, known to be secreted from cells such as erythrocytes (1), plays a key role in hemostasis by directly stimulating platelet aggregation, and through its secretion from platelet dense granules it potentiates the aggregation response induced by other agents (2, 3). Stimulation of platelets by ADP leads to a rapid influx of calcium from the external medium, the mobilization of calcium from intracellular stores and inhibition of adenylyl cyclase (3). Our understanding of the molecular basis of ADP-induced platelet activation has improved with the identification of purinoceptors involved in these processes. In previous work we showed that the metabotropic $P2Y_1$ receptor, originally thought to be an adenosine triphosphate (ATP) receptor (4), is in fact an ADP receptor antagonized by ATP and its analogues and is present on blood platelets (5, 6). Apart from platelets, this receptor is present in many other tissues including brain, heart, skeletal muscle, smooth muscle, endothelium, and placenta (7). The physiological roles of this widespread distribution is not known to date. However, in the cardiovascular system a critical role for extracellular nucleotides in the regulation of the vascular tone is well established (7).

The $P2Y_1$ receptor is necessary for ADP to induce platelet aggregation through an increase in intracellular calcium because its inhibition by selective antagonists such as adenosine-2′-phosphate-5′-phosphate (A2P5P) and adenosine-3′-phosphate-5′-phosphate (A3P5P) (8) totally abolishes ADP-induced platelet aggregation, shape change, and calcium mobilization (9–11). On the other hand, antithrombotic drugs like the thienopyridine clopidogrel or the ATP analogue AR-C66096 (12) inhibit ADP-induced platelet aggregation by acting on the adenylyl cyclase pathway, but without affecting calcium mobilization or shape change (13–17). This suggests that another P2 receptor could mediate ADP-induced inhibition of adenylyl cyclase (9–11, 13–18). Additional support for a second receptor is derived from studies of patients suffering from congenital bleeding disorders with a selective impairment of ADP-induced platelet aggregation resembling thienopyridine treatment (19, 20). These

patients appear to have a deficiency of the platelet ADP receptor coupled to inhibition of adenylyl cyclase; the $P2Y_1$ receptor has been shown to be normal in one of the patients (21). This still-unidentified P2 receptor is thought to be coupled to a G protein of α_{i2} subtype (22) and hence could belong to the P2Y metabotropic receptor family. A full aggregation response to ADP requires activation of both P2 receptors (10, 11, 23), but it is not yet clear how synergy between these 2 receptors may occur. In addition, to date no molecular entity has been reported to have the properties of a P2 receptor negatively coupled to adenylyl cyclase. Thus, doubts still remain as to whether the $P2Y_1$ receptor may exist in different conformations, which could account for a differential coupling to the Gq or Gi alpha subunits of G proteins. Platelets also express the ionotropic $P2X_1$ receptor (24–28). Its role in ADP-induced platelet aggregation is unknown and seems to be discrete, although this receptor is responsible for a unique fast calcium entry into platelets (24, 29). The aims of the present study were (a) to define the role of the $P2Y_1$ receptor in hemostasis and thrombosis and (b) to determine whether or not the unidentified P2 receptor coupled to inhibition of adenylyl cyclase is distinct from the $P2Y_1$ receptor and independently expressed in platelets. We report that targeted disruption of the $P2Y_1$ receptor results in impaired platelet aggregation and inhibition of acute thrombosis induced by infusion of ADP or collagen and adrenaline. We also show that platelets from $P2Y_1$-deficient mice are still responsive to ADP through the P2 receptor coupled to inhibition of adenylyl cyclase.

Methods

Materials. ADP was purchased from Roche Molecular Biochemicals (Meylan, France). U46619, thrombin, fura-2/acetoxymethyl ester (fura-2/AM), adrenaline, and type I bovine collagen (for aggregation studies) were from Sigma (Saint-Quentin Fallavier, France). Equine collagen (Kollagenreagent Horm), used in the thromboembolism experiments, was purchased from Hormon Chemie (München, Germany). Human fibrinogen was from Kabi (Stockholm, Sweden). AR-C69931MX was kindly provided by Astra Charnwood (Loughborough, United Kingdom), and clopidogrel was from Sanofi Recherche (Toulouse, France). The radioimmunoassay cAMP measurement kit was purchased from Amersham Pharmacia Biotech (Les Ulis, France).

Targeted inactivation of the $P2Y_1$ gene. A 0.57-kb cDNA probe corresponding to mouse $P2Y_1$ cDNA was prepared by PCR after reverse transcription of total RNA isolated from mouse hearts. The PCR primers were derived from the published sequence of mouse $P2Y_1$ cDNA (30) (sense primer: 5′-CCGGTTTCCAGTTCTACTACCTGCCG-3′; and antisense primer: 5′-CATAACAGCCCAAGATCAGCACCAAAGG-3′). This probe was used to screen a 129/Sv genomic library (kind gift of P. Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). A 12.6-kb fragment was cloned and entirely sequenced in both directions (accession no. AJ245636). The mouse $P2Y_1$ gene contains 1 intron as compared with the mouse cDNA cloned previously (30), located in the 3′ untranslated region at position 4228 to 6524. Six polyadenylation signals are present at base 4401, 4611, 9721, 10610, 10636, and 11842. The first 6 kb containing the entire coding sequence was subcloned into pBluescript-KS (Stratagene, La Jolla, California, USA), and the coding sequence was interrupted by insertion of a PGK-neo-poly(A) cassette (gift of M. McBurney, University of Ottawa, Ottawa, Ontario, Canada) in position 3208 (Figure 1a). The targeting vector was linearized and electroporated into the P1 embryonic stem (ES) cell line (129/Sv mouse strain; IGBMC, Strasbourg, France) and neomycin-resistant clones were isolated and screened by PCR for homologous recombination. Oligonucleotides used were: neomycin resistance gene 5′-ACTCTGGGGTTCGAAATGACCGACCAAGCG-3′ and 5′-GCCTTCTATCGCCTTCTTGACGAGTTCTTC-3′; $P2Y_1$ gene 5′-CTTCATGGACCTGAGTAATAAGACATTAGGC-3′ and 5′-AAATGGAAGCACTCCAAAAATCTCCCAGATC-3′. Southern blot analyses were performed to confirm the PCR results, using as a probe a fragment of the $P2Y_1$ gene from base 6847 to base 8179, outside the construction, which distinguishes between the wild-type allele (12 kb) and the recombinant allele (9.5 kb).

Generation of $P2Y_1$-deficient mice. Targeted ES cells were introduced into C57BL/6 mouse blastocysts as described by Lufkin et al. (31). Male chimeras were mated with C57BL/6 females and heterozygous offspring (50% 129/Sv and 50% C57BL/6 strain) were intercrossed to obtain animals homozygous for the null mutation ($P2Y_1^{-/-}$ mice) and homozygous for the wild-type allele ($P2Y_1^{+/+}$ mice). Both control $P2Y_1^{+/+}$ and $P2Y_1^{-/-}$ mice used in this study were at the F_3 generation, resulting from intercrossing between several homozygous F_2 littermates.

Bleeding time. Male and female mice (20–30 g) were anesthetized by intraperitoneal injection of 150 μL of a mixture of 0.08% xylazine base (Rompun, Bayer, France) and 1.6% ketamine (Imalgen 1000; Mérial, Lyon, France). A cut was made longitudinally in the mouse tail using a Simplate device (Organon Teknika B.V., Boxtel, The Netherlands). The tail was immediately immersed in 0.9% isotonic saline at 37°C. The bleeding time was defined as the time required for arrest.

Platelet aggregation. Blood was drawn from the abdominal aorta into acid-citrate-dextrose solution (ACD) (1 vol ACD/6 vol blood) and pooled (10 mL). Washed platelet aggregation was measured turbidimetrically as described previously (11). Final platelet suspension was adjusted to 2.10^5 platelets/μL and kept at 37°C. Fibrinogen (0.2 mg/mL) was added before stimulation by ADP.

Calcium measurements. Washed platelets were loaded with fura-2/AM, and intracellular calcium movements were measured in a spectrofluorometer PTI Deltascan (Photon Technology International Inc., South Brunswick, New Jersey, USA) using excitation wave-

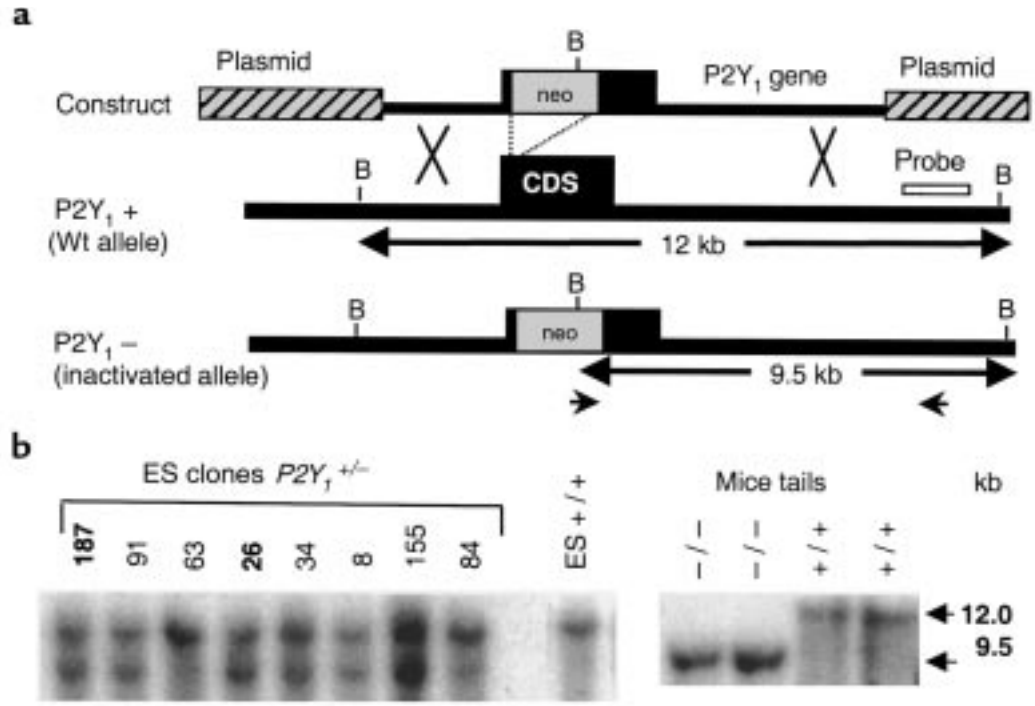


Figure 1
Disruption of the $P2Y_1$ receptor gene in mice. (**a**) Gene targeting strategy: structure and partial restriction map of the $P2Y_1$ gene, targeting construct, and targeted allele. The filled box represents the coding sequence. Restriction endonuclease sites: B, *Bam*HI; arrowheads, PCR primers. (**b**) Southern blot analyses of ES clones identified as positive by PCR and of tail DNA from $P2Y_1^{-/-}$ and $P2Y_1^{+/+}$ mice. The 12-kb wild-type and 9.5-kb targeted *Bam*HI fragments were identified using a probe located outside the 3′ limit of the construct (shown in **a**). Clones 187 and 26 (bold) were selected to be injected into blastocysts.

lengths of 340 and 380 nm and fluorescence emission detection at 510 nm, as described previously (11).

Cyclic AMP measurements. Platelets (8.10^4/μL) were stimulated at 30-second intervals with either vehicle (Tyrode's buffer without Ca^{2+} or Mg^{2+}) (11), or various agonists, or antagonists, in a final volume of 250 μL. The reaction was stopped by addition of 50 μL of 6.6 M perchloric acid, and intracellular cAMP was determined by radioimmunoassay (11).

Electron microscopy. Platelets were directly fixed in the aggregation cuvette with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 2% sucrose. Platelets were then processed as described previously (32). Ultrathin sections were observed under a Philips CM120 Biotwin electron microscope (Philips Consumer Electronics B.V., Eindhoven, The Netherlands) at 120 kV.

ADP-induced thromboembolism. The jugular vein of anesthetized male mice was exposed surgically, and increasing doses of ADP were injected intravenously within 3 to 4 seconds. After 50 to 60 seconds, blood was collected by intracardiac puncture, recovered in EDTA (6 mM), and platelets were counted optically under a phase-contrast microscope.

Thromboembolism induced by collagen and epinephrine. A mixture of collagen (0.5 mg/kg) and epinephrine (60 μg/kg) was injected into the jugular vein of anesthetized male mice (33). The incisions of surviving mice were stitched, and they were allowed to recover.

Results

Targeted disruption of the $P2Y_1$ receptor gene in ES cells by homologous recombination. The coding region of the mouse $P2Y_1$ gene was interrupted by insertion of a neomycin resistance gene cassette (Figure 1a), and homologous recombination events were screened. A targeting frequency of 4% was observed and 2 targeted ES clones were injected into blastocysts to generate chimeric mice. Mice homozygous for either the targeted allele or the wild-type allele (Figure 1b) were obtained by interbreeding the heterozygous offspring, with the expected mendelian frequency. Two strains of $P2Y_1$ knockout mice were generated with identical phenotypes. These mice are viable, have no apparent morphological or physiological abnormalities, and display normal development, survival, and reproduction. The platelet count as well as other hematological parameters are identical to those of wild-type mice (Table 1).

Table 1
Hematological parameters of $P2Y_1^{+/+}$ and $P2Y_1^{-/-}$ mice

Genotype	+/+	–/–
Platelets ($\times 10^4$/μL)	100 ± 12	98 ± 15
White blood cells ($\times 10^3$/μL)	4.9 ± 1.1	5.4 ± 1.0
Red blood cells ($\times 10^6$/μL)	9.1 ± 0.6	8.9 ± 1.2
Hematocrit (%)	46.4 ± 3.6	45.2 ± 5.4
Hemoglobin (g/dL)	14.6 ± 0.8	14.1 ± 1.1

Mean ± SD (n = 4, except for platelet counts where n = 10).

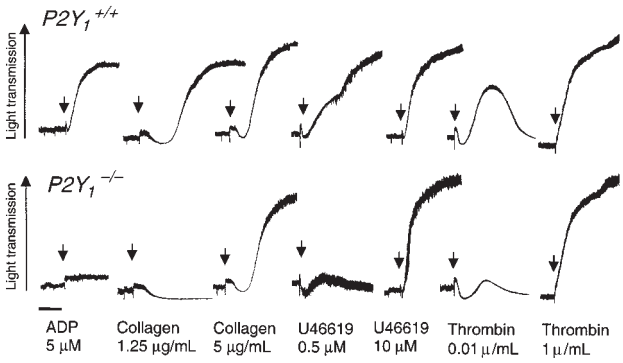


Figure 2
In vitro aggregation responses of $P2Y_1^{-/-}$ washed mice platelets as compared with wild-type. $P2Y_1^{-/-}$ mice platelet aggregation is lost in response to ADP and strongly impaired in response to 1.25 μg/mL collagen, whereas the lag phase, measured from the addition of the agonist to starting of aggregation, is prolonged in response to 5 μg/mL collagen (25 seconds in $P2Y_1^{-/-}$ versus 15 seconds in wild-type). In contrast, responses to thrombin and to U46619 were modified only at threshold concentrations. Bar: 1 minute.

Platelet aggregation is impaired in $P2Y_1$-deficient mice. Functional studies of platelets from $P2Y_1^{-/-}$ mice demonstrated a loss of ADP-induced aggregation and shape change at concentrations of ADP up to 10 μM, confirming the necessity of this receptor for ADP-induced platelet aggregation (Figure 2). Aggregation in response to 1.25 μg/mL collagen was strongly impaired, whereas the lag time in response to 5 μg/mL collagen was markedly prolonged in $P2Y_1^{-/-}$ mice (25 seconds) as compared with the wild type (15 seconds). Conversely, aggregation in response to the TXA_2 mimetic U46619 or to thrombin was affected only at subthreshold concentrations of agonists (0.5 μM and 0.01 U/mL, respectively) (Figure 2). Heterozygous mice displayed an intermediate phenotype, i.e., decreased responses to ADP and to collagen as compared with the wild-type mice, but unchanged responses to the other agonists whatever the concentration tested (data not shown).

A potentiation role of ADP in $P2Y_1^{-/-}$ mice through activation of the P2 receptor negatively coupled to adenylyl cyclase. Serotonin is a weak platelet agonist known to induce shape change only through activation of the $5HT_{2A}$ receptor and mobilization of intracellular calcium, which could be used to bypass the $P2Y_1$ receptor (10, 23). Simultaneous addition of ADP (5 μM) and serotonin (1 μM) restored aggregation to ADP in $P2Y_1^{-/-}$ platelets, suggesting the conservation of a potentiation role of ADP in $P2Y_1^{-/-}$ mice. (Figure 3a). This effect was due to activation of the P2 receptor negatively coupled to adenylyl cyclase because it was completely inhibited by addition of the ATP analogue AR-C69931MX (Figure 3a). This was further confirmed by the observation that whereas the calcium response induced by ADP was abolished in the knockout mice (Figure 3b), ADP still inhibited the PGE_1-stimulated accumulation of cAMP in intact platelets (Figure 3c), an effect inhibited by AR-C69931MX. These results demonstrate the existence of an ADP receptor, coupled to the inhibition of adenylyl cyclase, which is distinct from the $P2Y_1$ receptor.

Platelet aggregation by activation of the P2cyc receptor requires high concentrations of ADP. Addition of 100 μM ADP to washed platelets from $P2Y_1^{-/-}$ mice resulted in a slow, gradual increase of light transmission up to 20% above the baseline at 5 minutes (Figure 4a, upper-right tracing) because of formation of small platelet aggregates without optically detectable shape change. The number of platelets in the aggregates was estimated by transmission electron microscopy to be between 5 and 10 (Figure 4b, right panel). This "partial platelet aggregation" occurred without detectable calcium movements (Figure 3b) and was inhibited either by incubation with AR-C69931MX or by treatment of $P2Y_1^{-/-}$ mice with clopidogrel (Figure 4a, lower-right tracing),

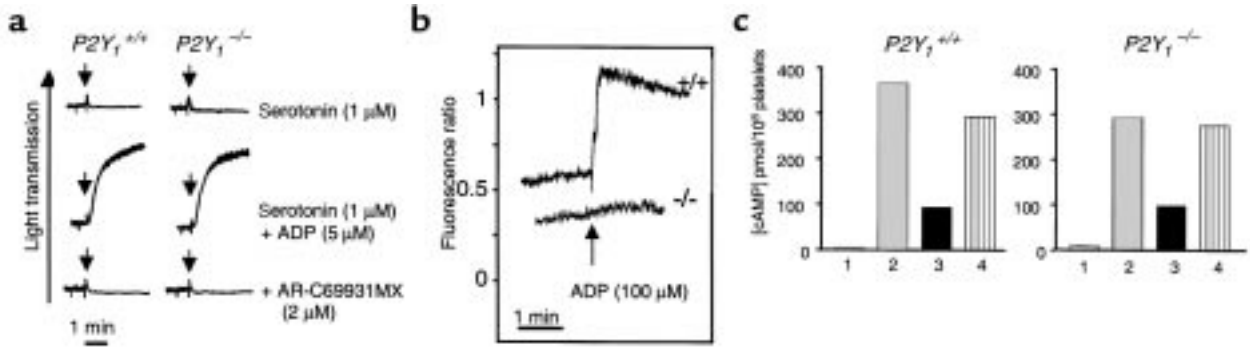
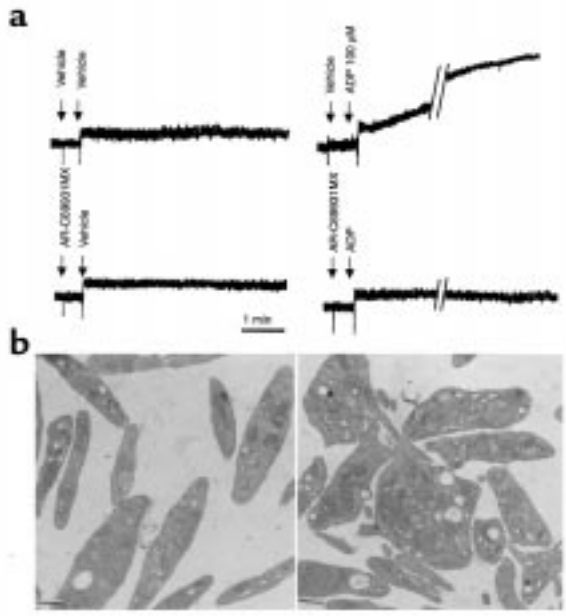


Figure 3
Conserved potentiating effect of ADP through activation of the P2cyc receptor. (**a**) Serotonin induces shape change only in both $P2Y_1^{+/+}$ and $P2Y_1^{-/-}$ platelets. Simultaneous addition of serotonin and ADP restores aggregation in $P2Y_1^{-/-}$ platelets, an effect inhibited by the selective P2cyc antagonist AR-C99631MX. (**b**) Intracellular calcium mobilization in response to ADP is lost in $P2Y_1$ knock-out mice. (**c**) ADP still inhibits cAMP formation in $P2Y_1^{-/-}$ mice. Cyclic AMP levels were measured in intact washed platelets exposed to: vehicle (open bars), 1 μM PGE_1 (gray bars), PGE_1 plus 5μM ADP (black bars), PGE_1 plus ADP in the presence of 2 μM AR-C69931MX (striped bars). These data represent 1 experiment performed in duplicate, representative of 3 separate experiments giving similar results.

Figure 4
P2cyc receptor–mediated platelet aggregation with high concentrations of ADP. (**a**) Addition of 100 μM ADP to $P2Y_1^{-/-}$ mice platelets induced a gradual increase of light transmission, without optically detectable shape change, up to 20% above the baseline at 5 minutes (upper-right tracing), which is inhibited by 2 μM AR-C69931MX (lower-right tracing). (**b**) Transmission electron microscopy of resting (left) or 100 μM ADP-induced small aggregates of 5–10 $P2Y_1^{-/-}$ mice platelets (right). Bars: 0.5 μm.



indicating that it occurred through activation of the P2cyc receptor. Epinephrine, even at 1 mM, did not induce such a response (data not shown).

$P2Y_1^{-/-}$ mice have a prolonged bleeding time and a strong reduction of thrombosis. No spontaneous bleeding was observed in $P2Y_1^{-/-}$ mice, but the bleeding time, which reflects in vivo primary hemostasis, was prolonged in $P2Y_1^{-/-}$ mice as compared with wild-type mice. Mean bleeding time ± SD was 225.7 ± 30 seconds for $P2Y_1^{-/-}$ (range 60–900 seconds, n = 43), and 126.6 ± 10 seconds for $P2Y_1^{+/+}$ (range 29–300 seconds, n = 80, with 1 mouse having a bleeding time of 800 seconds). The difference in bleeding time between the 2 populations is statistically significant (P < 0.0001, unpaired t test) (Figure 5). However, increasing doses of ADP up to 500 mg/kg infused intravenously had no effect on the platelet count in $P2Y_1^{-/-}$ mice as compared with wild-type mice where platelet counts were decreased by 50% (Figure 5b), suggesting that platelets from $P2Y_1^{-/-}$ mice are protected from in vivo ADP-induced platelet activation and removal from the circulation. Moreover, because collagen-induced platelet aggregation is strongly impaired, we measured the mortality induced by infusion of a mixture of collagen (0.5 mg/kg body weight) and epinephrine (60 μg/kg body weight). As shown in Figure 5c, 100% wild-type mice died within 4 minutes, whereas 50% of $P2Y_1^{-/-}$ mice survived. These results demonstrate the key role of the $P2Y_1$ receptor in these acute thrombotic processes.

Discussion

We have generated $P2Y_1$ receptor–null mice in order to characterize the function of the $P2Y_1$ receptor in hemostasis and in thrombosis. These mice are viable, have no apparent morphological or physiological abnormalities and, so far, display normal development, survival and reproduction. This viability was somewhat surprising, since the $P2Y_1$ receptor is expressed early in embryonic development and throughout adult life in a wide range of tissues (7, 34). It is possible that the role of the $P2Y_1$ receptor is discrete in all these tissues, or that its absence is overcome by the presence of other P2 receptors (7).

In platelets, the $P2Y_1$ receptor has been shown to be necessary for ADP-induced aggregation (9–11), whereas a separate P2 receptor coupled to inhibition of adenylyl cyclase is thought to complete and to amplify the response (11, 14, 16, 18). However, doubts still remained as to whether the $P2Y_1$ receptor might be coupled to adenylyl cyclase inhibition, at least in native cells (35). Functional studies of platelets from $P2Y_1^{-/-}$ mice demonstrated a loss of ADP-induced aggregation, firmly establishing the requirement for this receptor for ADP-induced platelet aggregation (Figure 2). Unexpectedly, aggregation in response to medium concentrations of collagen, able to sustain a full aggregation of wild-type platelets, was strongly impaired in $P2Y_1^{-/-}$ platelets, whereas at higher concentrations the typical lag phase observed during collagen-induced aggregation was significantly prolonged (Figure 2). Collagen-induced platelet activation is a complex process involving binding of collagen to at least 2 membrane proteins, the integrin $\alpha_2\beta_1$ and GPVI, tyrosine phosphorylation of cytosolic substrates, PLCγ2 activation, and finally generation of TXA_2 and release of ADP (36, 37). Our results suggest an unsuspected role of the $P2Y_1$ receptor in the early phase of collagen-induced platelet activation that will deserve further characterization. Conversely, aggregation in response to the TXA_2 mimetic U46619 or to thrombin was affected only at threshold concentrations of agonists (Figure 2). Strikingly, simultaneous addition of ADP and serotonin, used to bypass the $P2Y_1$ receptor (10, 23), restored aggregation in $P2Y_1^{-/-}$ platelets, demonstrating the conservation of a potentiation role of ADP in $P2Y_1^{-/-}$ mice (Figure 3a). This response was due to the P2cyc receptor because it was inhibited by the ATP analogue AR-C69931MX. Moreover, whereas the calcium response induced by ADP was abolished in the knock-out mice (Figure 3b), ADP still inhibited the PGE_1-stimulated accumulation of cAMP in intact platelets, this effect being blocked by AR-C69931MX (Figure 3c). These results unambiguously demonstrate the existence of the putative P2cyc (13) (or $P2T_{AC}$ [14] or $P2Y_{AC}$ [15, 16])

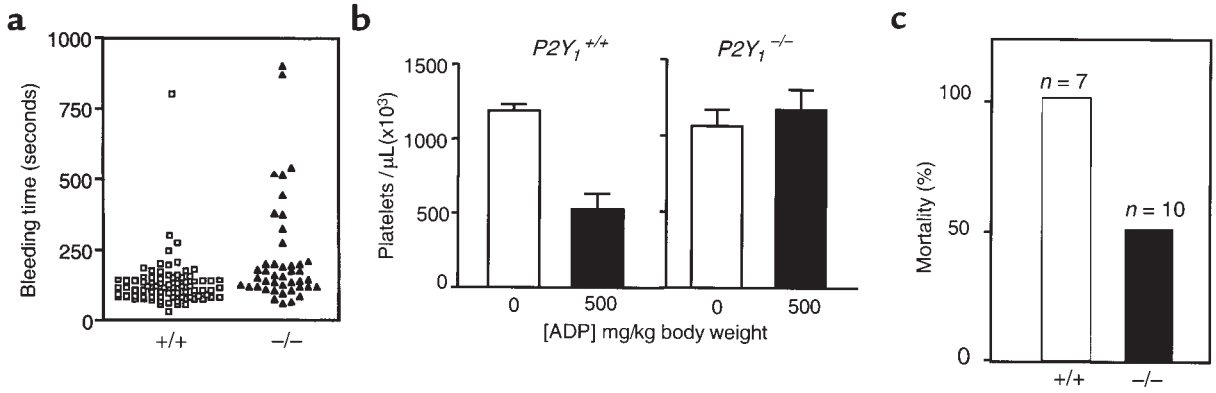


Figure 5
Hemostasis and thrombosis. (**a**) Bleeding time of $P2Y_1^{+/+}$ and $P2Y_1^{-/-}$ mice. Each point represents 1 individual ($P2Y_1^{+/+}$, $n = 43$; $P2Y_1^{-/-}$, $n = 80$). (**b**) $P2Y_1^{-/-}$ mice platelet counts are not modified after bolus injections of ADP whereas $P2Y_1^{+/+}$ mice platelet count is decreased by more then 50%. Each bar is the mean of 4 mice. (**c**) Mortality by thromboembolism in response to a bolus injection of a mixture of collagen (0.5 mg/kg body weight) and adrenaline (60 µg/kg body weight) is reduced by 50% in $P2Y_1^{-/-}$ mice. Results are expressed as the percentage of the total number of animals tested.

platelet ADP receptor, coupled to the inhibition of adenylyl cyclase, which is distinct from and independent of the $P2Y_1$ receptor.

The P2cyc receptor is believed to belong to the G protein–coupled receptor family on the basis of its coupling to the Gi_2 alpha subunit of heterotrimeric G proteins in photolabeling experiments (22) and its ability to inhibit cAMP accumulation. However, it is well-known that inhibition of adenylyl cyclase per se is not sufficient to trigger platelet aggregation (3), and agonists such as epinephrine, acting by activation of Gi only, do not promote platelet aggregation (32). However, we show here that a high concentration of ADP (100 µM) induces aggregation of $P2Y_1^{-/-}$ mice platelets through activation of the P2cyc receptor (Figure 4). This effect was observed in the absence of any calcium movement as measured using fura-2/AM–loaded platelets and without the classic disc-to-sphere transformation of platelets. In contrast to ADP, epinephrine did not induce such a response even at high concentrations (not shown). Together, these results indicate that activation of the P2cyc receptor triggers additional pathways in platelets, as compared with the α_{2A} adrenergic receptor, leading to the formation of microaggregates. Further studies should rapidly allow the fine characterization of the intracellular pathways involved when the P2cyc receptor is activated independently of the $P2Y_1$ receptor.

Despite the lack of platelet aggregation in response to ADP up to 10 µM, no bleeding, either spontaneous or during delivery, was observed in $P2Y_1^{-/-}$ mice, although mean bleeding time in the knockout mice was nearly twice that of the wild-type mice. $P2Y_1$-deficient mice displayed a strong resistance to thromboembolism induced by infusion of ADP (Figure 5b) or a mixture of collagen and epinephrine (Figure 5c) as compared with wild-type mice. These observations demonstrate that the $P2Y_1$ receptor must play a key role in primary hemostasis and in thrombosis. To date, antithrombotic drugs selectively inhibiting ADP-induced platelet activation act on the P2cyc receptor without affecting the $P2Y_1$ receptor activation. These drugs are the thienopyridine compounds ticlopidine and clopidogrel or the ATP analogues termed AR-C66096, AR-C67085, and AR-C69931MX (13–17). This study demonstrates that the $P2Y_1$ receptor is crucial under circumstances where collagen and ADP are the principal agents involved in platelet activation. Such circumstances are frequently encountered in small arteries where platelet interactions with the vessel wall are dependent mainly on collagen and the von Willebrand factor (38) during such clinical interventions as angioplasty, where deep injury of the vessel wall often results in vascular reocclusion (39). In addition, platelet interactions with the vessel wall are mostly dependent on extracellular nucleotides and are regulated by the presence of ectonucleotidases, among which, CD39, the ATP diphosphohydrolase, has proved to be of major importance (40, 41). Moreover, besides fibrin deposition in multiple organs because of deficiency in vascular protective effects, CD39 knock-out mice display an unexpected prolongation of the bleeding time because of selective desensitization of the $P2Y_1$ receptor (41). Thus, analogous to the unidentified P2 receptor sensitive to the thienopyridine clopidogrel, the $P2Y_1$ receptor represents a potential key pharmacological target for antithrombotic drugs. Such compounds would act in the early phase of platelet-vessel wall interactions through inhibition of ADP- and collagen-induced platelet activation. Finally, this strain of knockout mice should allow the investigation of other key roles that the $P2Y_1$ receptor may play, not only in the cardiovascular system, but also in all other tissues where it is expressed (7).

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