Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y₁ receptor–null mice

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ADP is a key agonist in hemostasis and thrombosis. ADP-induced platelet activation involves the purinergic P2Y₁ 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. P2Y₁-null mice were generated to define the role of the P2Y₁ receptor and to determine whether the unidentified P2cyc receptor is distinct from P2Y₁. 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 P2Y₁-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 P2Y₁. P2Y₁-null mice have no spontaneous bleeding tendency but are resistant to thromboembolism induced by intravenous injection of ADP or collagen and adrenaline. Hence, the P2Y₁ receptor plays an essential role in thrombotic states and represents a potential target for antithrombotic drugs.

patients appear to have a deficiency of the platelet
ADP receptor coupled to inhibition of adenylyl cyclase;
the P2Y1 receptor has been shown to be normal in one
of the patients (21). This still-identified P2 receptor is
thought to be coupled to a G protein of αq subtype
(22) and hence could belong to the P2Y metatropic
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 P2Y1 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 P2X1 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 P2Y1 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 P2Y1 receptor and independently
expressed in platelets. We report that targeted disruption
of the P2Y1 receptor results in impaired platelet
aggregation and inhibition of acute thrombosis
induced by infusion of ADP or collagen and adrena-
line. We also show that platelets from P2Y1-deficient
mice are still responsive to ADP through the P2 recep-
tor 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 throm-
boembolism 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 P2Y1 gene. A 0.57-kb cDNA
probe corresponding to mouse P2Y1 cDNA was pre-
pared by PCR after reverse transcription of total RNA
isolated from mouse hearts. The PCR primers were
derived from the published sequence of mouse P2Y1
RNA (sense primer: 5'-CCGGTTTCCACAGTTCTAC-
TACCTGCCG-3'; and antisense primer: 5'-CATAACAGC-
CAGGATCAGCAGGAAAGG-3'). This probe was used to
screen a 129/Sv genomic library (kind gift of P. Cham-
bon, Institut de Génétique et de Biologie Moléculaire
et Cellulaire, Strasbourg, France). A 12.6-kb fragment
was cloned and entirely sequenced in both directions
(cession no. AJ245636). The mouse P2Y1 gene con-
tains 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'-ACTCTGGGTTGATGAGGATGGAACGACGG-3' and
5'-GCCCTTCATAGCGCTTCTTACGGTCTC3'-3'; P2Y1 gene
5'-CTCTATGGACTGTTAAGACATTCAGGGC-3' and 5'-
AAATGGAAGCACTCCAAAAATCTCCCAGATC-3'. Southern
blot analyses were performed to confirm the PCR
results, using as a probe a fragment of the P2Y1 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 P2Y1-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
(P2Y1+/− mice) and homozygous for the wild-type allele
(P2Y1+/+ mice). Both control P2Y1+/+ and P2Y1+/− mice used
in this study were at the F3 generation, resulting from
intercrossing between several homozygous F2 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 Tekni-
ka 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 abdomi-
nal 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
2.105 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-
lengths of 340 and 380 nm and fluorescence emission detection at 510 nm, as described previously (11).

Cyclic AMP measurements. Platelets (8.10⁴/µL) were stimulated at 30-second intervals with either vehicle (Tyrode’s buffer without Ca²⁺ or Mg²⁺) (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.

Table 1
Hematological parameters of P2Y₁⁺/+ and P2Y₁⁻/⁻ mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>+/+</th>
<th>−/−</th>
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<tr>
<td>Platelets (× 10⁴/µL)</td>
<td>100 ± 12</td>
<td>98 ± 15</td>
</tr>
<tr>
<td>White blood cells (× 10⁴/µL)</td>
<td>4.9 ± 1.1</td>
<td>5.4 ± 1.0</td>
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<tr>
<td>Red blood cells (× 10⁵/µL)</td>
<td>9.1 ± 0.6</td>
<td>8.9 ± 1.2</td>
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<tr>
<td>Hematocrit (%)</td>
<td>46.4 ± 3.6</td>
<td>45.2 ± 5.4</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.6 ± 0.8</td>
<td>14.1 ± 1.1</td>
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Mean ± SD (n = 4, except for platelet counts where n = 10).
Platelet aggregation is impaired in P2Y1-deficient mice. Functional studies of platelets from P2Y1–/– 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 phase, measured from the addition of the agonist to starting of aggregation, is prolonged in response to 5 μg/mL collagen (25 seconds in P2Y1–/– versus 15 seconds in wild-type). In contrast, responses to thrombin and to U46619 were modified only at threshold concentrations. Bar: 1 minute.

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A potentiation role of ADP in P2Y1–/– 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 5HT2A receptor and mobilization of intracellular calcium, which could be used to bypass the P2Y1 receptor (10, 23). Simultaneous addition of ADP (5 μM) and serotonin (1 μM) restored aggregation to ADP in P2Y1–/– platelets, suggesting the conservation of a potentiation role of ADP in P2Y1–/– mice. (Figure 3a). This effect was due to activation of the P2 receptor negatively coupled to adenyl 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 PGE1-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 adenyl cyclase, which is distinct from the P2Y1 receptor.

Platelet aggregation by activation of the P2cyc receptor requires high concentrations of ADP. Addition of 100 μM ADP to washed platelets from P2Y1–/– 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 P2Y1–/– mice with clopidogrel (Figure 4a, lower-right tracing).

Figure 2
In vitro aggregation responses of P2Y1–/– washed mice platelets as compared with wild-type. P2Y1–/– 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 P2Y1–/– versus 15 seconds in wild-type). In contrast, responses to thrombin and to U46619 were modified only at threshold concentrations. Bar: 1 minute.

Figure 3
Conserved potentiating effect of ADP through activation of the P2cyc receptor. (a) Serotonin induces shape change only in both P2Y1+/+ and P2Y1–/– platelets. Simultaneous addition of serotonin and ADP restores aggregation in P2Y1–/– platelets, an effect inhibited by the selective P2cyc antagonist AR-C99631MX. (b) Intracellular calcium mobilization in response to ADP is lost in P2Y1 knock-out mice. (c) ADP still inhibits cAMP formation in P2Y1–/– mice. Cyclic AMP levels were measured in intact washed platelets exposed to: vehicle (open bars), 1 μM PGE1 (gray bars), PGE1 plus 5μM ADP (black bars), PGE1 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.
indicating that it occurred through activation of the P2yc receptor. Epinephrine, even at 1 mM, did not induce such a response (data not shown).

**P2Y1**−/− mice have a prolonged bleeding time and a strong reduction of thrombosis. No spontaneous bleeding was observed in P2Y1−/− mice, but the bleeding time, which reflects in vivo primary hemostasis, was prolonged in P2Y1−/− mice as compared with wild-type mice. Mean bleeding time ± SD was 225.7 ± 30 seconds for P2Y1−/− (range 60–900 seconds, n = 43), and 126.6 ± 10 seconds for P2Y1+/+ (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 P2Y1−/− mice as compared with wild-type mice where platelet counts were decreased by 50% (Figure 5b), suggesting that platelets from P2Y1−/− 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 P2Y1−/− mice survived. These results demonstrate the key role of the P2Y1 receptor in these acute thrombotic processes.

**Discussion**

We have generated P2Y1 receptor–null mice in order to characterize the function of the P2Y1 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 P2Y1 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 P2Y1 receptor is discrete in all these tissues, or that its absence is overcome by the presence of other P2 receptors (7).

In platelets, the P2Y1 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 P2Y1 receptor might be coupled to adenylyl cyclase inhibition, at least in native cells (35). Functional studies of platelets from P2Y1−/− 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 P2Y1−/− 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 αβ1 and GPVI, tyrosine phosphorylation of cytosolic substrates, PLCγ2 activation, and finally generation of TXA2, and release of ADP (36, 37). Our results suggest an unsuspected role of the P2Y1 receptor in the early phase of collagen-induced platelet activation that will deserve further characterization. Conversely, aggregation in response to the TXA2 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 P2Y1 receptor (10, 23), restored aggregation in P2Y1−/− platelets, demonstrating the conservation of a potentiation role of ADP in P2Y1−/− mice (Figure 3a). This response was due to the P2yc 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 PGE1-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 P2yc (13) (or P2TAC [14] or P2YAC [15, 16])
platelet ADP receptor, coupled to the inhibition of adenylly cyclase, which is distinct from and independent of the P2Y₁ receptor.

The P2cyc receptor is believed to belong to the G protein–coupled receptor family on the basis of its coupling to the Gₛ 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 adenylly 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 P2Y₁–/– 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 α₂A 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 P2Y₁ 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 P2Y₁–/– mice, although mean bleeding time in the knockout mice was nearly twice that of the wild-type mice. P2Y₁-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 P2Y₁ 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 P2Y₁ 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 P2Y₁ 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 ectonucleotidas, 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 P2Y₁ receptor (41). Thus, analogous to the unidentified P2 receptor sensitive to the thienopyridine clopidogrel, the P2Y₁ 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 P2Y₁ receptor may play, not only in the cardiovascular system, but also in all other tissues where it is expressed (7).
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