Overexpression of the platelet $P2X_1$ ion channel in transgenic mice generates a novel prothrombotic phenotype

Cécile Oury, Marijke J. E. Kuijpers, Emese Toth-Zsamboki, Arnaud Bonnefoy, Sophie Danloy, Ingrid Vreys, Marion A. H. Feijge, Rita De Vos, Jos Vermylen, Johan W. M. Heemskerk, and Marc F. Hoylaerts

We have generated transgenic mice overexpressing the human $P2X_1$ ion channel in the megakaryocytic cell lineage. Platelets from transgenic mice exhibited a gain of $P2X_1$ ionotropic activity as determined by more prominent $P2X_1$-mediated Ca^{2+} influx and platelet shape change. $P2X_1$ overexpression enhanced platelet secretion and aggregation evoked by low doses of collagen, convulxin, or the thromboxane A_2 mimetic U46619. In contrast, transgenic platelet responses to adenosine diphosphate (ADP) or thrombin were normal. Perfusing whole blood from transgenic mice over collagen fibers at a shear rate of 1000 seconds^{-1} resulted in increased $P2X_1$-dependent aggregate formation and phosphatidylserine exposure. Platelet hyperreactivity to collagen was correlated with up-regulated extracellular signal-regulated kinase 2 (ERK2) phosphorylation. Accordingly, the MEK1/2 inhibitor U0126 potently inhibited the collagen-induced aggregation of transgenic platelets when stirred or when perfused over a collagen surface. In a viscometer, shear stress caused potent aggregation of transgenic platelets under conditions in which wild-type platelets did not aggregate. In an in vivo model of thromboembolism consisting of intravenous injection of a low dose of collagen plus epinephrine, transgenic mice died more readily than wild-type mice. Preinjection of U0126 not only fully protected transgenic mice against thrombosis, it also enhanced the survival of wild-type mice injected with a higher collagen dose. Hence, the platelet $P2X_1$ ion channel plays a role in hemostasis and thrombosis through its participation in collagen-, thromboxane A_2-, and shear stress–triggered platelet responses. Activation of the ERK2 pathway is instrumental in these processes. (Blood. 2003; 101:3969-3976)

Introduction

Adenosine triphosphate (ATP) is released as a cotransmitter from the sympathetic nerve endings, endothelium, and activated platelets. It is now established that ATP and other nucleotides act as extracellular signaling molecules.[1] The receptors that mediate the action of the adenine nucleotides belong to 2 classes, the G-protein–coupled P2Y receptors and the P2X receptors, a family of ligand-gated ion channels.[2] Seven distinct P2X purinergic receptors have been cloned from mammalian species ($P2X_{1-7}$) and have been found to be widely expressed in excitable and nonexcitable cells.[3] Subunits of these receptors can assemble to form homomeric and heteromeric functional channels. All P2X receptors are cation-selective channels with almost equal permeability to Na^+ and K^+ and significant permeability to Ca^{2+}.[3] The Ca^{2+} permeation through P2X receptors is considered to be an important component of the physiologic and pathophysiologic responses mediated by these receptors in vivo (reviewed by Burnstock[4] and North[5]).

In platelets, ATP and adenosine diphosphate (ADP) are present at high concentrations in the dense granules and are coreleased during platelet activation.[6] ADP has long been recognized as an important platelet activator, playing an essential role in enhancing secretion and in amplifying platelet aggregation induced by other agonists. Biologic effects of ADP are mediated by 2 distinct metabotropic receptors, the G_q-protein–coupled $P2Y_1$ receptor and the G_i-protein–coupled $P2Y_{12}$ receptor. The latter is the target for specific antithrombotic drugs (reviewed by Gachet[7]). Platelets express the $P2X_1$ member of the P2X family of ligand-gated ion channels,[8] which mediates a rapid ATP-induced Ca^{2+} influx.[9,10] Because of the fast desensitizing property of the $P2X_1$ ion channel[11] and the lack of specific platelet $P2X_1$ antagonists and because platelet studies have mainly been performed ex vivo at low extracellular Ca^{2+} concentrations in citrated plasma, the function of $P2X_1$ in platelet activation only recently started to be unraveled, and a physiologic role of ATP in this process is now being considered.

In human platelets, the selective $P2X_1$ agonists αβ-methylene adenosine 5′-triphosphate (αβ-meATP) and βγ-methylene adenosine 5′-triphosphate (βγ-meATP) were shown to evoke a transient Ca^{2+} increase accompanied by reversible platelet shape change, provided that measures had been taken to avoid the activation and desensitization of $P2X_1$ by ATP spontaneously released during platelet preparation.[12,13] Despite the fact that the sole $P2X_1$ activation cannot cause platelet aggregation, the use of $P2X_1$ desensitization strategies indicated that this ion channel contributes significantly to human platelet aggregation induced by collagen, a

From the Center for Molecular and Vascular Biology and the Laboratory of Morphology and Molecular Pathology, University of Leuven, Belgium; and the Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, the Netherlands.

Submitted October 24, 2002; accepted January 4, 2003. Prepublished online as *Blood* First Edition Paper, January 9, 2003; DOI 10.1182/blood-2002-10-3215.

Supported by the bilateral scientific and technological cooperation between Flanders and Hungary (BIL00/12) and from Fonds voor Wetenschappelijk Onderzoek (FWO) project G.0227.03. C.O. is holder of a postdoctoral research mandate of the FWO. E.T.Z. is recipient of a doctoral KULeuven scholarship. J.V. is holder of the Aventis Chair of Hemostasis Research.

Reprints: Marc Hoylaerts, Center for Molecular and Vascular Biology, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium; e-mail: marc.hoylaerts@med.kuleuven.ac.be.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

major platelet agonist able to trigger dense granule release.[13] Recently, we have reported that this effect depends on $P2X_1$-mediated activation of the extracellular signal-regulated kinase 2 (ERK2) mitogen-activated protein kinase (MAPK), which enhances platelet secretion initiated by collagen.[14] According to our model, platelet stimulation with low doses of collagen rapidly causes minor dense granule release; ATP secreted during this early event activates a Ca^{2+}- and a protein kinase C (PKC)–dependent $P2X_1$-ERK2 signaling cascade needed to complete platelet aggregation by enhancing release from collagen-primed dense granules.[14]

To further investigate the physiologic role of the platelet $P2X_1$ ion channel, we have generated transgenic mice overexpressing human $P2X_1$ in the megakaryocytic cell lineage. Platelets from these mice displayed a gain of $P2X_1$ functionality accompanied by a mild prothrombotic phenotype. Combining ex vivo and in vivo analyses of platelet function, this mouse model enabled us to demonstrate the involvement of $P2X_1$-mediated Ca^{2+} influx and the coupled ERK2 activation in platelet responses to collagen. We found that $P2X_1$ overexpression promotes platelet secretion induced by the thromboxane A_2 mimetic U46619 and thereby enhances platelet aggregation. Moreover, $P2X_1$ overexpression also increases platelet activation and aggregate formation under shear stress. Together, our findings suggest a regulatory role for $P2X_1$ during in vivo hemostasis and thrombosis.

Materials and methods

DNA constructs

Polymerase chain reaction (PCR) cloning of the murine GPIIb promoter fragment extending from +23 to −508 relative to the initiation start site (TOPO TA cloning kit; Invitrogen, Carlsbad, CA) was performed using the following primers: sense, 5′-AGGAAGTGGGTAAATGTCCTACTC-3′; antisense, 5′-TCCCAAACGTCCTAAACAGGAATGG-3′. The *Xho*I–*Hin*dIII promoter fragment was excised from the mGPIIb-PCR2.1–TOPO plasmid and inserted into the pGL3-basic luciferase reporter vector (Promega, Leiden, the Netherlands) digested with *Xho*I and *Hin*dIII. Megakaryocytic human erythroblastic leukemia (HEL) and nonmegakaryocytic HeLa cell lines were transiently transfected with the resultant reporter construct, and megakaryocytic-specific promoter activity was verified as described[15]; promoter-driven increases of luciferase expression were only measured in HEL cells, as expected. The promoter fragment was excised from the mGPIIb-pGL3 plasmid by digestion with *Kpn*I and *Bam*HI and inserted into the *Kpn*I-*Bam*HI–digested $P2X_1$-pcDNA3 vector[16] in front of the human $P2X_1$ ($hP2X_1$) cDNA. DNA constructs were verified by sequencing on the automated A.L.F. sequencer (Pharmacia Biotech, Uppsala, Sweden). The 2.2-kilobase (kb) *Kpn*I–*Dra*III fragment (GPIIb-$hP2X_1$) was excised and purified for zygote injection. The GPIIb promoter has been successfully used to restrict transgene expression to the megakaryocytic cell lineage of mice.[17]

Generation of transgenic mice

Transgenic mice expressing human $P2X_1$ were generated by zygote injection into the Friend leukemia virus, strain B (FVB) background according to previously published procedures.[18] Transgenic offspring were identified by PCR screening using genomic DNA extracted from tail samples. The following primer pair was used: sense, mGPIIb promoter, 5′-AGGAAGTGGGTAAATGTCCTACTC-3′; antisense, $hP2X_1$, 5′-TCAGGATGTCCTCATGTTCTCCTGCAGG-3′.

RNA isolation and RT-PCR

Total RNA was extracted from mouse washed platelets and leukocytes isolated from freshly drawn citrated blood using the High Pure RNA isolation kit (Roche Diagnostics, Brussels, Belgium). During reverse transcription–polymerase chain reaction (RT-PCR), specific amplification of the human $P2X_1$ cDNA in transgenic mouse samples was accomplished with the following primers: sense, $hP2X_1$, 5′-GTTCCAGGAGGAGCTG-GCCGCCTTCC-3′; antisense, $hP2X_1$, 5′-GGTCTTCATGTGGGCAGCAT-TCAC-3′. For the specific amplification of $mP2X_1$ cDNA, the following primers were used: sense, $mP2X_1$, 5′-CTGCAGGATGAGCTGTCAGCCT-TCTTC-3′; antisense, $mP2X_1$, 5′-GTAGAGGCATTTCTTCATGTAGGT-3′.

Materials

Adenosine 5′-diphosphate (ADP), αβ-meATP, βγ-meATP, apyrase (EC 3.6.1.5, grade 1: mixture of both high and low ATPase/ADPase ratio isoenzymes), and the thromboxane A_2 mimetic U46619 were from Sigma (St Louis, MO). ADP, αβ-meATP, and βγ-meATP were purified by high-performance liquid chromatography (HPLC) on an Adsorbosphere HS C18 7-μm, 250 × 4.6-mm column (Alltech, Bad Segeberg, Germany) as described.[12] Fibrillar collagen (Horm-type 1 collagen) was from Nycomed (Munich, Germany) and thrombin (Dade Thrombin Reagent) was from Dade Behring (Marburg, Germany). The MEK1/2 inhibitor U0126 was purchased from BioMol Research Laboratories (Plymouth Meeting, MA), and D-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (San Diego, CA). OG 488–annexin V was from Nexins Research (Hoeven, the Netherlands). Fura-2 acetoxymethyl ester and Pluronic F-127 came from Molecular Probes (Leiden, the Netherlands). Recombinant saratin was produced in the yeast *Hansenula polymorpha* as described.[19]

Preparation of platelet-rich plasma and washed platelets

Eight- to 12-week-old mice were bled under sodium pentobarbital anesthesia (6 mg/kg) from the retro-orbital plexus. Mouse blood was collected in a saline solution containing either 4 U/mL heparin, 20 μM PPACK, and 0.1 U/mL apyrase or 20 μg/mL hirudin. Platelet-rich plasma (PRP) was obtained by centrifugation at 800*g* for 30 seconds followed by 5 minutes at 150*g*. PRP from 3 animals were pooled, and the platelet counts were adjusted to 2.5×10^5 platelets/μL with autologous platelet-poor plasma (PPP). Mouse washed platelets were prepared as previously described,[14] using apyrase (1 U/mL) throughout the procedure. Platelets were resuspended in Ca^{2+}-free Tyrode buffer containing 0.35% (wt/vol) human or bovine serum albumin and 1 U/mL apyrase, at a density of 2.5×10^5 platelets/μL.

Electron microscopy

Platelet-rich fractions were immediately fixed overnight at 4°C in 2.5% (wt/vol) glutaraldehyde and 0.1 M phosphate buffer, pH 7.2. After centrifugation at 800*g* for 10 minutes, a condensed pellet of platelets was formed. After fixation in 1% OsO_4 (wt/vol), 0.1 M phosphate buffer, pH 7.2, and dehydration in a graded series of ethanol, the pellets were embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate before examination with a Zeiss EM 10 electron microscope (Oberkochen, Germany).

Platelet aggregation and ATP secretion analyses

Light transmission during mouse platelet aggregation was recorded using apyrase-treated washed platelets in the presence of 2 mM $CaCl_2$ on an ELVI 840 aggregometer (Elvi Logos, Milan, Italy). Shear-induced platelet aggregations were performed in an annular ring-shaped viscometer generating laminar shear (Ravenfield viscometer; Heywood, Lancashire, United Kingdom) using mouse heparinized PRP. After 3 minutes, platelet samples were collected and fixed in 1% paraformaldehyde; the percentage of platelet aggregation was calculated by comparing single platelet counts before and after shearing. ATP secretion was monitored in hirudinized PRP in parallel with platelet aggregation by adding firefly luciferase and luciferin and comparing the luminescence generated by platelet ATP release or by an ATP standard (Chrono-Lume, Kordia, The Netherlands) as previously described.[14]

Immunoblotting

Western blot detection of the human $P2X_1$ protein in transgenic mouse platelets (8×10^8 platelets) was performed by using a polyclonal rabbit

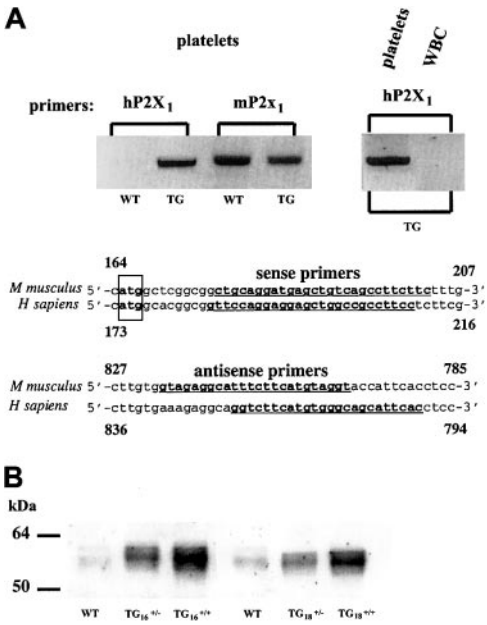

Figure 1. $hP2X_1$ overexpression in transgenic mouse platelets. (A) RT-PCR performed on total RNA extracted from platelets or total leukocytes (WBCs) of wild-type (WT) or transgenic (TG) mice. Identical results were found for 2 independent transgenic founder lines. Partial sequences of the human and mouse $P2X_1$ cDNAs are shown. Primers used to specifically amplify the $hP2X_1$ and $mP2X_1$ cDNAs are underlined and in boldface type. The translation initiation codon is boxed. (B) Western blotting detection of the $hP2X_1$ protein in TG platelet whole-cell lysate with a polyclonal anti-$hP2X_1$ antibody.[12] The $hP2X_1$ expression levels in identical numbers of platelets from heterozygous or homozygous transgenic mice originating from 2 independent transgenic founder lines (TG_{16} and TG_{18}) are shown in parallel with those of the endogenous $mP2X_1$ detected in WT mouse platelets.

anti-$hP2X_1$ antibody.[16] Detection of ERK1/2 phosphorylation in human or mouse washed platelets (1×10^7 platelets) was accomplished with the PhosphoPlus p44/42 MAP Kinase Antibody kit (New England Biolabs, Hitchin, United Kingdom) according to the instructions of the manufacturer.

Ca^{2+} measurements

Apyrase (2 U/mL)–treated mouse washed platelets (2×10^5 platelets/μL) were loaded with 3.5 μM fura-2 acetoxymethyl ester in the presence of Pluronic F-127 for 15 minutes as described.[20] The measurements were performed between 30 and 90 minutes after final platelet resuspension (0.7×10^5 platelets/μL). $CaCl_2$ (2 mM) was added before the agonist. Fura-2 fluorescence was recorded from 0.2 mL aliquots of platelet suspension stirred at 37°C in an SLM-Aminco spectrofluorimeter (SLM Instruments, Rochester, NY) with excitation wavelengths of 340 and 380 nm and emission of 500 nm. Changes in intracellular Ca^{2+} concentration were monitored using the fura-2 340/380 fluorescence ratio and were calibrated according to the method of Grynkiewicz et al.[21] The Ca^{2+} signals evoked by αβ-meATP were assessed as the peak amplitude of the intracellular Ca^{2+} rise occurring within a few milliseconds after agonist application and returning to basal levels after 10 seconds.

Adhesion under flow conditions

Adhesion experiments under flow conditions were performed with anticoagulated mouse blood (4 U/mL heparin, 20 μM PPACK), basically as described.[22] Whole blood was perfused for 4 minutes over a collagen-coated coverslip through a parallel-plate transparent flow chamber using a pulse-free pump, at a wall-shear rate of 1000 $seconds^{-1}$. During the perfusion, high-resolution microscopic transmission or fluorescent images were recorded in real-time with a Visitech digital imaging system (Sunderland, United Kingdom). Exposure of phosphatidylserine (PS) was detected by postperfusion with the heparinized rinsing buffer containing OG488-labeled annexin V (1 μg/mL). Phase-contrast and fluorescent images were obtained from at least 10 different collagen-containing microscopic fields that were arbitrarily chosen. When indicated, apyrase (0.1 U/mL) was added during blood sampling; in some experiments, blood was incubated with saratin (10 μg/mL) blocking von Willebrand factor (VWF) binding to collagen[19] 1 minute before perfusion. Area coverage from phase-contrast images was analyzed off-line using ImagePro software (Media Cybernetics, Silver Spring, MD). Area coverage by platelets stained with OG488-annexin V was determined with Quanticell software (Visitech).

In vivo experiments

Thromboembolism was induced by injection of a mixture of collagen (0.125 or 0.06 mg/kg) and epinephrine (60 μg/kg) into the jugular veins of anesthetized mice. When indicated, mice received 200 μg/kg U0126 1 minute before the induction of thromboembolism. For bleeding time measurements, mice were anesthetized, and 3 mm of the tail tip was amputated with a scalpel. The tail was then blotted with filter paper every 15 seconds until the paper was no longer blood stained.

Statistical analyses

Statistical analyses of the data were made using the nonpaired Student *t* test and the 2-tailed Tukey-Kramer multiple comparisons test. Survival data were analyzed using 2×2 contingency tables.

Results

Generation of transgenic mice overexpressing the human $P2X_1$ ion channel in the megakaryocytic cell lineage

On zygote injection of a constructed GPIIb-$hP2X_1$ transgene, among 19 offspring mice obtained, 4 animals were found to be transgenic by PCR screening. All founders transmitted the transgene in a Mendelian fashion. RT-PCR analyses using primer pairs that selectively amplify $hP2X_1$ versus $mP2X_1$ mRNAs revealed the presence of $hP2X_1$ transcripts in the platelets of the transgenic (TG) mice (Figure 1A). The expression of the endogenous platelet $mP2X_1$ remained comparable to that of wild-type (WT) platelets (Figure 1A). No transgene expression was found in the leukocytes (Figure 1A). Overexpression of the $hP2X_1$ protein in platelets was demonstrated by immunoblotting of heterozygous ($TG^{+/-}$) and homozygous ($TG^{+/+}$) mouse platelet extracts (Figure 1B); similarly, immunohistochemistry of bone marrow sections revealed increased $P2X_1$ staining in the TG megakaryocyte membranes (not shown). The homozygous mice of 2 founder lines (denominated TG_{16} and TG_{18}) overexpressing similar amounts of platelet $hP2X_1$ were characterized and showed identical phenotypes. These mice had no apparent physiologic abnormalities and displayed normal development, survival, and reproduction. Platelet count (Table 1) and morphology (not shown), as well as other hematologic parameters, were identical to those of WT mice, with the exception of mild leukocytosis (Table 1). Values are represented as mean ± SD ($P = .003$).

Table 1. Hematologic parameters of wild-type and transgenic mice

Parameter	Wild-type	Transgenic
Platelet count, $\times 10^4/\mu L$	69.5 ± 8.9 (n = 13)	64.8 ± 2.8 (n = 9)
White blood cell count, $\times 10^3/\mu L$	1.7 ± 0.7 (n = 12)	2.9 ± 0.5 (n = 10)*
Red blood cell count, $\times 10^6/\mu L$	7.07 ± 0.18	7.25 ± 0.15
Hematocrit, %	31.5 ± 0.9	32.9 ± 0.9
Hemoglobin level, g/dL	10.9 ± 0.3	11.7 ± 0.2

*$P = .03$.

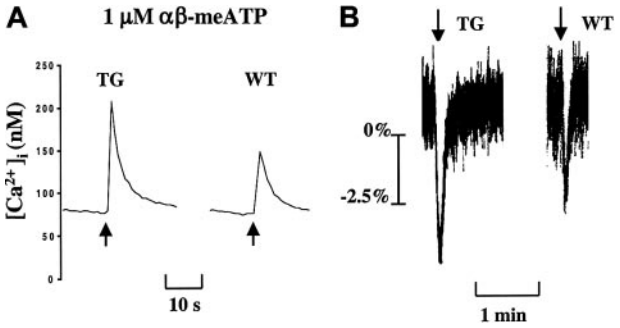

Figure 2. Gain of $P2X_1$ functionality in TG platelets. (A) Increased $P2X_1$-mediated Ca^{2+} influx in TG platelets. (B) Enhanced $P2X_1$-mediated TG platelet shape change. Apyrase (2 U/mL)–treated washed platelets were stimulated with αβ-meATP (1 μM) in the presence of 2 mM $CaCl_2$. Data are representative curves of at least 2 separate experiments performed in triplicate on platelet pools from 3 animals. Arrows indicate the addition of αβ-meATP (1 μM).

Overexpression of $hP2X_1$ results in a gain of $P2X_1$ functionality in the transgenic platelets

Apyrase, by degrading ATP spontaneously released during blood sample handling, is needed ex vivo to protect platelet $P2X_1$ channels from artificial desensitization.[12,13] In the presence of this ectonucleotidase and physiologic Ca^{2+} concentrations (2 mM $CaCl_2$), the nonhydrolyzable $P2X_1$ selective agonists αβ-meATP (1 μM, defined to be the optimal concentration) (Figure 2) and βγ-meATP (not shown) evoked a rapid Ca^{2+} influx in mouse platelets. The peak value of this intracellular Ca^{2+} rise was increased by approximately 50% in TG platelets compared with WT platelets (TG, 146.5 ± 23.6 nM, n = 8; WT, 93.1 ± 21.4 nM, n = 8; $P = .0003$) (Figure 2). In the same experimental conditions, the WT and TG platelets displayed identical ADP-induced Ca^{2+} increases, reflecting $P2Y_1$-mediated Ca^{2+} responses (WT, 399 ± 66 nM, n = 5; TG, 478 ± 74 nM, n = 5; $P = .112$).

In human platelets, $P2X_1$ stimulation causes Ca^{2+} influx and subsequent platelet shape change.[12,13] Similarly, αβ-meATP and βγ-meATP induced a quickly reversible shape change of WT mouse platelets proportional to Ca^{2+} influx. Platelets from TG mice exhibited a more prominent αβ-meATP–induced platelet shape change compared with WT platelet response (Figure 2B). Taken together, these data are compatible with a significant gain of $P2X_1$ functionality in the TG platelets.

Enhanced platelet aggregation induced by collagen in $hP2X_1$ transgenic mice: role of the ERK2 signaling pathway

In an aggregometer, the $P2X_1$ selective agonists αβ-meATP and βγ-meATP did not cause platelet aggregation or secretion either in WT or in TG mice. Functional studies of apyrase-treated platelets from TG mice, where $P2X_1$ desensitization is prevented, yet demonstrated strongly enhanced platelet aggregation evoked by low and intermediate doses of collagen (1 to 2 μg/mL) compared with WT platelets (Figure 3A). A similar increase of TG platelet aggregation was observed after platelet stimulation with low concentrations of convulxin, a glycoprotein VI (GPVI)–selective agonist (0.025 to 0.04 μg/mL) (Figure 3A, right panel), indicating that $P2X_1$ overexpression enhances platelet aggregation mediated by the collagen receptor GPVI. Platelet aggregations induced by higher collagen (4 μg/mL or greater) or convulxin (0.05 μg/mL or greater) concentrations were identical for WT and TG platelets (not shown).

Threshold concentrations of collagen, which only caused shape change of WT platelets, produced full aggregation of TG platelets (Figure 3A). Consistent with an event requiring functional $P2X_1$ channels, the enhanced reactivity to collagen was abrogated when the apyrase treatment was omitted (Figure 3B) or when these platelets were pretreated with αβ-meATP (1 μM) to selectively desensitize $P2X_1$ (Figure 3B). Blockade of ERK2 activation with the selective MEK1/2 inhibitor, U0126 (1 μM) (Figure 3C, last 2 lanes) abolished collagen-induced platelet aggregation for TG (1 μg/mL) and WT (2 μg/mL) platelets (Figure 3B). Interestingly, full aggregation of TG platelets already evoked by 1 μg/mL collagen coincided with maximal ERK2 phosphorylation (Figure 3C). These data demonstrated that $P2X_1$ and ERK2 are proximal and distal components of a common signaling cascade contributing to collagen-induced platelet aggregation and that this pathway has been up-regulated in TG platelets as a consequence of $P2X_1$ overexpression.

Further analyses of TG platelet aggregation revealed an increased response to low concentrations of the thromboxane A_2 mimetic U46619 (less than 2 μM) (Figure 4A). In contrast, TG platelet aggregation provoked by any concentration of HPLC-purified ADP or of thrombin occurred normally (Figure 4B).

Enhanced platelet secretion induced by collagen and U46619 in $hP2X_1$ transgenic mice

To investigate the mechanism responsible for the observed increased aggregation of TG platelets, we compared platelet secretion induced by low concentrations of collagen or of U46619 in WT and TG PRP. As in human platelets, the desensitization of $P2X_1$ with αβ-meATP strongly inhibited ATP secretion from WT platelets, indicating a $P2X_1$ contribution to this platelet response (not shown). Figure 5 shows increased ATP secretion of TG platelets (1.9 ± 0.6- and 3.3 ± 0.2-fold increases in response to 0.5 μg/mL collagen and 4 μM U46619, respectively [$P < .001$; n = 3])

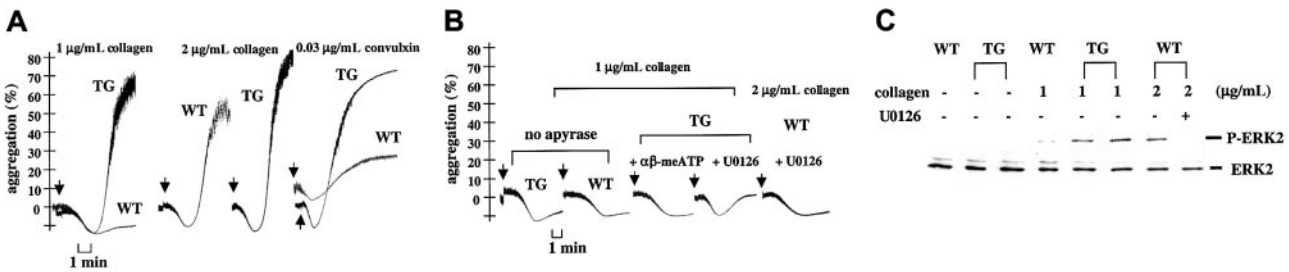

Figure 3. Increased GPVI-mediated collagen-induced platelet aggregation and ERK2 activation with transgenic platelets. (A) Light transmission (%) recordings in apyrase (1 U/mL)–treated washed WT or TG mouse platelets stimulated with collagen (1-2 μg/mL) or convulxin (0.03 μg/mL). (B) Platelet aggregations were induced with collagen (1 μg/mL) in the absence of apyrase (2 curves on the left). The other curves represent collagen-induced platelet aggregations performed in the presence of apyrase after 1-minute preincubation with αβ-meATP (1 μM) or with the MEK1/2 inhibitor U0126 (1 μM), as indicated. Arrows depict the time of collagen application. (C) Western blot analyses of ERK2 phosphorylation (P-ERK2) evoked by collagen in WT or TG apyrase-treated platelets. For TG, data from 2 independent platelet pools are shown. Samples were analyzed 3 minutes after collagen addition. Blockade of collagen-induced ERK2 phosphorylation with U0126 (1 μM) is shown for WT platelets in parallel with its effect on WT platelet aggregation (panel B). Data are representative of at least 3 independent experiments performed in duplicate on platelets from WT and TG mice.

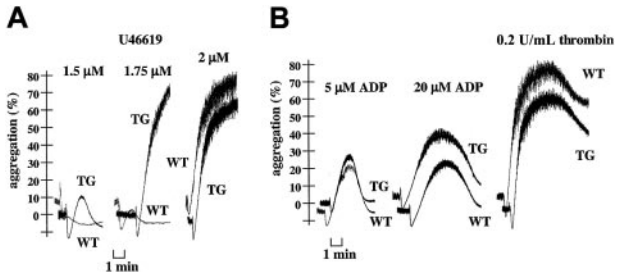

Figure 4. Increased U46619-induced platelet aggregation and normal responses to ADP and thrombin with transgenic platelets. Light transmission (%) recordings in apyrase (1 U/mL)–treated washed WT or TG mouse platelets stimulated with thromboxane A2 mimetic U46619 (1.5-1.75-2 μM) (A) or HPLC-purified ADP (5-20 μM) and thrombin (0.2 U/mL) (B). Data are representative of at least 3 independent experiments performed in duplicate on platelets from WT and TG mice.

paralleled with enhanced platelet aggregation. Thus, P2X$_1$ overexpression in TG platelets potentiates platelet-dense granule release initiated by low doses of collagen or of the thromboxane A$_2$ mimetic U46619, leading to the amplification of the initial platelet response and the completion of aggregation. ATP secretion triggered by ADP or thrombin was found to be normal (not shown).

Increased whole blood aggregate formation and platelet phosphatidylserine exposure on a collagen-coated surface under flow

To further investigate the TG platelet hyperreactivity to collagen, aggregate formation and exposure of coagulation-active negatively charged phosphatidylserine (PS) on a collagen surface were analyzed in whole mouse blood under conditions of flow. Using videomicroscopy, aggregate formation was monitored from phase-contrast images, and PS exposure (procoagulant activity) was imaged after annexin V staining. As previously described,[22] when WT mouse blood was perfused over collagen at a shear rate of 1000 seconds^{-1}, platelets tethered, adhered, and assembled on the surface, with the adherent cells responding by a rapid increase in cytosolic Ca^{2+},[22] and subsequent surface-exposure of PS (Figure 6). A similar picture was observed following perfusion of TG blood (Figure 6). The use of ATP/ADP-degrading apyrase, needed to protect P2X$_1$ from ATP-provoked desensitization, considerably reduced aggregate formation and platelet PS exposure after the perfusion of WT blood (Figure 6A-B). This is compatible with the reported role of the P2Y$_1$ and P2Y$_{12}$ receptors interacting with released ADP during aggregate formation on collagen.[23,24] Perfusion of apyrase-treated TG blood yet resulted in prominent platelet aggregation coinciding with high PS exposure (Figure 6A-B), suggesting that P2X$_1$ overexpression in TG platelets has compensated the inhibitory effects of apyrase by promoting ATP-dependent platelet adhesion, activation, and aggregation under these flow conditions.

Figure 6 also shows how aggregate formation and PS exposure by apyrase-treated TG platelets were abrogated by saratin (Figure 6A-B), which, by blocking VWF binding to collagen, prevents GPIbα-VWF–dependent platelet adhesion,[19] suggestive of a role for P2X$_1$ in the initial control of platelet activation and aggregation on surface-bound VWF at the shear rate investigated.

Interestingly, area coverage by platelet aggregates and PS exposure was reduced by 30% to 35% during the perfusion of apyrase-treated TG blood in the presence of U0126 (Figure 6C-D). These data indicate the involvement of the ERK2 pathway in platelet aggregate formation on collagen under flow. U0126 did not affect aggregate formation or PS exposure when WT blood was perfused in P2X$_1$ nonprotective conditions (no apyrase) (not shown). These observations thus support the existence of a P2X$_1$-ERK2 pathway contributing to platelet activation by collagen and surface-bound VWF.

Potent shear-induced aggregation of TG platelets

Platelet aggregation induced by shear stress can be measured in a viscometer producing a laminar flow. At high shear stress, VWF binding to GPIbα is essential to induce $\alpha_{IIb}\beta_3$-dependent platelet aggregation.[25] We have investigated whether P2X$_1$ would contribute to shear-dependent platelet aggregation. For this purpose, platelet aggregations were performed in apyrase-treated heparinized PRP from WT and TG mice, at a shear rate of 9000 seconds^{-1} corresponding to a shear stress of 124 dyne/cm^2. At this shear rate, the transgenic platelets underwent potent aggregation (52.4% ± 15.5% of aggregation) compared with negligible aggregation of WT platelets (9.9% ± 6.3%) (Figure 7), showing that the TG platelets exhibited increased ability to respond to shear stress.

Increased thrombotic tendency in transgenic mice

Bleeding times, considered to reflect primary hemostasis in vivo, were identical for WT and TG mice (WT, 5.07 ± 2.05 minutes, n = 22; TG, 4.98 ± 2.41 minutes, n = 12). Because in vitro collagen-induced transgenic platelet activation and aggregation were greatly enhanced, we used an in vivo model of pulmonary thromboembolism by intravenous injection of a mixture of a low dose of collagen (0.06 mg/kg body weight) and epinephrine (60 μg/kg). As shown in Figure 8A, 80% of the TG mice died after 4 minutes compared with only a 30% mortality rate for WT mice. These results indicate that P2X$_1$ overexpression generates a prothrombotic phenotype.

Antithrombotic protection by blockade of the ERK2 pathway

To further examine the importance of the P2X$_1$-coupled ERK2 signaling pathway for platelet function in vivo, we analyzed the effect of a 1-minute pretreatment with U0126 (200 μg/kg) on the mortality of TG mice. All mice pretreated with U0126 survived the injection of collagen (0.06 mg/kg) plus epinephrine (60 μg/kg) (Figure 8A). Inhibition of ERK2 also conferred antithrombotic protection to WT mice injected with a mixture of a higher dose of collagen (0.125 mg/kg) plus epinephrine (60 μg/kg); the number of survivors reached 90% compared with 40% in controls (Figure 8B).

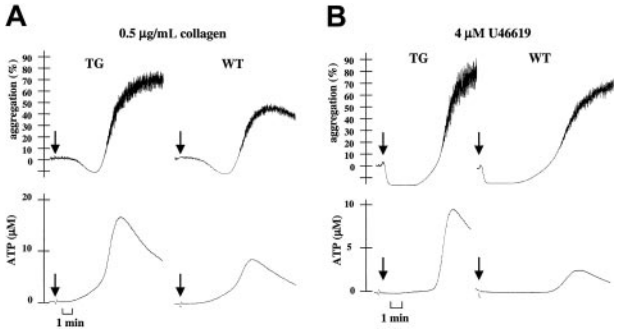

Figure 5. Increased collagen- or U46619-induced platelet ATP secretion with transgenic platelets. (A) Parallel recordings of light transmission (%) and ATP secretion (μM) in WT or TG mouse hirudinized PRP during platelet stimulation with 0.5 μg/mL collagen. (B) Identical experiment after platelet stimulation with 4 μM U46619. Data are representative of at least 3 independent experiments performed in duplicate on platelets from WT and TG mice.

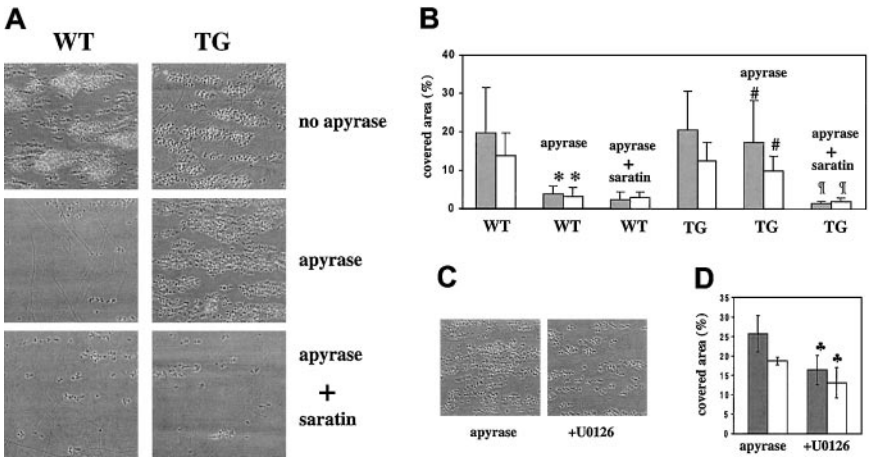

Figure 6. Increased aggregate formation and PS exposure over a collagen surface under flow with transgenic platelets. Inhibitory effect of U0126. Whole blood from WT or TG mice was perfused at a wall shear rate of 1000 seconds^{-1} for 4 minutes over a fibrillar collagen-coated surface. Blood from these 2 groups was assessed in the absence or presence of apyrase (0.1 U/mL) with or without saratin (10 μg/mL) and with or without U0126 (1 μM) for TG blood, as indicated. (A,C) Representative phase-contrast microscope images after perfusion. (B,D) Gray bars (▨) represent the surface area coverage by platelets calculated from the phase-contrast images at the end of the perfusion period. White bars (□) represent the area coverage by platelets binding OG488-annexin V after perfusion (platelets exposing negatively charged PS) (n = 3-6) (*$P < .001$ vs WT; #$P < .001$ vs WT + apyrase; ¶$P < .001$ vs TG + apyrase; ♣$P < 0.035$ vs TG + apyrase).

Discussion

We have generated transgenic mice overexpressing the human ATP-gated $P2X_1$ ion channel in the megakaryocytic cell lineage. Platelets from these mice displayed a gain of $P2X_1$ ionotropic activity, as shown by more prominent Ca^{2+} influx and platelet shape change triggered by the 2 $P2X_1$-selective agonists, αβ-meATP and βγ-meATP. Transgenic platelets underwent enhanced aggregation in response to low doses of collagen or of the thromboxane A_2 mimetic U46619. We showed that the increased collagen- and U46619-induced platelet aggregations resulted from enhanced platelet-dense granule release. $P2X_1$ overexpression also increased platelet reactivity to shear stress. Transgenic mice manifested increased in vivo thrombosis after injections of a mixture of low-dose collagen and epinephrine. Thus, the overexpression of $P2X_1$ in mouse platelets has generated a novel, mild, prothrombotic phenotype.

Enhanced secretion and aggregation of the transgenic platelets in response to collagen corroborate our previous findings in healthy human platelets.[13,14] We have reported that low concentrations of collagen cause early minor ATP release that elicits a rapid $P2X_1$-mediated Ca^{2+} influx, contributing to enhancement of the platelet release reaction, thus completing platelet aggregation. It is therefore likely that the enhanced TG platelet response to collagen results from an increased number of functional ATP-responsive $P2X_1$ channels expressed at the surfaces of TG platelets. The higher level of $P2X_1$ activation thereby leads to enhanced platelet secretion and aggregation. Similarly, the increased TG platelet aggregation induced by low concentrations of the thromboxane A_2 mimetic U46619 was related to enhanced platelet secretion induced by this agonist. This is in agreement with earlier studies on the importance of secreted products, mainly ADP, during U46619-induced platelet aggregation.[26] Collagen activates platelets by transducing signals through glycoprotein VI (GPVI). We investigated whether the observed enhanced platelet aggregation induced by collagen depended on this glycoprotein by using the GPVI-selective agonist convulxin. We found that TG platelet aggregation induced by low concentrations of convulxin was similarly enhanced, as with collagen, suggesting cooperation between secreted ATP- and GPVI-mediated signaling under mild stimulation of this receptor. In agreement with this finding and using ADP receptor antagonists, Quinton et al[27] have reported a role for secreted ADP during platelet aggregation provoked by low concentrations of convulxin, whereas platelet aggregation at higher concentrations of convulxin was unaffected by these agents.

In human platelets, we have identified the ERK2 signaling pathway as an intracellular mechanism subserving the function of the ATP-gated $P2X_1$ ion channel during platelet aggregation induced by low concentrations of collagen. We have shown that the $P2X_1$-mediated ERK2 activation is needed to amplify dense-granule release initiated by this agonist. As in human platelets, the aggregation of wild-type mouse platelets evoked by low concentrations of collagen was abolished after blockade of the ERK2 pathway with the selective MEK1/2 inhibitor U0126. Accordingly, the increased collagen-induced aggregation of transgenic platelets coincided with the up-regulation of ERK2 and could equally be abolished by U0126. It appears that $P2X_1$ and ERK2 are 2

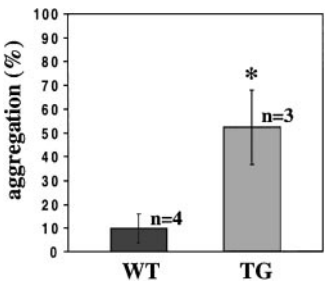

Figure 7. Potentiated shear-induced TG platelet aggregation. Aggregation of platelets from WT or TG mice induced at a shear rate of 9000 seconds^{-1} in a viscometer for 3 minutes. WT and TG platelet aggregations were analyzed in apyrase (0.1 U/mL)–treated heparinized PRP (*$P = .0039$). At least 3 independent experiments were performed on PRP pools prepared from 2 mice.

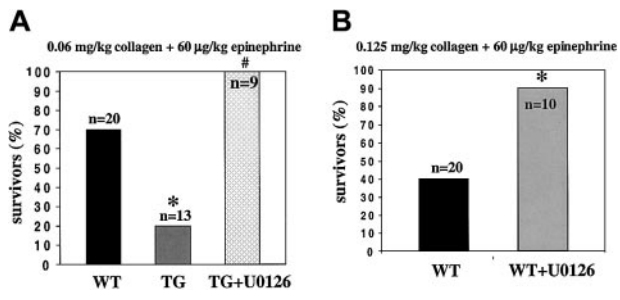

Figure 8. Pulmonary thromboembolism. (A) Increased lethal thrombosis in transgenic mice after the intravenous injection of collagen and epinephrine and protection against thrombosis by the inhibition of ERK2. Survival in WT and TG mice is shown as a percentage of animals treated (*$P = .017$ vs WT; #$P = .017$ vs TG). (B) Antithrombotic protection after the inhibition of ERK2 in WT mice by the preinjection of U0126 (200 μg/kg) versus control (*$P = .0187$).

components of a common signaling cascade in human and mouse platelets.

Thus, even though the $P2X_1$-mediated Ca^{2+} influx seems to be small, this necessarily local signal close to the plasma membrane, where ERK2 is also translocated, is likely to generate a significant local trigger for the promotion of Ca^{2+}- and ERK2-dependent secretion.

To investigate the physiologic relevance of the $P2X_1$-coupled ERK2 pathway in thrombosis, we used an in vivo model of pulmonary thromboembolism by injection of a mixture of collagen and epinephrine. We showed that inhibition of the ERK2 pathway by U0126 fully protected transgenic mice against lethal thrombosis induced by a low dose of collagen plus epinephrine. Wild-type mice were also partly protected after the administration of a higher dose of collagen. Together with the observation that $P2X_1$-overexpressing transgenic platelets were hyperreactive to collagen in an ERK2-dependent fashion, our in vivo data support the existence of a $P2X_1$-ERK2 signaling axis involved—among several other components—in the control of platelet function during hemostasis.

Shear-induced platelet aggregation requires initial VWF binding to platelet GPIbα and subsequent $\alpha_{IIb}\beta_3$ activation.[25] The fact that shear stress triggered potent aggregation of transgenic platelets whereas wild-type platelets hardly aggregated supports an additional role for the $P2X_1$ ion channel in this process. Because the sustained elevation of cytosolic Ca^{2+} occurs in association with shear-induced platelet aggregation, possibly as the consequence of a rapid transmembrane ion flux,[28] the contribution of $P2X_1$-mediated Ca^{2+} influx to platelet activation can be hypothesized. This would be in analogy to the shear stress–activated Ca^{2+} influx into human endothelial cells through another member of the P2X family, $P2X_4$.[29] The authors proposed that $P2X_4$ has a "shear-transducer" property through which shear stress is perceived directly or indirectly (shear-induced ATP release) and is transmitted to the cell through Ca^{2+} signaling. Further analyses of transgenic platelet responses to shear stress will enable us to determine whether $P2X_1$ represents a platelet shear-transducer acting in conjunction with GPIb.

Where rapid blood flow creates high wall shear rates, such as in arterioles in the healthy circulation or in atherosclerotic arteries with restricted lumen, platelet thrombus formation depends on VWF immobilized on extracellular matrix components, in particular collagens.[30] Compatible with the enhanced reactivity of transgenic platelets to collagen and shear stress, perfusion of whole blood from transgenic mice over a collagen surface at a shear rate of 1000 $seconds^{-1}$ resulted in greatly enhanced aggregate formation and surface exposure of negatively charged phosphatidylserine, which is instrumental in coagulation activation.[31] These platelet responses were inhibited by saratin, which blocks the binding of VWF to collagen,[19] indicating enhancement of an event requiring collagen-bound VWF. Furthermore, consistent with the existence of the $P2X_1$-ERK2 pathway, transgenic platelet aggregation on such a surface was significantly reduced after inhibition of the ERK2 pathway with U0126, possibly because of reduced secretion.

In vitro, because of the rapid desensitization of $P2X_1$ by spontaneously released ATP, a high concentration of apyrase is often required to demonstrate this ion channel function.[12-14] In addition, in the present experiments with isolated mouse platelets, apyrase was needed to detect αβ-meATP–induced Ca^{2+} influx and platelet shape change. Using this ectonucleotidase leads to the simultaneous degradation of actively secreted ATP, the major agonist of $P2X_1$, but also of ADP, acting at $P2Y_1$ and $P2Y_{12}$. Therefore, the use of apyrase for in vitro studies always constitutes a compromise between receptor protection and ligand breakdown. In vivo experiments in the transgenic mouse model, where endogenous ecto-ATPases prevent $P2X_1$ desensitization, essentially confirm the conclusions of our in vitro analyses of this ion channel function and thus demonstrate a physiologic role for $P2X_1$ in platelets. This implies that, in vivo, mechanisms relying on fine-tuning ATPases[32] must operate to control $P2X_1$ activity (desensitization and resensitization). The importance of these mechanisms for platelet function has been highlighted in mice lacking the endothelial ecto-nucleoside triphosphate diphosphohydrolase CD39 (NTPDase 1). Indeed, these mice showed disordered hemostasis and thromboregulation.[33]

$P2X_1$ knock-out mice display male infertility resulting from reduced neurogenic vas deferens contraction.[34] In agreement with our study, preliminary data recently presented indicate impaired in vitro platelet aggregation induced by low doses of collagen.[35] Furthermore, the perfusion of whole blood from these mice over a collagen surface revealed reduced aggregate formation under flow conditions.[35]

Finally, because $P2X_1$ overexpression in platelets generates a prothrombotic tendency, we can speculate that pathologic deregulation of $P2X_1$ expression may have a significant impact on platelet activation and may contribute to abnormal thrombosis. It is noteworthy that, in other tissues, the up-regulation of $P2X_1$ mRNA levels has been described in pathophysiologic conditions (reviewed by Burnstock[4]). Thus, $P2X_1$, as the predominant P2 receptor subtype in bladder smooth muscle, showed a considerably increased expression in the symptomatically obstructed bladder. The up-regulation of $P2X_1$ mRNA in the hearts of rats with congestive heart failure has been reported.

Overall, the present study provides evidence that the $P2X_1$ ion channel plays a role in mediating the biologic effects of ATP during platelet activation. Platelet overexpression of $P2X_1$ resulted in increased platelet secretion and aggregation triggered by collagen and thromboxane A_2 but also enhanced platelet responses under shear stress. A novel physiologic role of the $P2X_1$-ERK2 signaling pathway in hemostasis and thrombosis is also proposed.

References

1. Ravelic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998;50:413-492.
2. Abbracchio MP, Burnstock G. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther. 1994;64:445-475.
3. Buell G, Collo G, Rassendren F. P2X receptors: an emerging channel family. Eur J Neurosci. 1996;8:2221-2228.
4. Burnstock G. Potential therapeutic targets in the rapidly expanding field of purinergic signaling. Clin Med. 2002;2:45-53.
5. North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002;82:1013-1067.
6. Reimers HJ. Adenine nucleotides in blood platelets. In: Longenecker GL, ed. The Platelets: Physiology and Pharmacology. Orlando, FL: Academic Press; 1985:85-98.
7. Gachet C. ADP receptors of platelets and their inhibition. Thromb Haemost. 2001;86:222-232.
8. Savi P, Bornia J, Sahel V, Delfaud M, Herbert J-M. Characterization of $P2X_1$ purinoceptors on rat platelets: effect of clopidogrel. Br J Haematol. 1997;98:880-886.
9. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via $P2X_1$ not P2T purinoceptors in human platelets. J Biol Chem 1996;271:2879-2881.
10. Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at $P2X_1$ receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. Br J Pharmacol. 2000;131:108-114.
11. Evans RJ, Lewis C, Buell G, Valera S, North RA, Surprenant A. Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2X purinoceptors). Mol Pharmacol. 1995;48:178-183.
12. Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of $P2X_1$ purinoceptors with α,β-methylene ATP. Thromb Haemost. 2001;85:303-308.

13. Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts MF. The ATP-gated $P2X_1$ ion channel acts as a positive regulator of platelet responses to collagen. Thromb Haemost. 2001; 86:1264-1271.
14. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. $P2X_1$-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. Blood. 2002;100:2499-2505.
15. Denarier E, Martin F, Martineau S, Marguerie G. PCR cloning and sequence of the murine GPIIb gene promoter. Biochem Biophys Res Commun. 1993; 195:1360-1364.
16. Oury C, Toth-Zsamboki E, Van Geet C, et al. A natural dominant negative $P2X_1$ receptor due to deletion of a single amino acid residue. J Biol Chem. 2000;275:22611-22614.
17. Tronick-Le Roux D, Roullot V, Schweitzer A, Berthier R, Marguerie G. Suppression of erythromegakaryocytopoiesis and the induction of reversible thrombocytopenia in mice transgenic for the thymidine kinase gene targeted by the platelet glycoprotein αIIb promoter. J Exp Med. 1995; 181:2141-2151.
18. Holvoet P, Danloy S, Collen D. Role of the carboxy-terminal domain of human apolipoprotein AI in high-density-lipoprotein metabolism: a study based on deletion and substitution variants in transgenic mice. Eur J Biochem. 1997;245:642-647.
19. Barnes CS, Krafft B, Frech M, et al. Production and characterization of saratin, an inhibitor of von Willebrand's factor-dependent platelet adhesion to collagen. Semin Thromb Hemost. 2001;27: 337-346.
20. Heemskerk JWM, Feijge MAH, Sage SO, Walter U. Indirect regulation of Ca^{2+} entry by cAMP-dependent and cGMP-dependent protein kinases and phospholipase C in rat platelets. Eur J Biochem. 1994;223:543-551.
21. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicator dyes with greatly improved fluorescence properties. J Biol Chem. 1985;260:3440-3450.
22. Nieswandt B, Brakebusch C, Bergmeier W, et al. Glycoprotein VI but not $\alpha_2\beta_1$ integrin is essential for platelet interaction with collagen. EMBO J. 2001;20:2120-2130.
23. Remijn JA, Wu Y-P, Jeninga EH, et al. Role of ADP receptor $P2Y_{12}$ in platelet adhesion and thrombus formation in flowing blood. Arterioscler Thromb Vasc Biol. 2002;22:686-691.
24. Turner NA, Moake JL, McIntire LV. Blockade of adenosine diphosphate receptors $P2Y_{12}$ and $P2Y_1$ is required to inhibit platelet aggregation in whole blood under flow. Blood. 2001;98:3340-3345.
25. Ikeda Y, Handa M, Kawano K, et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest. 1991;87:1234-1240.
26. Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A_2-induced platelet aggregation: essential role for $P2T_{AC}$ and α_{2A} receptors. J Biol Chem 1999;274:29108-29114.
27. Quinton TM, Ozdener F, Dangelmaier C, Daniel JL, Kunapuli SP. Glycoprotein VI-mediated platelet fibrinogen receptor activation occurs through calcium-sensitive and PKC-sensitive pathways without a requirement for secreted ADP. Blood. 2002;99:3228-3234.
28. Ikeda Y, Handa M, Kamata T, et al. Transmembrane calcium influx associated with von Willebrand factor binding to GPIb in the initiation of shear-induced platelet aggregation. Thromb Haemost. 1993;69:496-502.
29. Yamamoto K, Korenaga R, Kamiya A, Ando J. Fluid shear stress activates Ca^{2+} influx into human endothelial cells via $P2X_4$ purinoceptors. Circ Res. 2000;87:385-391.
30. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. Cell. 1998;94:657-666.
31. Heemskerk JWM, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thromb Haemost. 2002;88:186-193.
32. Coade SB, Pearson JD. Metabolism of adenine nucleotides in human blood. Circ Res. 1989;65: 531-537.
33. Enjyoji K, Sevigny J, Lin Y, et al. Targeted disruption of CD39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med. 1999;5:1010-1017.
34. Mulryan K, Gitterman DP, Lewis CJ, et al. Reduced vas deferens contraction and male infertility in mice lacking $P2X_1$ receptors. Nature. 2000; 403:86-89.
35. Hechler B, Vial C, Freund M, Cazenave J-P, Evans RJ, Gachet C. A minor role of the platelet $P2X_1$ receptor in hemostasis: studies in $P2X_1$ knock-out mice [abstract]. J Hematol. 2002;87: 17.