Differential Involvement of the P2Y$_1$ and P2Y$_{12}$ Receptors in Platelet Procoagulant Activity

Catherine Léon, Catherine Ravanat, Monique Freund, Jean-Pierre Cazenave, Christian Gachet

Objective—In vivo, activated platelets contribute to the initiation of thrombin generation through the exposure of phosphatidylserine to form a procoagulant catalytic surface and through platelet–leukocyte interactions, which lead to the exposure of leukocyte tissue factor (TF). On the basis of observations that the platelet P2Y$_1$ and P2Y$_{12}$ receptors both contribute to thrombosis and thrombin formation in an in vivo model of TF-induced thromboembolism, we further characterized the role of these receptors in thrombin generation.

Methods and Results—By using the selective P2 antagonists MRS2179 and AR-C69931MX, the P2Y$_{12}$ receptor was found to be involved in thrombin-induced exposure of PS on isolated platelets and consequently in TF-induced thrombin formation in platelet-rich plasma. By contrast, the P2Y$_1$ receptor was not involved in phosphatidylserine exposure nor in thrombin generation in platelet-rich plasma. In addition, both receptors were found to contribute to the interactions between platelets and leukocytes mediated by platelet P-selectin exposure, which result in TF exposure at the surface of leukocytes.

Conclusions—Overall, these results point to a differential involvement of the 2 platelet ADP receptors in the generation of thrombin and provide further evidence for the relevance of molecules targeting these receptors as antithrombotic agents. (Arterioscler Thromb Vasc Biol. 2003;23:1941-1947.)

Key Words: P2 receptor ■ ADP ■ thrombin generation ■ thrombosis ■ platelets
thrombin–antithrombin complexes (TAT), suggesting that this receptor might also be involved in the generation of thrombin.

The aim of the present study was to characterize the respective roles of the P2Y1 and P2Y12 receptors in thrombin generation. In vivo studies were performed in P2Y1-deficient mice treated or not treated with clopidogrel. The contributions of the P2Y1 and P2Y12 receptors to different aspects of thrombin formation were distinguished using their respective selective antagonists, MRS2179 and AR-C69931MX. The direct real-time in vitro thrombin generation in PRP was measured by the thrombogram method19 and the exposure of leukocytes, the exposure of platelet P-selectin, and (3) interactions between platelets and leukocytes was determined in whole blood by flow cytometry. Overall, our data indicate that both ADP receptors are involved, although differentially, in the generation of thrombin in vivo and in vitro.

Materials and Methods

Materials
ADP, MRS2179, and fatty acid free human serum albumin were from Sigma-Aldrich (Saint Quentin-Fallavier, France). Purified human α-thrombin was from EFS-Alsace (Strasbourg, France), thrombin receptor activating peptide (TRAP) from Néosystem (Strasbourg, France), clopidogrel (Plavix) from Sanofi-Synthélabo (Toulouse, France) and FITC Annexin V from Roche (Meylan, France). Recombinant hirudin was kindly provided by Transgène (Strasbourg, France). Monoclonal antibodies against human antigens were FITC-CD142 (TF, clone 4508) from American Diagnostica (Greenwich, CT); FITC-CD42b (GPIibα, clone SZ2) and FITC-CD62P (P-selectin, clone CLB-thromb/6) from Immunotech Coulter (Marseille, France); and PE-CD45 (clone 30-F11) and PE-CD42b (clone HIP1) from Pharmingen (France). Human thromboplastin (Thromborel S) and recombinant human TF (Innovin) were from Dade Behring (Mannheim, Germany). AR-C69931MX was from Astra Charnwood (Loughborough, UK) and the fluorogenic substrate Z-GGR-AMC (400 μmol/L) was added in Fluo buffer (20 mmol/L HEPES, 60 μg/mL bovine serum albumin, pH 7.35 containing CaCl2 (17 mmol/L). AR-C69931MX, which is a potent P2Y1 antagonist, was nevertheless used at a concentration of 10 μmol/L in all experiments so as to block all P2Y1 receptors. At this concentration, AR-C69931MX is still selective with no effect on the P2Y1 receptor. Thrombin generation was started by injection of recombinant human tissue factor (Innovin, 1/12,000 final dilution) and recorded for 70 minutes as described.19 Concentrations of the thrombin generated were calculated from a calibration curve constructed with known amounts of calibrated thrombin.

Measurement of PI Exposure by FITC Annexin V Binding
Washed platelet suspensions in Tyrode’s buffer were prepared from human and mouse blood as previously described.17,21 Platelets (3×10^5/mL) were incubated with MRS2179 (100 μmol/L), AR-C69931MX (10 μmol/L), or vehicle and activated with thrombin (1 U/mL) for 5 minutes at 37°C, without stirring. A 5-μL aliquot was then labeled with FITC Annexin V in the presence of hirudin (10 U/mL) for 10 minutes at room temperature, diluted, and analyzed by flow cytometry.

Whole Blood Stimulation and Double-Labeling Flow Cytometry
Immediately after collection, a 70-μL sample of blood anticoagulated with hirudin (100 U/mL) was incubated with MRS2179 (100 μmol/L), AR-C69931MX (10 μmol/L), or vehicle at 37°C for 2 minutes without stirring. The sample was stimulated with ADP (100 μmol/L) or TRAP (10 μmol/L) at 37°C for 15 minutes. A 5-μL aliquot was then incubated with Tyrode’s buffer (45 μL) containing fluorescent antibodies (1/10 final dilution) for 20 minutes, diluted, and fixed in saline containing 0.5% (vol/vol) formaldehyde. All flow cytometric parameters were acquired on a logarithmic scale, and the forward and side scatter parameters were used to identify the leukocyte and isolated platelet populations. TF exposure on leukocytes was measured by recording a total of 1500 events in the leukocyte population gated as CD45-positive and determining the percentage of FITC-CD42b-positive cells in this population. Platelet-leukocyte aggregates were determined as the percentage of FITC-CD42b-positive events in the leukocyte population. Platelet P-selectin expression was measured by recording 10,000 events in the platelet population (FITC-CD42b-positive) and calculating the percentage of FITC-CD62P-positive cells in this population. Fluorescein isothiocyanate- and PE-conjugated isotype controls were used to quantify the background labeling.

Statistical Analyses
Results were expressed as the mean (± SEM) and the data were compared by one-way analysis of variance or repeated measures one-way analysis of variance where appropriate, followed by a Dunnet post-test.

Results
Intravascular Thromboembolism
The role of the P2Y1 and P2Y12 receptors in systemic intravascular thrombosis was evaluated by measuring the platelet consumption caused by massive occlusion of the microcirculation of the lungs with platelet thromboemboli.22 Platelet consumption is a more sensitive marker of thromboembolism than mortality and allows the simultaneous measurement of plasma TAT complexes.18 As previously reported,18 intravenous injection of TF induced less platelet consumption in P2Y1-deficient mice (40.6±5.6%) than in wild-type mice (88.7±1.4%, P<0.01) (Figure 1A). Wild-type mice treated with clopidogrel displayed a significantly reduced platelet consumption similar to that of P2Y1-deficient mice (38.6±7.6%, P<0.01 versus untreated wild-type mice). Finally, only 13.7±2.3% platelet consumption was observed in...
P2Y₁-deficient mice treated with clopidogrel, demonstrating an additive effect when the 2 ADP receptors were blocked (*P < 0.01 versus clopidogrel-treated wild-type mice or non-treated P2Y₁-deficient mice; Figure 1A).

The contribution of the receptors to thrombin generation was evaluated in this model by measuring levels of TAT complexes. After TF injection, levels of TAT were lower in P2Y₁-deficient mice than in wild-type mice (193.1 ± 6.7 and 274.2 ± 40.5 μg/L, respectively, *P < 0.01), confirming previous data (Figure 1B). Clopidogrel treatment of wild-type mice also reduced TAT formation (176.1 ± 10.9 μg/L, *P < 0.01 versus untreated wild-type mice), but no additive effect on TAT complexes was observed in P2Y₁-deficient mice treated with clopidogrel (151.6 ± 7.1 μg/L; Figure 1B).

These results indicate that the P2Y₁ and P2Y₁₂ receptors both play a role in thrombin generation in vivo. To characterize the part played by each receptor, we next determined their involvement in the in vitro production of thrombin in PRP, the exposure of procoagulant phospholipids on platelets, and the exposure of TF on leukocytes.

In Vitro Thrombin Generation Induced by TF in Human and Mouse PRP

The respective roles of the P2Y₁ and P2Y₁₂ receptors in the thrombin production induced by TF in human and mouse PRP were investigated using the thrombogram method. The thrombin generation curve (thrombogram) is characterized by several parameters: (1) the lag phase reflecting the clotting time, (2) the peak thrombin height, which represents the maximum velocity of net thrombin production and reflects the maximum prothrombinase activity attained, (3) the time to this peak, and (4) the endogenous thrombin potential (ETP), which is the area under the curve representing the total amount of active thrombin generated, but is a less sensitive parameter than the peak thrombin production. The TF dilution (1/12,000) was selected so that the formation of thrombin depended on the presence of platelets. Thus, the thrombin generated in platelet-poor plasma was undetectable under these conditions (data not shown). The effects on the thrombogram parameters of the P2Y₁₂ antagonist AR-C69931MX (10 μmol/L) and the P2Y₁ antagonist MRS2179 (100 μmol/L) on thrombin formation in human PRP (1 × 10⁸ platelets/mL) from wild-type and P2Y₁-deficient mice. Results are from one experiment performed in quadruplicate, representative of at least 3 independent experiments.
Effects of MRS2179 and AR-C69931MX on Thrombin Generation in Human and Mouse PRP

<table>
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<th>Vehicle (n=4)</th>
<th>MRS 100 μmol/L (n=4)</th>
<th>ARC 10 μmol/L (n=4)</th>
<th>MRS+ARC (n=4)</th>
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<tr>
<td><strong>Human PRP</strong></td>
<td></td>
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<tr>
<td>Lag time, min</td>
<td>11.75±1.0</td>
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<td>Time to peak, min</td>
<td>23.1±1.3</td>
<td>24.5±1.6</td>
<td>28.8±1.6†</td>
<td>28.6±2.0†</td>
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<tr>
<td>Peak height, nM</td>
<td>77.0±13.2</td>
<td>71.5±14.4</td>
<td>60.4±12.5†</td>
<td>59.6±8.7†</td>
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<td>ETP, nM thrombin×min</td>
<td>1399.5±150.3</td>
<td>1382.3±147.8</td>
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<th>Wild-Type Vehicle (n=3)</th>
<th>Wild-Type ARC 10 μmol/L (n=3)</th>
<th>P2Y1−/− Vehicle (n=3)</th>
<th>P2Y1−/− ARC 10 μmol/L (n=3)</th>
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<td>Lag time, min</td>
<td>5.1±0.8</td>
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<td>Time to peak, min</td>
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<td>10.4±1.0</td>
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<td>Peak height, nM</td>
<td>60.1±10.2</td>
<td>41.9±7.8</td>
<td>55.3±8.8</td>
<td>39.8±8.2*</td>
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<td>ETP, nM thrombin×min</td>
<td>550.0±34.8</td>
<td>452.2±34.2</td>
<td>514.2±67.3</td>
<td>440.2±83.8</td>
</tr>
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</table>

Values are the mean±SEM of 3 or 4 experiments performed in quadruplicate.
*P<0.05, †P<0.01 vs vehicle.

The part played by the P2Y1 and P2Y12 receptors in platelet PS exposure was investigated by measuring the binding of FITC Annexin V by flow cytometry.

**Effects of P2 Years1 and P2 Tears12 Antagonists on PS Exposure on Human and Mouse Platelets**

The part played by the P2Y1 and P2Y12 receptors in the exposure of PS at the surface of platelets was investigated by measuring the binding of FITC Annexin V by flow cytometry. Human platelets were stimulated with thrombin (1 U/mL) in the presence or absence of the P2Y1 antagonist MRS2179 or the P2Y12 antagonist AR-C69931MX. Thrombin treatment increased the percentage of FITC Annexin V-labeled platelets from 1.9±0.8% in the resting state to 31.4±4.8% after stimulation, reflecting the exposure of negative membrane phospholipids. Conversely, no significant increase was observed when platelets were stimulated with ADP (100 μmol/L) or collagen (10 μg/mL; data not shown). AR-C69931MX (10 μmol/L) significantly decreased thrombin-induced PS exposure (12.6±2.8% FITC Annexin V-labeled platelets, P<0.01; Figure 3A), whereas MRS2179 (100 μmol/L) had no significant effect (25.9±3.5% labeled platelets).

Similarly to human platelets, wild-type mouse platelets stimulated with thrombin (1 U/mL) displayed a significant increase in FITC Annexin V binding (42.3±11.7% labeled platelets versus 0.7±0.1% in the resting state). P2Y1-deficient mouse platelets had a PS exposure comparable to that of wild-type platelets (37.1±11.0% versus 1.0±0.1% in the resting state; Figure 3B). AR-C69931MX decreased the percentage of labeled wild-type or P2Y1-deficient mouse platelets to 16.5±3.0% (P<0.05) and 19.3±4.2% (P<0.05), respectively. These results suggest that in isolated human or mouse platelets, the P2Y12 receptor is involved in PS exposure after thrombin stimulation whereas the P2Y1 receptor is not, which probably explains the thrombogram observations.

**Platelet P-Selectin Exposure in Whole Blood**

The involvement of the P2Y1 and P2Y12 receptors in platelet P-selectin exposure was evaluated in whole blood stimulated with TF and MRS2179 or AR-C69931MX. Figure 3A and 3B show that the percentage of annexin V-labeled isolated platelets or CD42b-positive cells (mean±SEM). *P<0.05, **P<0.01 versus vehicle (human) or vehicle/wild-type (mouse).
with either ADP (100 μmol/L) or TRAP (10 μmol/L). Double-labeling flow cytometry was used to determine the percentage of P-selectin-positive events in the isolated platelet population (CD42b positive). In the resting state, less than 0.05% of platelets expressed P-selectin, whereas ADP weakly increased this expression to up to 6.6%. The effect of ADP was blocked by either MRS2179 (0.8±0.4% P-selectin-positive platelets, P<0.01) or ARC69931MX (0.6±0.2% P-selectin-positive platelets, P<0.01; Figure 4A). TRAP markedly increased P-selectin exposure to 41.0±5.5% of platelets. Blockade of the P2Y₁ receptor decreased TRAP-induced P-selectin expression by 50% (19.3±4.5% P-selectin-positive platelets, P<0.01), whereas blockade of the P2Y₁₂ receptor reduced this expression by more than 90% (2.9±0.5% P-selectin-positive platelets, P<0.01; Figure 4B). Thus, the 2 ADP receptors would both appear to be involved in platelet P-selectin exposure.

Platelet–Leukocyte Aggregates (PLAs)

PLAs were determined as the percentage of CD42b-positive events in the leukocyte population (CD45 positive). In the absence of stimulation, less than 5% of leukocytes were TF positive, whereas ADP (100 μmol/L) increased TF exposure to 21.0±2.9% TF-positive leukocytes. MRS2179 and ARC-69931MX both inhibited ADP-induced TF expression (6.5±1.4%, P<0.01, and 5.1±0.8%, P<0.01, respectively; Figure 6A). TRAP stimulation led to 23.2±2.9% TF-positive leukocytes, and this percentage decreased to 9.9±0.7% (P<0.01) or 3.4±0.2% (P<0.01) in the presence of MRS2179 or ARC-69931MX, respectively.

Leukocyte TF Expression

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When both ADP receptors were blocked, no further decrease was observed using either agonist (Figure 6B).

Discussion

The aim of the present study was to characterize the involvement of the 2 platelet ADP receptors in thrombin generation. In vivo, the blocking of either the P2Y1 or the P2Y12 receptor was equally effective with regard to both platelet consumption and thrombin generation as measured by TAT complex formation. However, the blocking of both receptors had an additive effect on platelet consumption but not on thrombin generation, suggesting that separate pathways are responsible for these 2 phenomena.

Activated platelets contribute to fibrin formation by providing a catalytic surface for the assembly of enzyme complexes involved in the sequential reactions leading to thrombin formation.2 The P2Y12 receptor appeared to play a significant part in the exposure of a procoagulant surface on platelets because the P2Y1 antagonist AR-C69931MX decreased the exposure of PS and delayed the course of thrombin generation in PRP. The total amount of thrombin generated (ETP), although delayed, was nevertheless not significantly affected by AR-C69931MX in either human or mouse PRP. Similarly, when platelet GPIIbIIIa is absent as in Glanzmann’s thrombasthenia or inhibited with abciximab at a therapeutic dose, the kinetics of thrombin formation are strongly retarded, the lag time, peak time, and peak height being affected, whereas the endogenous thrombin potential is not or only slightly decreased (C. Ravanat, unpublished data, 2002).8,23 This is consistent with previous observations showing that blocking platelet activation affects primarily the kinetics of the reaction rather than the total amount of thrombin produced, unlike direct anticoagulants, which considerably decrease the total thrombin generation.24 These results also confirm earlier studies demonstrating a role of the P2Y12 receptor in the exposure of negative phospholipids and the formation of thrombin in PRP.7,25,26 In contrast, the P2Y1 receptor did not appear to be significantly involved in either platelet consumption or thrombin generation, suggesting that separate pathways are responsible for these 2 phenomena.

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However, the in vivo thromboembolism studies supporting a role of the P2Y1 receptor in thrombin formation suggest that either the in vivo context is important in highlighting the involvement of this receptor or that other mechanisms could account for its participation in thrombin generation. We therefore investigated in whole blood the role of the P2Y1 and P2Y12 receptors in interactions between platelets and leukocytes. Activation of platelets leads to the expression of adhesion molecules, among which P-selectin has been shown to be involved in the formation of conjugates with leukocytes resulting in TF exposure, the physiological initiator of thrombin generation, at the surface of these conjugates.3,4 Whole blood was stimulated with TRAP or ADP. ADP was used at 100 µmol/L to compensate for nucleotide degradation resulting from the presence of ectonucleotidase activities in blood. The exposure of P-selectin at the surface of platelets increased within minutes, and P2Y1 and P2Y12 antagonists inhibited not only ADP-induced but also TRAP-induced P-selectin exposure. Similar effects were observed when examining platelet TF exposure and aggregate formation, the P2Y12 antagonist having a more pronounced effect than the P2Y1 antagonist. These results are consistent with previous studies showing that the blocking of the P2Y12 receptor with clopidogrel or AR-C69931MX decreases both P-selectin expression and PLA formation in response to TRAP or ADP.27–30 Our findings confirm the potentiating role of the P2Y12 receptor in such responses and suggest that this receptor is further involved in the generation of thrombin through platelet–leukocyte interactions. In addition, our results also demonstrate that the P2Y1 receptor contributes to these interactions and to the exposure of TF at the surface of the platelet–leukocyte conjugates, in accordance with our in vivo observations. However, the P2Y1 antagonist failed to decrease the formation of PLA while it inhibited platelet P-selectin exposure, suggesting alternative mechanisms for the role of the P2Y1 receptor in TF exposure. P-selectin exposure is a measure of α-granule secretion and therefore platelet-released products, such as chemokines, known to attract leukocytes and to potentiate platelet activation, may contribute to TF exposure.31,32 Moreover, although it is admitted that platelets are involved in the rapid TF exposure on leukocytes without de novo transcription, it is not yet clear whether TF originates from platelets or from leukocytes or both and by what mechanisms it may be transferred from one cell to another. In our whole blood experiments, we could not come to a conclusion regarding the origin of the TF exposed at the surface of the platelet–leukocytes aggregates. However, recent evidences have shown that TF may also be of intraplatelet origin and may transfer this TF to leukocytes.33–35 Both P2Y1 and P2Y12 receptors participate in the exposure of TF at the platelet surface on activation (C. Léon, unpublished data, 2003) probably contributing also to the in vivo generation of thrombin. However, because the P2Y1 receptor is also expressed by endothelial cells and other blood cells,36 we nevertheless cannot exclude a role of endothelial and leukocyte P2Y1 receptors, which might participate to and reinforce these interactions, leading to platelet activation and TF exposure.

In conclusion, we show here that the P2Y1 and P2Y12 receptors are differentially involved in the generation of thrombin. Both receptors participate in leukocyte TF exposure in whole blood, whereas mostly the P2Y12 receptor plays a role in the TF-induced formation of thrombin in PRP, probably by mediating negative PS exposure on platelets. These results, together with the increased thromboresistance observed in mice when both ADP receptors are blocked, provide further evidence for the relevance of molecules targeting these receptors as antithrombotic agents.
Acknowledgments

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References

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