Prostaglandin Endoperoxide Analogues Stimulate Phospholipase C and Protein Phosphorylation During Platelet Shape Change

By Wolfgang Siess, Barbara Boehlig, Peter C. Weber, and Eduardo G. Lapetina

We have studied the effects of two stable prostaglandin endoperoxide analogues on platelet lipid metabolism in relation to specific platelet functional changes. During platelet shape change, the endoperoxide analogues induce the formation of 1,2-diacylglycerol and phosphatidic acid, indicating the activation of a phosphoinositide-specific phospholipase C. In parallel, they stimulate the phosphorylation of a 40-kd and a 20-kd protein. During platelet shape change, arachidonic acid is released, but not metabolized by platelet cyclo-oxygenase or lipooxygenase. Phospholipase C activation and platelet shape change are independent of extracellular Ca++ and Mg++, arachidonic metabolism, and release of adenosine diphosphate (ADP). Activation of phospholipase C during platelet aggregation seems, however, to be mediated partly by release of ADP. We conclude that endoperoxide analogues initially stimulate in platelets the formation of products derived from phospholipase C activation, which might serve as intracellular messengers for phosphorylation of specific proteins related to platelet shape change.

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Materials and Methods

Reagents

Prostaglandin endoperoxide analogues, U 44069 (15s-hydroxy-9,11-epoxymethano prostaglandin F15) and U 46619 (15s-hydroxy-11,9-epoxymethano prostaglandin F15), prostacyclin (PGI2), and thromboxane (TXB2) were kindly provided by Dr J. Pike (Upjohn Co, Kalamazoo, Mich). Stock solutions of U 44069 or U 46619 (10 mmol/L) in ethanol were stored at –20 °C. Dilutions in isotonic saline were freshly prepared before each experiment. [1H]Arachidonic acid (57 Ci/mmol) was obtained from Amersham (Braunschweig, FRG). Arachidonic acid, 1,2-diolein, phosphatidic acid, phosphatidylcholinostol, adenosine triphosphate (ATP), creatine phosphate (CP), creatine phosphokinase (CPK), potato aaprase, and prostaglandin E2 were obtained from Sigma Chemical Co, St Louis. Indomethacin was a gift from Merck Sharp & Dohme (München, FRG). Two 2-channel aggregometers were from Fresenius (Bad Homburg, FRG). Unlabeled 12-hydroxy-5,8,10-heptadecatnienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosaatetraenoic acid (12-HETE) were prepared by incubation of human platelets with arachidonic acid. Silica gel 60 DC plates were purchased from Merck (Darmstadt, FRG).

Platelet Preparation

Human platelets from 200 mL of blood were prelabeled in platelet-rich plasma with 400 Ci/mCi of [1H]arachidonic acid at 37 °C for two hours in the presence of prostaglandin E1 (1 μg/mL). Platelets were then washed twice with a modified Ca++- and Mg++-free Tyrode-HEPES buffer containing 1 mmol/L ethylene glycol-bis(β-aminoethanol) ether)N,N′-tetraacetate acid (EGTA). Prostaglandin inhibitors were included to prevent platelet activation as detailed previously. Platelet suspensions were centrifuged and kept at room temperature.

This platelet preparation technique was modified in experiments in which the influence of ADP and extracellular divalent cations on platelet lipid metabolism was studied. The washing and resuspension buffer contained 1.5 mmol/L CaCl2 and 1 mmol/L MgCl2 instead of EGTA, since the activity of the ADP-scavenging enzymes apyrase and CPK depends on divalent cations. Either apyrase (0.2 mg/mL) or CP/CPK (0.065 mmol/L 0.2 U/mL) was added to

Key Points

- Prostaglandin endoperoxide analogues induce the activation of phospholipase C.
- During platelet shape change, arachidonic acid is released.
- Platelet shape change, activation of phospholipase C, and release of ADP are independent processes.
- Endoperoxide analogues stimulate the formation of products derived from phospholipase C activation.

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Supported by the Deutsche Forschungsgemeinschaft.

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0006–4971/85/6505–0014$03.00/0
remove trace amounts of ADP that could originate from leaky red cells or platelets damaged during the platelet resuspension procedure. Heparin (25 U/mL) and prostacyclin (300 ng/mL) were added to the washing buffer. Washing and resuspension buffer were kept at 37°C for optimal activity of ADP-scavenging enzymes.

Platelet Shape Change and Aggregation

Suspensions of washed platelets were adjusted to 4 to 6 x 10^9 platelets per milliliter, and the experiments were carried out between 30 and 90 minutes after final resuspension. Platelet responsiveness to the endoperoxide analogues was constant during that period. Platelet suspensions (1.6 mL) were placed into aggregometer cuvettes, stirred for three minutes at 37°C in the aggregometer, and then exposed to U 44069 or U 46619. Shape change and aggregation of platelets were recorded. Inhibitors (indomethacin, prostacyclin, ATP, high concentrations of apyrase or CP/CPK) were added two minutes before platelet stimulation.

Platelet Lipid Metabolism

Incubations (0.2 mL) of platelet suspensions before (control) or at various times after addition of the endoperoxide analogues were stopped by transferring the samples into 0.75 mL of chloroform/methanol 1:2. Unlabeled standards of phosphatidic acid and 1,2-diolein were added, and samples were then partitioned after addition of 0.25 mL chloroform and 0.25 mL of 0.2% formic acid. Lipids were then extracted and separated on thin-layer chromatography as detailed previously. Samples were counted in a scintillation counter (Beckman type LS 330, Fullerton, Calif; efficiency 60%) for ten to 20 minutes to reach a counting error lower than 3%. In some experiments, TXB2 was measured by a specific radioimmunoassay in unlabeled platelet suspensions.

Protein Phosphorylation

Platelets were labeled with ^32P and proteins were separated by electrophoresis through an 11% sodium dodecyl sulfate-polyacrylamide gel.

Data Presentation

Assays were done in duplicates. The radioactivities of the unstimulated control values were either set to 100% or subtracted from the stimulated values (A cpm). In the control samples the ranges of radioactivity (cpm ^3H) for the different lipids were as follows: 600 to 2,000 for phosphatidic acid, 100 to 300 for 1,2-diacylglycerol, 200 to 600 for arachidonic acid, and 80 to 200 for TXB2, HHT, or 12-HETE. These variations were due to the differences of incorporation of [3H]AA into platelet phospholipids (range, 20% to 50%). Data are presented as mean ± SE of individual experiments from different blood donors.

RESULTS

Effect of Endoperoxide Analogues on Platelet Lipid Metabolism

Washed human platelets, prelabeled with [3H]arachidonic acid and resuspended in a Ca<sup>2+</sup>, Mg<sup>2+</sup>-free buffer containing 1 mmol/L EGTA, were exposed to U 44069 or U 46619. Platelet functional responses and changes of [3H]arachidonic acid-labeled platelet lipids were monitored simultaneously. Low concentrations (10<sup>-7</sup> mol/L) of the endoperoxide analogues induce only platelet shape change without subsequent aggregation, whereas higher concentrations (10<sup>-6</sup> to 10<sup>-5</sup> mol/L) induce platelet aggregation after platelet shape change (Fig 1a through c). U 44069 at 10<sup>-5</sup> mol/L induces the rapid formation of 1,2-diacylglycerol and phosphatidic acid, indicating the activation of phospholipase C, and stimulates the release of arachidonic acid and the production of TXB2, HHT, and 12-HETE (Fig 2). Low concentrations of U 44069 or U 46619 (10<sup>-6</sup> mol/L, and in some experiments even 10<sup>-7</sup> mol/L) induce a higher amount of phosphatidic acid and of arachidonic acid, which is metabolized to TXB2, HHT, and 12-HETE. U 46619 at 10<sup>-6</sup> and 10<sup>-5</sup> mol/L has a stronger effect on platelet lipid metabolism than U 44069 (Fig 3), and it also induces a higher rate of platelet aggregation than U 44069 (data not shown). Radioimmunologic determination of TXB2 also revealed that TXB2 is not formed during platelet shape change induced by low concentrations (10<sup>-8</sup> to 10<sup>-7</sup> mol/L) of endoperoxide analogues. 

![Figure 1](https://www.bloodjournal.org)
44069 cuvettes and exposed to U13H-labeled arachidonic acid were placed into aggregometer Suspensions of washed human platelets prelabeled with decatrienoic acid; HETE. 1,2-OH-5,8,10-heptadecatrienoic acid; HHT. 12-OH-5,8,10-heptadecatrienoic acid; HETE. 12-OH-5,8,10,14-eicosатетраен.
In parallel, the formation of 1,2-diacylglycerol and phosphatic acid and the release of arachidonic acid induced by U 44069 are reduced, but not blocked. The formation of TXB$_2$ is, however, almost abolished (Table 1).

ADP-scavenger enzymes, such as apyrase and CP/CPK, require for optimal activity the presence of extracellular Ca$^{2+}$ and Mg$^{2+}$. Low concentrations of apyrase (0.2 mg/mL) or CP/CPK (0.065 mmol/L/0.2 U/mL) do not block the aggregation response to U 44069 (10$^{-5}$ mol/L). High concentrations of CP/CPK (2 mmol/L/20 U/mL) or apyrase (4.5 mg/mL) reduce platelet aggregation induced by U 44069, but platelet shape change is not affected. The changes in the formation of phosphatic acid and arachidonic acid parallel the extent of the platelet functional responses induced by U 44069 (see Table 2). The results show that release of ADP may be an important factor in mediating the aggregation response and, in part, the activation of phospholipase C induced by endoperoxide analogues. The initial activation of phospholipase C during platelet shape change is, however, directly induced by the endoperoxide analogues and independent of ADP.

**Effect of Extracellular Divalent Cations**

Endoperoxide analogues induce a much more rapid and large platelet aggregation response in the presence of 1.5 mmol/L of Ca$^{2+}$ and 1 mmol/L of Mg$^{2+}$ (Fig 1e). The formation of phosphatic acid and the release of arachidonic acid induced by U 44069 (10$^{-5}$ mol/L) are, however, not affected by Ca$^{2+}$ and Mg$^{2+}$. Interestingly, in the presence of Ca$^{2+}$ and Mg$^{2+}$, the released arachidonic acid is not metabolized by cyclooxygenase and lipoxygenase activities (Table 3).

**DISCUSSION**

Low concentrations of the stable prostaglandin endoperoxide analogues U 44069 or U 46619 only
induce platelet shape change and stimulate the formation of diacylglycerol and phosphatidic acid, indicating the activation of phospholipase C. This effect is independent of formation of cyclooxygenase products or release of ADP. Formation of diacylglycerol and phosphatidic acid during platelet shape change occurs without liberation of arachidonic acid metabolites, which indicates a clear dissociation of phospholipase C activation and arachidonic acid metabolism. We recently reported similar results with thrombin and platelet-activating factor as agonists. A possible link between the activation of phospholipase C and the expression of platelet shape change is the phosphorylation of specific proteins: We found that during platelet shape change, endoperoxide analogues stimulate the phosphorylation of two proteins, a 20-kd and a 40-kd protein, respectively. Products derived from phospholipase C activation may be intimately linked to the phosphorylation of the 20-kd and 40-kd proteins. Diacylglycerol activates the Ca⁺⁺- and phospholipid-dependent protein kinase C, which phosphorylates the 40-kd protein.

Table 1. Effect of ATP on Platelet Lipid Metabolism and Platelet Function Stimulated by U 44069

<table>
<thead>
<tr>
<th>Addition</th>
<th>1,2-Diacylglycerol</th>
<th>Phosphatidic Acid</th>
<th>Arachidonic Acid</th>
<th>Thromboxane B₂</th>
<th>Platelet Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>203 ± 18</td>
<td>878 ± 78</td>
<td>367 ± 35</td>
<td>114 ± 20</td>
<td>0</td>
</tr>
<tr>
<td>ATP, 1 mmol/L</td>
<td>215 ± 14</td>
<td>769 ± 96</td>
<td>384 ± 31</td>
<td>111 ± 14</td>
<td>0</td>
</tr>
<tr>
<td>U 44069, 1 μmol/L</td>
<td>292 ± 20</td>
<td>1,972 ± 215</td>
<td>1,118 ± 168</td>
<td>339 ± 20</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>U 44069, 10 μmol/L</td>
<td>280 ± 30</td>
<td>2,061 ± 236</td>
<td>1,108 ± 156</td>
<td>395 ± 12</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>U 44069, 1 μmol/L plus ATP, 1 mmol/L</td>
<td>266 ± 7</td>
<td>1,301 ± 135</td>
<td>692 ± 20</td>
<td>168 ± 25</td>
<td>Shape change</td>
</tr>
<tr>
<td>U 44069, 10 μmol/L plus ATP, 1 mmol/L</td>
<td>251 ± 25</td>
<td>1,410 ± 79</td>
<td>878 ± 21</td>
<td>129 ± 18</td>
<td>Shape change</td>
</tr>
</tbody>
</table>

Washed platelet suspensions (1 mmol/L EGTA) prelabeled with [³H]-arachidonic acid were preincubated for three minutes with saline or ATP before addition of U 44069. [³H]-labeled 1,2-diacylglycerol was measured at ten seconds, [³H]-labeled phosphatidic acid and arachidonic acid were measured at 30 seconds, and thromboxane B₂ at 60 seconds after addition of U 44069. Values are mean ± SD from three experiments.

Table 2. Effect of ADP Scavengers on Platelet Lipid Metabolism and Platelet Aggregation Induced by U 44069

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphatidic Acid</th>
<th>Arachidonic Acid</th>
<th>Thromboxane B₂</th>
<th>12-HETE</th>
<th>Platelet Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>U 44069, 10 μmol/L plus apyrase, 0.2 mg/mL</td>
<td>694 ± 140</td>
<td>161 ± 66</td>
<td>32 ± 23</td>
<td>48 ± 22</td>
<td>35 ± 12*</td>
</tr>
<tr>
<td>U 44069, 10 μmol/L plus apyrase, 4.5 mg/mL</td>
<td>264 ± 54</td>
<td>66 ± 32</td>
<td>10 ± 13</td>
<td>122 ± 67</td>
<td>Shape change</td>
</tr>
<tr>
<td>U 44069, 10 μmol/L plus CP/CPK, 0.065 mmol/L/0.2 U/mL</td>
<td>783 ± 89</td>
<td>356 ± 132</td>
<td>10 ± 32</td>
<td>57 ± 63</td>
<td>70 ± 25</td>
</tr>
<tr>
<td>U 44069, 10 μmol/L plus CP/CPK, 2 mmol/L/20 U/mL</td>
<td>446 ± 59</td>
<td>87 ± 23</td>
<td>11 ± 42</td>
<td>2 ± 11</td>
<td>13 ± 18*</td>
</tr>
</tbody>
</table>

Human platelets prelabeled with [³H]-arachidonic acid were washed and resuspended in a buffer containing 1.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, and either apyrase or CP/CPK. For each lipid, the respective peak levels after stimulation—as revealed from kinetic studies—are given as Δ cpm (stimulated minus control) of [³H]-radioactivity. Arachidonic acid peaked at ten seconds, phosphatidic acid at 30 seconds, thromboxane B₂ at 60 seconds after addition of U 44069. Values are mean ± SD from three experiments.

*Reversible aggregation.

Table 3. Influence of Extracellular Ca⁺⁺ and Mg⁺⁺ on Platelet Lipid Metabolism Stimulated by U 44069

<table>
<thead>
<tr>
<th>Addition</th>
<th>Platelet Function</th>
<th>Phosphatidic Acid</th>
<th>Arachidonic Acid</th>
<th>Thromboxane B₂</th>
<th>HHT</th>
<th>12-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>U 44069</td>
<td>Ca⁺⁺, 1.5 mmol/L plus Mg⁺⁺, 1 mmol/L</td>
<td>619 ± 41</td>
<td>250 ± 62</td>
<td>10 ± 22</td>
<td>10 ± 10</td>
<td>70 ± 34</td>
</tr>
<tr>
<td>U 44069</td>
<td>Citrate, 22 mmol/L plus EDTA, 5 mmol/L</td>
<td>664 ± 56</td>
<td>450 ± 54</td>
<td>60 ± 20*</td>
<td>155 ± 50*</td>
<td>305 ± 87*</td>
</tr>
</tbody>
</table>

Washed human platelets prelabeled with [³H]-arachidonic acid were resuspended in buffer containing 1.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, and apyrase (0.2 mg/mL). Saline or citrate (22 mmol/L, pH 7.35) and EDTA (5 mmol/L, pH 8.5) were added three minutes before addition of U 44069 (10⁻⁵ mmol/L). Results are expressed as Δ cpm (stimulated minus control) of [³H]-radioactivity. For other details see Table 2. Values are mean ± SE from three experiments.

*P < .01.
phosphate, one of the products of phospholipase C attack on phosphatidylinositol 4,5-bisphosphate, may mobilize \( \text{Ca}^{2+} \) from intracellular stores. Intracellular mobilization of \( \text{Ca}^{2+} \) activates the calmodulin-dependent myosin light chain kinase, which phosphorylates the 20-kd protein. Myosin light chain phosphorylation seems to be important for the interaction of myosin with the platelet cytoskeleton and may thereby be directly related to platelet shape change.

Prostacyclin prevents both platelet shape change and phospholipase C activation induced by prostaglandin endoperoxide analogues. A similar effect of prostacyclin has previously been observed for other platelet stimuli. Prostacyclin increases cyclic adenosine monophosphate (cAMP) levels, which counteract \( \text{Ca}^{2+} \) mobilization induced by platelet agonists, and phospholipase C activation in vitro is dependent on \( \text{Ca}^{2+} \). How prostacyclin prevents the activation of phospholipase C in intact platelets is, however, not clear.

Arachidonic acid is released, but not metabolized, during platelet shape change induced by prostaglandin endoperoxide analogues. Metabolites of arachidonic acid are formed subsequently during platelet aggregation. Such an association of arachidonic acid metabolism and platelet aggregation suggests a role of cyclooxygenase products in inducing platelet aggregation and phospholipase C activation, as we have previously shown for collagen and low concentrations of thrombin. If endoperoxide analogues are the platelet stimuli, cyclooxygenase products do, however, not mediate platelet aggregation and phospholipase C activation. The synthetic endoperoxide analogues may occupy the endoperoxide/thromboxane receptors and inhibit binding of endogenously formed endoperoxides and thromboxane \( \text{A}_2 \).

Release and metabolism of arachidonic acid are uncoupled not only during platelet shape change, but also in the presence of physiologic concentrations of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). Under those conditions, platelet aggregation induced by endoperoxide analogues is greatly facilitated, since divalent cations allow binding of fibrinogen to their receptors and cross-linking of fibrinogen molecules. A possible explanation for the suppressed metabolism of released arachidonic acid by extracellular \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) could be the activation of \( \text{Ca}^{2+} \)-dependent proteases, which degrade two cytoskeletal proteins, the actin-binding protein and the P235 protein. Both of these proteins affect the polymerization of actin in vitro and we have previously shown that polymerization of actin may be important in coupling liberation and metabolism of arachidonic acid.

Prostaglandin endoperoxides stimulate the platelet release reaction independently of cyclo-oxygenase activity. We evaluated the possible role of released ADP in mediating platelet responses induced by endoperoxide analogues by blocking the ADP receptor with ATP or by removing ADP with scavenger enzymes. Low concentrations of apyrase or CP/CPK sufficient to remove small amounts of ADP (<1 \( \mu \text{mol/L} \)) do not inhibit platelet aggregation and lipid changes induced by endoperoxide analogues. This indicates that trace amounts of extracellular ADP may not be important for platelet stimulation induced by endoperoxide analogues. However, ADP released during platelet aggregation could mediate some effects of endoperoxide analogues on platelets, since high concentrations of ADP scavengers or ATP produced a strong inhibition of platelet aggregation and reduced phospholipase C activation and arachidonic acid metabolism. Platelet shape change and the initial phospholipase C activation, were, however, not affected. These results partly support a previous study showing that platelet aggregation and exposure of fibrinogen receptors induced by endoperoxide analogues may be mediated by released ADP.

Shape change and phospholipase C activation by endoperoxide analogues are induced in the absence of extracellular \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). Addition of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) does not change the degree of phospholipase C activation induced by endoperoxide analogues. We therefore conclude that endoperoxide analogues are agonists similar to thrombin, PAF-acether, collagen, arachidonic acid, and dihomogammalinolenic acid, which can activate platelets independently of extracellular divalent cations. Activation of phospholipase C may therefore be a mechanism to stimulate platelets in the absence of divalent cations.

**ACKNOWLEDGMENT**

We thank Mrs. I. Drössel for the careful preparation of the manuscript.

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