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 Ca²⁺ and Mg²⁺, 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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Reagents

Prostaglandin endoperoxide analogues, U 44069 (15s-hydroxy-9,11-epoxymethano prosta-S2, 13E-dienioic acid), U 46619 (15s-hydroxy-11,9-[epoxymethano] prosta-S2, 13E-dienioic acid), prosta
tacyclin (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. [3H]arachidonic acid (57 Ci/mmol) was obtained from Amersham (Braunschweig, FRG). Arachidonic acid, 1,2-diolen, phosphatidic acid, phosphatidylinositol, adenosine triphosphate (ATP), creatine phosphate (CP), creatine phosphokinase (CPK), potato apyrase, and prostaglandin E₁ 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-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic 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 μCi of [³H]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 Ca²⁺ free Tyrode-HEPES buffer containing 1 mmol/L ethylene glycol-bis(β-aminoethyl ether) N,N' -tetraacetic acid (EGTA). Prostacyclin was 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

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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 × 10⁸ 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 and 0.25 mL of 0.2% formic acid. Lipids were exposed to U 44069 on U 46619. Shape change and aggregation of platelet suspensions (1.6 mL) were placed into aggregometer cuvettes, and samples were then partitioned after addition of various times after addition of 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.

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**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 (Δ 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 Ca²⁺-, Mg²⁺-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 (10⁻⁷ mol/L) of the endoperoxide analogues induce only platelet shape change without subsequent aggrega-
mol/L) of endoperoxide analogues: In control samples, 3.44 ± 0.73 ng of TXB₂ are found vs. 3.56 ± 0.30 ng in endoperoxide (10⁻⁶ to 10⁻⁵ mol/L)-stimulated samples (mean ± SD per 10⁶ platelets, n = 3). The results indicate that platelet shape change is related to the formation of 1,2-diacylglycerol and phosphatidic acid, but not to the metabolism of arachidonic acid.

Effect of Endoperoxide Analogues on Protein Phosphorylation

The 40-kd protein is substrate for protein kinase C, and the 20-kd protein is phosphorylated by the Ca²⁺-dependent myosin light chain kinase. Low concentrations of U 44069 (10⁻⁶, 10⁻⁵ mol/L), which only induce platelet shape change, stimulate the rapid phosphorylation of the 40-kd and the 20-kd protein, respectively (Fig 5). Maximal phosphorylation of the 40-kd protein is achieved with a concentration of 10⁻⁴ mol/L of U 44069 (data not shown). We also found that 40-kd and 20-kd protein phosphorylation is not inhibited by pretreatment of platelets with aspirin (data not shown).

Effects of Indomethacin and Prostacyclin

Pretreatment of platelets with indomethacin (10 μmol/L), which completely blocks the formation of TXB₂, does not affect platelet aggregation and phospholipase C activation induced by U 44069 (Fig 1d and 6). Prostacyclin (100 ng/mL) prevents platelet shape change (Fig 1g) and all platelet lipid changes induced by U 44069 or U 46619. The effects of prostacyclin are concentration dependent: Formation of 1,2-diacylglycerol, phosphatidic acid, arachidonic acid, and TXB₂ induced by 10⁻⁵ mol/L of U 44069 or U 46619 is completely abolished by 10 ng/mL prostacyclin. Platelet lipid metabolism stimulated by U 44069, but not by U 46619, is already inhibited by 1 ng/mL prostacyclin (data not shown).

Role of ADP

ATP, a competitive antagonist of the ADP receptor, and ADP scavengers (apyrase, CP/CPK) were used to evaluate the role of ADP in mediating platelet effects induced by prostaglandin endoperoxide analogues. ATP (1 mmol/L) prevents platelet aggregation, but not platelet shape change induced by U 44069 (Fig 1f).
In parallel, the formation of 1,2-diacylglycerol and phosphatidic acid and the release of arachidonic acid induced by U 44069 are reduced, but not blocked. The formation of TXB₂ is, however, almost abolished (Table 1).

ADP-scavenger enzymes, such as apyrase and CP/CPK, require for optimal activity the presence of extracellular Ca²⁺ and Mg²⁺. 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 phosphatidic 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 Ca²⁺ and 1 mmol/L of Mg²⁺ (Fig 1e). The formation of phosphatidic acid and the release of arachidonic acid induced by U 44069 (10⁻⁵ mol/L) are, however, not affected by Ca²⁺ and Mg²⁺. Interestingly, in the presence of Ca²⁺ and Mg²⁺, 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 cyclo-oxygenase 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. Inositol-1,4,5-triphos-

<table>
<thead>
<tr>
<th>Table 1. Effect of ATP on Platelet Lipid Metabolism and Platelet Function Stimulated by U 44069</th>
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<td><strong>cpm $[^{3}H]$-radioactivity</strong></td>
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<tr>
<td><strong>Addition</strong></td>
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<tr>
<td>Saline, 1 mmol/L</td>
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<tr>
<td>ATP, 1 mmol/L</td>
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<tr>
<td>U 44069, 10 mmol/L</td>
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<tr>
<td>U 44069, 10 mmol/L</td>
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<td>U 44069, 1 mmol/L plus ATP, 1 mmol/L</td>
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<td>U 44069, 10 mmol/L plus ATP, 1 mmol/L</td>
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Washed platelet suspensions (1 mmol/L EGTA) prelabeled with $[^{3}H]$-arachidonic acid were preincubated for three minutes with saline or ATP before addition of U 44069. $[^{3}H]$-labeled 1,2-diacylglycerol was measured at ten seconds, $[^{3}H]$-labeled phosphatidic acid and arachidonic acid were measured at 30 seconds, and thromboxane B$_2$ at 60 seconds after addition of U 44069. Values are mean ± SD from three experiments.

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<th>Table 2. Effect of ADP Scavengers on Platelet Lipid Metabolism and Platelet Function Induced by U 44069</th>
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<tr>
<td><strong>cpm $[^{3}H]$-radioactivity</strong></td>
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<tr>
<td><strong>Addition</strong></td>
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<tr>
<td>U 44069, 10 mmol/L plus apyrase, 0.2 mmol/L</td>
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<td>plus apyrase, 4.5 mmol/L</td>
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<tr>
<td>U 44069, 10 mmol/L plus CP/CPK, 0.065 mmol/L/0.2 U/mL</td>
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<tr>
<td>plus CP/CPK, 2 mmol/L/20 U/mL</td>
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Human platelets prelabeled with $[^{3}H]$-arachidonic acid were washed and resuspended in a buffer containing 1.5 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 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 $[^{3}H]$-radioactivity. Arachidonic acid peaked at ten seconds, phosphatidic acid at 30 seconds, thromboxane B$_2$ and 12-HETE at 90 seconds. Platelet aggregation is indicated as percentage of light transmission. Values are mean ± SD from three experiments.

*Reversible aggregation.

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<th>Table 3. Influence of Extracellular Ca**+** and Mg**++** on Platelet Lipid Metabolism Stimulated by U 44069</th>
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<tr>
<td><strong>cpm $[^{3}H]$-radioactivity</strong></td>
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<td><strong>Addition</strong></td>
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<tr>
<td>U 44069 Ca**+-<strong>1.5 mmol/L plus Mg</strong>++**, 1 mmol/L</td>
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<td>EDTA, 5 mmol/L</td>
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Washed human platelets prelabeled with $[^{3}H]$-arachidonic acid were resuspended in buffer containing 1.5 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 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 $[^{3}H]$-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-biphosphate, may mobilize Ca$^{2+}$ from intracellular stores.\textsuperscript{9,32-34} Intracellular mobilization of Ca$^{2+}$ activates the calmodulin-dependent myosin light chain kinase, which phosphorylates the 20-kd protein.\textsuperscript{10} 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.\textsuperscript{30,31}

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.\textsuperscript{11,12} Prostacyclin increases cyclic adenosine monophosphate (cAMP) levels, which counteract Ca$^{2+}$ mobilization induced by platelet agonists, and phospholipase C activation in vitro is dependent on Ca$^{2+}$.\textsuperscript{35-38} How prostacyclin prevents the activation of phospholipase C in intact platelets is, however, not clear.\textsuperscript{39-40}

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.\textsuperscript{10} If endoperoxide analogues are the platelet stimuli, cyclooxygenase products do 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 A$_2$.\textsuperscript{1,34,42}

Release and metabolism of arachidonic acid are uncoupled not only during platelet shape change, but also in the presence of physiologic concentrations of Ca$^{2+}$ and 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.\textsuperscript{43,44} A possible explanation for the suppressed metabolism of released arachidonic acid by extracellular Ca$^{2+}$ and Mg$^{2+}$ could be the activation of Ca$^{2+}$-dependent proteases, which degrade two cytoskeletal proteins, the actin-binding protein and the P235 protein.\textsuperscript{45,46} Both of these proteins affect the polymerization of actin in vitro,\textsuperscript{47,48} and we have previously shown that polymerization of actin may be important in coupling liberation and metabolism of arachidonic acid.\textsuperscript{49}

Prostaglandin endoperoxides stimulate the platelet release reaction independently of cyclo-oxygenase activity.\textsuperscript{50} 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 ($<1 \mu$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.\textsuperscript{51}

Shape change and phospholipase C activation by endoperoxide analogues are induced in the absence of extracellular Ca$^{2+}$ and Mg$^{2+}$. Addition of Ca$^{2+}$ and 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 dihomogammarilonic acid, which can activate platelets independently of extracellular divalent cations.\textsuperscript{50-10,52} Activation of phospholipase C may therefore be a mechanism to stimulate platelets in the absence of divalent cations.

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