Activation of Phospholipase C Is Dissociated from Arachidonate Metabolism during Platelet Shape Change Induced by Thrombin or Platelet-activating Factor

EPINEPHRINE DOES NOT INDUCE PHOSPHOLIPASE C ACTIVATION OR PLATELET SHAPE CHANGE*

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The present study compares the molecular mechanism by which thrombin, platelet-activating factor, and epinephrine induce platelet activation. Thrombin and platelet-activating factor induce an initial activation of phospholipase C, as measured by formation of 1,2-diacylglycerol and phosphatic acid, during platelet shape change which is independent of and dissociated from metabolism of arachidonic acid. Phospholipase C activation and shape change are independent of extracellular Ca** and Mg**. Formation of cyclooxygenase products occurs subsequent to the initial activation of phospholipase C and those metabolites are associated with platelet aggregation and further activation of phospholipase C. On the other hand, epinephrine is an unique platelet stimulus since it requires extracellular divalent cations and does not induce platelet shape change or activation of phospholipase C. Our results indicate that activation of phospholipase C may be a mechanism by which physiological agonists can activate platelets independently of extracellular divalent cations.

In various cell types, the binding of hormones to their specific receptors induces distinct changes in the membrane phospholipids. In platelets, both the stimulation of the degradation of the inositol phospholipids and the release of arachidonic acid from phospholipids occur following platelet stimulation with various agonists such as thrombin, collagen, ADP, and platelet-activating factor (1–8). Degradation of inositol-containing phospholipids by phospholipase C sequentially leads to the formation of 1,2-diacylglycerol and its phosphorylated product phosphatic acid (9, 10). These products remain inside the cell and might moderate platelet activation, since 1,2-diacylglycerol can activate protein kinase C (11, 12) and phosphatic acid can act as a Ca** ionophore and a fusogen at low concentrations of calcium (13–16). Intracellular accumulation of phosphatic acid could also activate phospholipase A2 (17) leading to the liberation of arachidonic acid from various phospholipids (5). Arachidonic acid is metabolized by cyclooxygenase and thromboxane synthase to the biologically active endoperoxides and thromboxane A2 which can act inside the platelet or are released to the outside where they can activate other platelets (18). The significance of these changes in lipid metabolism for platelet function is, however, not clearly defined. Experiments in which platelets were stimulated in citrated platelet-rich plasma by ADP, platelet-activating factor, or l-epinephrine showed that cyclooxygenase products (endoperoxides and thromboxane A2) are involved in later physiological platelet responses such as release reaction and second wave of aggregation, whereas they are not involved in primary aggregation (19–24).

Platelet shape change is the first measurable physiological platelet response preceding other responses such as platelet aggregation and release reaction (25). The present study indicates that platelet shape change induced by thrombin or platelet-activating factor is closely related to the activation of phospholipase C, but independent of the liberation and metabolism of arachidonic acid. In contrast, epinephrine does not induce phospholipase C activation or platelet shape change.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin, aspirin, trifluoperazine, quinacrine (mepacrine), arachidonic acid, 1,2-diolen, phosphatic acid, prostaglandin E2, bovine fibrinogen (Type I-S, lot F8630), creatine phosphate, creatine phosphokinase, hirudin, and potato apyrase (Grade I, lot A6132) were all obtained from Sigma. Platelet-activating factor (PAF) was purchased from Calbiochem (Frankfurt, FRG). l-EPinephrine was purchased from Serva (Heidelberg, FRG), dissolved as a 10 mM solution in tartaric acid (10 mM), and stored at -20 °C. [3H]Arachidonic acid (57 Ci/mmol) was obtained from Amersham Corp. Indomethacin was a gift from Merck Sharp and Dohme. Silica Gel G-25 plates without gypsum were purchased from Merck (Darmstadt, FRG). Prostaglandin D3, thromboxane B2, and prostacyclin were a gift from The Upjohn Co. Unlabeled HHT* and HETE were prepared by incubation of human platelets with arachidonic acid. Two 2-channel aggregometers were from Fresenius (Friedrichsdorf, FRG). Platelet Preparation for Stimulation with Thrombin or Platelet-activating Factor—Blood (200 ml) was obtained from healthy male volunteers (age 25–45 years) who had not received any medication in

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1 The abbreviations used are: HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-hydroxy-5,8,10-icosatetraenoic acid; Hepes, 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
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the previous 4 weeks. Blood was anticoagulated with 0.15 volume of ACD buffer (85 mm trisodium citrate, 111 mm dextrose, 71 mm citric acid, pH 5.5), and platelet-rich plasma (pH 6.8) was obtained by centrifugation at 200 × g for 20 min. Platelet-rich plasma (40–80 ml) was incubated with 200 μCi of [3H]arachidonic acid at 37 °C for 2 h in the presence of prostaglandin D3 or prostaglandin E1 (1 μg/ml) to prevent platelet activation. Platelets were then separated from plasma after addition of heparin (300 ng/ml) and washed twice with a modified Ca2+- and Mg2+-free Tyrode-Hepes buffer containing 1 mM EGTA and prostaglandin I2 (300 ng/ml) to prevent platelet activation as detailed previously (26, 27). Polypropylene material was used throughout the preparation procedure. In experiments in which the role of extracellular Ca2+ and Mg2+ for platelet lipid metabolism stimulated by platelet-activating factor was studied, the washing and resuspending buffer contained 1.5 mM CaCl₂ and 1 mM MgCl₂ instead of 1 mM EGTA. Platelets were washed once in that buffer to which heparin (25 units/ml), prostacyclin (300 ng/ml), and EDTA/Tris buffer (pH 8.65) were added to a final concentration of EDTA/Tris of 5 mM/2.5 mM.

Platelet Preparation for Stimulation with Epinephrine—A different washing procedure was developed for platelet stimulation with epinephrine since the effect of epinephrine is dependent on the presence of extracellular Ca2+ and Mg2+, and epinephrine can act synergistically with other agonists such as ADP or thrombin which could be present in trace amounts in the platelet suspension (28–35). Platelets were prelabeled with [3H]arachidonic acid as described above, and aspirin (1 mM final concentration) was added 15 min prior to the centrifugation of the platelets. Platelets from 60–80 ml of platelet-rich plasma were then washed once in 30 ml of the Tyrode-Hepes buffer which contained 0.1 mM CaCl₂, 1 mM MgCl₂, potash pyruvate (200 μg/ml), heparin (25 units/ml), and prostacyclin (300 ng/ml). After centrifugation, platelets were resuspended in the same buffer without heparin or prostacyclin. The temperature of the washing and resuspending buffer was kept at 37 °C, and platelets were stored at this temperature (35). Hurdin (2.5 units/ml) and creatine phosphate/creatine phosphokinase (2 mM/20 units/ml) were added to the platelet suspension 2 min before addition of epinephrine in order to avoid possible synergisms of epinephrine with trace amounts of endogenous thrombin or ADP. Thrombin (0.025 units/ml) or ADP (<1 μM) added to those platelet suspensions did not induce platelet aggregation. Fibrinogen (50 μg/ml) was added 1 min before epinephrine. Epinephrine (100 μM) consistently induced platelet aggregation under those conditions. In some experiments, pyruvate was omitted from the washing and resuspending buffer, and the buffer contained 1.5 mM CaCl₂ and 1 mM MgCl₂. In these experiments the platelets lost their discoid shape and sometimes spontaneously aggregated.

Platelet Shape Change and Aggregation—Suspensions of washed platelets were adjusted to 4–6 × 10⁷ platelets per ml and the experiments carried out between 30 and 90 min after final resuspension. Responsiveness of the platelets to the various agonists decreased slowly during that period. Platelet suspensions (0.5 ml) were placed into aggregometer tubes, stirred (1100 rpm) for 2 min at 37 °C in the aggregometer, and then exposed to thrombin, platelet-activating factor, or epinephrine for various times. Shape change and aggregation of platelets were recorded (22, 36). The use of two 2-channel aggregometers allowed the simultaneous study of control and stimulated platelet suspensions. In some experiments, platelet shape change was examined by scanning electron microscopy (37). In studies in which inhibitors were used, platelet suspensions were preincubated for 2 min at 37 °C with indomethacin (dissolved in ethanol), trifluoperazine (dissolved in ethanol/water, 50/50), or quinacrine (dissolved in HZO). The final ethanol concentration was 0.2% and did not affect platelet responses. Aspirinated platelets were prepared as described above.

Platelet Lipid Metabolism—Incubations (0.5 ml) were stopped by addition of 0.5 ml of methanol to the samples which were then transferred into 1.4 ml of chloroform/methanol (1:1.2). Unlabeled standards of phosphatidic acid and 1,2-diolein were added, and samples were then partitioned after addition of 0.62 ml of chloroform and 0.62 ml of 0.2% formic acid. The lower organic phases were evaporated under N₂, split into two halves, and lipids were separated on thin layer chromatography with two different solvent systems. Benzene/diethyl ether/acetone (5:3:2) was used to separate phosphatidic acid, thromboxane B₂, HHT, HETE, and arachidonic acid (1). Substances were localized by co-chromatography with unlabeled standards which were visualized by iodine vapor. After evaporation of iodine, the specific areas were scraped, transferred into scintillation vials, and ³H radioactivity was measured by liquid scintillation counting. Samples were counted in a Beckman β-scintillation counter (type LS 330; efficiency 60%) for 10 to 20 min to reach a counting error lower than 3%.

Data Presentation—Assays were done in duplicates or triplicates (for 1,2-diacylglycerol and arachidonic acid) and compared to unstimulated control samples which were set to 100%. In the control samples the range of radioactivity (counts per min of ³H) for the different lipids was as followed: 500 to 1500 for phosphatidic acid, 100 to 300 for 1,2-diacylglycerol, 200 to 500 for arachidonic acid, and 80 to 180 for thromboxane B₂, HHT, or HETE. These variations were due to differences of incorporation of [3H]arachidonic acid into platelet phospholipids (range 20–55%). Data are presented as mean ± S.E. of individual experiments from different blood donors (unless otherwise indicated). Statistical significance was calculated by the paired student t test using the data of the single assays of the individual experiments.

RESULTS

Platelet Stimulation with Thrombin and Platelet-activating Factor—Human platelets were prelabeled with [3H]arachidonic acid and washed in the presence of prostacyclin to prevent any platelet activation and to obtain the platelets in unstimulated discoid shape (26, 27, 37). The platelets were resuspended in buffer containing 1 mM EGTA and exposed to low concentrations of thrombin or platelet-activating factor, and platelet shape change and alterations of ³H-labeled platelet lipids were monitored simultaneously. Thrombin at low concentrations (0.05–0.075 unit/ml) induces only shape change (Fig. 1a) without subsequent platelet aggregation and an increase in the formation of 1,2-diacylglycerol and phosphatic acid (Fig. 2). Free arachidonic acid is not significantly increased up to 60 s, and metabolism of arachidonic acid via platelet cyclooxygenase or lipoygenase is not detectable.

![Graph](image-url)

**FIG. 1. Effects of trifluoperazine, aspirin, and indomethacin on platelet shape change induced by thrombin or platelet-activating factor.** Platelet suspensions (0.5 ml) were incubated at 37 °C with or without trifluoperazine (40 μM) or indomethacin (5 μM) before exposure to thrombin or platelet-activating factor. Aspirin-pretreated platelets for each thrombin dose were preincubated with 16 μM of aspirin for 1 min. A thrombin concentration of 0.06 unit/ml; b, 0.06 unit/ml; c, 0.06 unit/ml; d, 0.1 unit/ml; e, platelet-activating factor (10⁻7 M); f, aspirin plus platelet-activating factor (10⁻7 M).
Trifluoperazine (30 to 50 μM) which inhibits liberation of arachidonic acid from platelet phospholipids does not inhibit platelet shape change (Fig. 1b). Formation of phosphatidic acid and 1,2-diacylglycerol induced by low concentrations of thrombin was not different in the absence or presence of trifluoperazine: 1,2-diacylglycerol at 15 s was 117 ± 5 versus 126 ± 3; phosphatidic acid at 30 s was 127 ± 14 versus 130 ± 16 (values as per cent of control, means ± S.D. of four separate experiments). In addition, aspirin does not affect platelet shape change or the formation of 1,2-diacylglycerol and phosphatidic acid induced by thrombin (data not shown). A small increase in the concentration of thrombin (0.09–0.12 unit/ml) results in platelet aggregation which occurs after platelet shape change (Fig. 1c). The formation of 1,2-diacylglycerol and phosphatidic acid shows a characteristic biphasic pattern, i.e. an initial small increase of 1,2-diacylglycerol and phosphatidic acid in parallel to platelet shape change, and a subsequent sharp increase of 1,2-diacylglycerol and phosphatidic acid observed after 30 s that coincides with platelet aggregation and formation of arachidonate metabolites (Fig. 3). Indomethacin or aspirin reduces the second increase of 1,2-diacylglycerol and phosphatidic acid, and also platelet aggregation (Figs. 3 and 1d).

The differences between the changes in 3H-labeled lipids occurring during the selective platelet shape change induced by the lower thrombin concentrations (Fig. 2) and the shape change which precedes aggregation induced by the higher thrombin concentrations (Fig. 3) are summarized in Table I. First, it indicates that formation of 1,2-diacylglycerol and phosphatidic acid occurs more rapidly and is higher during the platelet shape change that precedes aggregation as compared to the platelet shape change induced by the lower thrombin concentrations. Secondly, free arachidonic acid only increases during the platelet shape change that precedes aggregation.

The lipid changes induced by platelet-activating factor during platelet shape change are similar to those induced by thrombin. Platelet-activating factor (0.1–1 μM) stimulates the formation of 1,2-diacylglycerol and phosphatidic acid during platelet shape change (see Figs. 4 and 5). It was noticed that the increase of free arachidonic acid during platelet shape change occurs in some of the experiments but not in others (Fig. 4, a and b). However, in all experiments metabolism of

**Table I**

Formation of 1,2-diacylglycerol, phosphatidic acid, and arachidonic acid during the platelet shape change induced by thrombin concentrations that only induce shape change (a) or that induce shape change followed by platelet aggregation (b).

| Thrombin | Time | 1,2-Diacylglycerol | Phosphatidic acid | Arachidonic acid |
|----------|------|-------------------|------------------|------------------|
|          | unit/ml |                  |                  |                  |
|          | 15    | 114 ± 2           | 133 ± 2          |
|          | 30    | 120 ± 3           | 139 ± 5          |
| a, 0.05-0.075 | 188 ± 14* | 165 ± 8*          |
| b, 0.09-0.12 | 103 ± 3 | 105 ± 4           |
| c, 0.05-0.075 | 170 ± 8* | 230 ± 21*         |

* p < 0.05 if compared to the values in a.
* p < 0.01 if compared to the values in a.
arachidonic acid by platelet cyclooxygenase or lipoxygenase is not detectable. In experiments with platelet-activating factor in which free arachidonic acid increases, pretreatment with trifluoperazine or mepacrine prevents the increase of free arachidonic acid without affecting the formation of phosphatidic acid or platelet shape change (Table II). Similarly, aspirin does not affect the formation of phosphatidic acid or platelet shape change (Fig. 1, e and f, Table II). In some experiments, platelet shape change induced by thrombin or platelet-activating factor was analyzed by scanning electron microscopy and found to be comparable to previously reported results (37) (data not shown). The presence of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) does not significantly affect the formation of 1,2-diacylglycerol, phosphatidic acid, or arachidonic acid induced by platelet-activating factor (1 \(\mu\)M); activation of phospholipase C and release of arachidonic acid induced by platelet-activating factor are comparable whether the platelet buffer contains either Ca\(^{2+}\) plus Mg\(^{2+}\) or EDTA (see Fig. 5).

**TABLE II**

Effects of trifluoperazine, mepacrine, or aspirin on the formation of phosphatidic acid and arachidonic acid during platelet shape change induced by platelet-activating factor

| Experiment | Addition     | Phosphatidic acid | Arachidonic acid |
|------------|--------------|-------------------|------------------|
| 1          | None         | 220 ± 44          | 191 ± 31         |
|            | Trifluoperazine | 244 ± 39         | 197 ± 32         |
|            | Aspirin      | 187 ± 20         | 212 ± 40         |
| 2          | None         | 176 ± 36          | 137 ± 5          |
|            | Mepacrine    | 156 ± 12          | 96 ± 4           |
|            | Aspirin      | 157 ± 20          | 198 ± 3          |
| 3          | None         | 172 ± 32          | 111 ± 10         |
|            | Aspirin      | 154 ± 20          | 107 ± 22         |

**Fig. 4.** Effect of platelet-activating factor on the formation of phosphatidic acid and arachidonic acid during platelet shape change. Washed human platelets prelabeled with \(^{3}{\text{H}}\)arachidonic acid were exposed to platelet-activating factor (0.1 or 1 \(\mu\)M) for various times. Some experiments as shown in a (n = 5) showed no increase of free arachidonic acid, but in some experiments as shown in b (n = 5) liberation of arachidonic acid was detected. Values are mean ± S.E. PA, phosphatidic acid; AA, arachidonic acid; TXB\(_2\), thromboxane B\(_2\); HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

**Fig. 5.** Effect of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) on the formation of 1,2-diacylglycerol, phosphatidic acid, and arachidonic acid induced by platelet-activating factor. Washed human platelets prelabeled with \(^{3}{\text{H}}\)arachidonic acid were washed and resuspended in buffer containing 1.5 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) (see "Experimental Procedures"). Platelet suspensions were preincubated without (—) or with addition of 5 mM of EDTA (O—O) for 2 min before exposure to platelet-activating factor (1 \(\mu\)M). The EDTA solution (200 mM) was buffered with Tris (100 mM, pH 8.65) to avoid pH changes of the platelet suspension. Formation of phosphatidic acid and 1,2-diacylglycerol is significantly different from controls (2 \(p < 0.001\)). Values are mean ± S.E. from seven experiments. PA, phosphatidic acid; 1,2-DG, 1,2-diacylglycerol; AA, arachidonic acid.

Again, release of arachidonic acid occurred in some experiments but not in others. Formation of arachidonic acid metabolites was found neither in the presence nor in the absence of Ca\(^{2+}\) and Mg\(^{2+}\) (data not shown). Platelet-activating factor induces rapid platelet aggregation in the presence of Ca\(^{2+}\) and Mg\(^{2+}\), whereas in the presence of EDTA only shape change occurs.

**Platelet Stimulation with Epinephrine**—The platelet-stimulating effect of epinephrine is critically dependent on the presence of extracellular divalent cations and especially on the ratio of Ca\(^{2+}\) to Mg\(^{2+}\) (28–34). We developed a system to study the direct effect of epinephrine on platelets. Possible synergistic effects of trace amounts of ADP or thrombin with epinephrine were precluded by the addition of ADP scavengers and hirudin. In addition, platelets were aspirinized. As shown in Fig. 6, epinephrine induces a slow aggregation response without a preceding platelet shape change. In contrast, platelet-activating factor rapidly induces platelet shape change and reversible aggregation. Doses of platelet-activating factor (10 or 100 nM) and epinephrine (100 nM) that induce a similar degree of platelet aggregation were chosen to compare the effects of these two agonists. Epinephrine has no effects on platelet lipid metabolism; formation of phosphatidic acid is not significantly enhanced, and production of 1,2-diacylglycerol, release of arachidonic acid, and formation of HETE are not detectable. In contrast, platelet-activating factor stimulates the formation of 1,2-diacylglycerol and phosphatidic acid (Fig. 7). The platelet-aggregating effect of epinephrine and platelet-activating factor is dependent on extracellular divalent cations; the addition of 5 mM EDTA (after buffering with citrate to avoid shape change induced by EDTA (38, 39)) abolishes platelet aggregation induced by epinephrine and platelet-activating factor, but does not affect platelet shape change induced by platelet-activating factor (Fig. 6). Epinephrine, in the presence of EDTA, induces only a continuous slow decrease of light transmission without changing the amplitude of the oscillations of the platelet suspension.

We further found that epinephrine could induce formation of phosphatidic acid and stimulate arachidonic acid metabolism, if the platelet suspension did not contain ADP scavengers and if the platelet cyclooxygenase was not blocked by...
aspirin. Epinephrine could then induce considerable production of thromboxane B₂ and phosphatidic acid (Table III). Indomethacin suppressed the formation of thromboxane B₂, and in parallel it reduced (but not abolished) the amount of phosphatidic acid formed. Scavengers of ADP, however, completely blocked the effect of epinephrine on formation of phosphatidic acid (Table III).

**DISCUSSION**

Platelet shape change is the first measurable platelet response. Our study shows that low concentrations of thrombin or platelet-activating factor which only induce platelet shape change increase the formation of 1,2-diacylglycerol and phosphatidic acid indicating the activation of the phosphodiesterase cleavage of the inositol phospholipids (phosphatidylinositol, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate; Refs. 1, 2, 6, 9, 40-46). Epinephrine which does not induce platelet shape change does not activate phospholipase C. Metabolism of arachidonic acid by platelet cyclooxygenase or lipoxygenase during platelet shape change does not occur. Agents which block the release of arachidonic acid from platelet phospholipids (trifluoperazine, mepacrine) or inhibit platelet cyclooxygenase (indomethacin, aspirin) do not affect the formation of 1,2-diacylglycerol and phosphatidic acid and the shape change of platelets induced by thrombin or platelet-activating factor. The results indicate that physiological agonists such as thrombin and platelet-activating factor induce initial platelet responses, i.e. platelet shape change and activation of phospholipase C without the participation of arachidonate metabolism.

![Graph showing the effects of platelet-activating factor and epinephrine on platelet lipid metabolism](image)

**FIG. 7.** Differential effects of platelet-activating factor and epinephrine on platelet lipid metabolism. Platelets were stimulated by doses of platelet-activating factor (10 or 100 nM) or epinephrine (100 μM) which induced comparable aggregation responses (see Fig. 6). Platelet preparation and incubation buffer are detailed in the legend of Fig. 6 and under "Experimental Procedures." Values are mean ± S.E. of six experiments. Formation of phosphatidic acid induced by epinephrine is not significantly different from control (2 p > 0.1). Formation of phosphatidic acid and 1,2-diacylglycerol induced by platelet-activating factor is significantly different from control samples and epinephrine-stimulated samples (2 p < 0.001 for phosphatidic acid and 2 p < 0.01 for 1,2-diacylglycerol at 15 and 60 s). PA, phosphatidic acid; 1,2-DG, 1,2-diacylglycerol; AA, arachidonic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

**TABLE III**

| Addition                                  | Phosphatidic acid | Thromboxane B₂ |
|-------------------------------------------|-------------------|----------------|
| None                                      | 100               | 100            |
| Epinephrine, 1 μM                         | 96                | 2980           |
| Epinephrine, 1 μM plus indomethacin, 10 μM| 109               | 98             |
| Epinephrine, 100 μM                       | 145               | 108            |
| Epinephrine, 100 μM plus indomethacin, 10 μM| 144               | 96             |
| Epinephrine, 100 μM plus apyrase (200 μg/ml)| 83                | 92             |
| Epinephrine, 100 μM plus creatine phosphate/creatine phosphokinase (2 mM, 20 units/ml)| 90               | 102            |

| Addition                                  | 1 min | 5 min | 1 min | 5 min |
|-------------------------------------------|-------|-------|-------|-------|
| None                                      | 100   | 100   | 100   | 100   |
| Epinephrine, 1 μM                         | 96    | 168   | 101   | 2980  |
| Epinephrine, 1 μM plus indomethacin, 10 μM| 109   | 128   | 98    | 97    |
| Epinephrine, 100 μM                       | 145   | 315   | 108   | 3938  |
| Epinephrine, 100 μM plus indomethacin, 10 μM| 144   | 200   | 96    | 98    |
| Epinephrine, 100 μM plus apyrase (200 μg/ml)| 83    | 87    | 92    | 93    |
| Epinephrine, 100 μM plus creatine phosphate/creatine phosphokinase (2 mM, 20 units/ml)| 90    | 93    | 101   | 102   |

Although the increase in the formation of 1,2-diacylglycerol and phosphatidic acid during platelet shape change is small (Figs. 2-4, Refs. 27, 37 and 47), the actual increase of those substances could occur in specific compartments of the cell resulting in high concentrations at critical sites. 1,2-Diacylglycerol may activate protein kinase C which phosphorylates a 40,000-dalton protein in platelets (11, 12). Phosphorylation of that protein during platelet shape change has been observed (37, 47, 48); its function is, however, not yet known. Another protein which is phosphorylated during platelet shape change...
is the myosin light chain which is directly involved in the contractile events of the cytoskeleton occurring during platelet shape change (48-50). The myosin light chain kinase is under control of Ca\(^{2+}\) which could be mobilized by phosphatidylinositol 4,5-bisphosphate (51), one of the products of phospholipase C attack on phosphatidylinositol 4,5-bisphosphate (46).

The present results strengthen the hypothesis of the sequential stimulation of phospholipases C and A\(_2\) (1, 17). It is possible to dissociate two levels of platelet activation by using different concentrations of thrombin; low concentrations of thrombin (0.05-0.075 unit/ml) which induce platelet shape change without subsequent aggregation increase the formation of a small amount of 1,2-diacylglycerol and phosphatidic acid without an increase of free arachidonic acid. Higher concentrations of thrombin (0.09-0.12 unit/ml) leading sequentially to shape change and platelet aggregation induce during platelet shape change a more effective formation of 1,2-diacylglycerol and phosphatidic acid, and, in addition, an increase of free arachidonic acid. Cyclooxygenase activity appears to play a triggering role for the initiation of platelet aggregation induced by these concentrations of thrombin (0.09 to 0.12 unit/ml, see Figs. 1d and 3). Cyclooxygenase products, such as endoperoxides and thromboxane A\(_2\), may be responsible for the sharp increase of 1,2-diacylglycerol and phosphatidic acid during aggregation, since inhibition of cyclooxygenase by indomethacin or aspirin reduces this second increase of 1,2-diacylglycerol and phosphatidic acid. Thus, the cyclooxygenase products formed at the onset of aggregation might act as positive feedback promoters for stimulation of phospholipase C (27, 47). The increase of free arachidonic acid and the formation of the lipoxigenase product HETE which are observed during platelet aggregation are also reduced by inhibition of cyclooxygenase, but only partly. One would have expected that inhibition of cyclooxygenase would lead to an increased formation of arachidonic acid and HETE; the contrary is, however, observed. It thus appears that the inhibition of cyclooxygenase has also removed a stimulating effect of endogenous endoperoxides/thromboxane A\(_2\) on the release of arachidonic acid. This effect of active cyclooxygenase products on the release of arachidonic acid may not be mediated directly, but via activation of phospholipase C, since it has been previously shown that phosphatidic acid may trigger the release of arachidonic acid (17). These observations seem to indicate that the release of arachidonic acid which is observed during platelet aggregation induced by low concentrations of thrombin is somehow dependent on the activity of phospholipase C.

It is important to note that liberation of arachidonic acid can already be observed during platelet shape change. The liberated arachidonic acid is, however, not metabolized. An increase of free arachidonic acid during shape change was consistently found with the higher concentrations of thrombin (0.09-0.12 unit/ml) and in some experiments with platelet-activating factor (Table I, Figs. 3 and 4). Extracellular Ca\(^{2+}\) and Mg\(^{2+}\) did not influence the release of arachidonic acid induced by platelet-activating factor (Fig. 5). At present we cannot explain why the arachidonic acid released during shape change is not metabolized. The findings imply that some kind of coupling mechanism between the liberation and metabolism of arachidonic acid must exist which is not readily operative during platelet shape change. Recently we obtained evidence that the polymerization of actin might be involved in the coupling of arachidonic acid release to arachidonic acid metabolism (52).

Platelet aggregation induced by epinephrine is not preceded by platelet shape change, and epinephrine does not stimulate phospholipase C and metabolism of arachidonic acid. Epinephrine is known to bind to \(\alpha_2\) receptors on platelets, to inhibit adenylate cyclase, and to increase the Ca\(^{2+}\) uptake of intact human platelets (30, 53, 54). It can directly induce exposure of fibrinogen receptors to which fibrinogen binds in the presence of Ca\(^{2+}\) or Mg\(^{2+}\) leading to platelet aggregation (33). Since under those conditions formation of phosphatidic acid or 1,2-diacylglycerol was not observed, it seems that exposure of fibrinogen receptors and aggregation can occur in the absence of phospholipase C activation. The results further indicate that \(\alpha_2\) receptors (in contrast to \(\alpha_1\) receptors) are not coupled to the metabolism of the inositol phospholipids, and inhibition of adenylate cyclase might not be related to phospholipase C activation. Under certain conditions, if trace amounts of ADP were present in the platelet suspensions and platelet cyclooxygenase was not inhibited, epinephrine could, however, induce formation of phosphatidic acid. The effect was not due to a direct action of epinephrine, since the formation of phosphatidic acid was partly inhibited by cyclooxygenase products on the release of arachidonic acid. This effect of active cyclooxygenase is not explained by the sharp increase of 1,2-diacylglycerol and phosphatidic acid which is then partly mediated by cyclooxygenase products. Epinephrine and ADP have synergistic effects on exposure of fibrinogen receptors, platelet aggregation, and release reaction (30-34). A possible source for trace amounts of extracellular ADP could be the contamination of the platelet suspension with red cells which become leaky during the washing and resuspending procedure (35).

We have previously observed that the level of phospholipase C activation induced by various agonists correlates with the degree of the physiological platelet response (27, 47, 49). The present study shows that platelet shape change and activation of phospholipase C are induced in the absence of extracellular Ca\(^{2+}\) or Mg\(^{2+}\). Addition of Ca\(^{2+}\) and Mg\(^{2+}\) does not affect platelet shape change or phospholipase C activation, but only allows binding of fibrinogen to their receptors and cross-linking of fibrinogen molecules, thereby leading to platelet aggregation (55). It is not clear if platelet shape change requires mobilization of intracellular Ca\(^{2+}\) or not (56-58). The present results strengthen the significance of phospholipase C activation for platelet shape change. Epinephrine is an unique platelet stimulus as it requires extracellular divalent ions and does not activate phospholipase C. Activation of phospholipase C could, therefore, be a mechanism by which agonists can rapidly stimulate platelets independently of extracellular divalent cations.

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