Platelet Agonists Enhance the Import of Phosphatidylethanolamine into Human Platelets*

Bernd Engelmann§§, Barbara Schaipp§, Petra Dobner‡, Mechthild Stoeckelhuber‡,
Christine Kögl‡, Wolfgang Siess§, and Albin Hermetter¶

From the §Physiologisches Institut der Universität München, Pettenkoferstrasse 12, D-80336 München, Germany;
¶Institut für Prophylaxe der Kreislaufkrankheiten, Universität München, D-80336 München, Germany;
and the iInstitut für Biochemie und Lebensmittelchemie, Technische Universität Graz, A-8010 Graz, Austria

It is unknown whether the endocytosis-independent transfer of phospholipids from lipoproteins to platelets is regulated by platelet agonists such as thrombin. The movements of the choline phospholipids phosphatidylcholine and sphingomyelin (labeled with either $^{14}$C or the fluorescent pyrenedecanoic acid) between low density lipoproteins and platelets were unaffected by thrombin (0.5 unit/ml). In contrast, thrombin accelerated the import of diacyl phosphatidylethanolamine (PE) and alkenylacyl phosphatidylethanolamine into platelets by about 4-fold. Similarly, thrombin receptor-activating peptide (15 $\mu$M), collagen (10 $\mu$g/ml), and ADP (10 $\mu$M) enhanced PE uptake. High density lipoprotein particles and egg phosphatidylcholine vesicles were also donors for stimulation of platelet PE import. Part of the $^{14}$C-labeled PE transferred from low density lipoprotein to platelets activated by thrombin and collagen was metabolized to $^{14}$C-eicosanoids. Inhibitors of protein kinase C partially prevented thrombin-induced $^{14}$CPE uptake, while direct activators of protein kinase C increased incorporation of $^{14}$CPE into platelets. Proteinaceous factor(s) recovered in the extracellular medium from ADP- and thrombin-activated platelet suspensions were found to accelerate the transfer of pyrenedecanoic-acid-labeled PE between donor and acceptor lipid vesicles. The stimulation of import of ethanolamine phospholipids led to a 2-fold enhancement of the prothrombinase activity of thrombin-activated platelets. Our study demonstrates that physiological platelet stimuli increase specifically the transfer of ethanolamine phospholipids from lipoproteins to platelets through a secretion-dependent mechanism. This might contribute to the increase of procoagulant activity of stimulated platelets.

The processes of phospholipid transfer between different plasma lipoproteins (1), between the two leaflets of biological membranes (2), as well as between different intracellular membranes (3) have been elucidated in considerable detail in recent years. Much less is known about the characteristics of phospholipid transfer between plasma lipoproteins and cells. Lipoproteins may supply their phospholipids by endocytosis of whole lipoprotein particles or by selective transfer of phospholipids to the acceptor cells. Within the plasma compartment, phospholipids (and cholesterol) carry major portions of highly polyunsaturated fatty acids with four and more double bonds such as, e.g., arachidonic acid. Only very small quantities of these fatty acids are present in the free form in plasma (4). Previous work indicates that arachidonic acid esterified to cholesterol within LDL (5) is taken up by endocytosis of the lipoproteins and further processed to bioactive eicosanoids in growth factor-activated fibroblasts (5). Apart from the potential role of phospholipid transfer in supplying cells with polyunsaturated fatty acids, the newly imported phospholipids may serve other functions in intracellular signaling mechanisms. Second messengers such as diacylglycerol and ceramide are generated in part by phospholipase C- and sphingomyelinase-mediated hydrolysis of membrane phosphatidylcholine (PC) and sphingomyelin (SM), respectively. Changes in import of precursor phospholipids may well modulate the intracellular signaling pathways regulated by these messenger molecules.

Phospholipids of the plasma membranes of cells are also known to be indispensable elements of the coagulation cascade. Within this process, interaction of extracellular coagulation factors with binding proteins on the plasma membrane of platelets is greatly facilitated by distinct phospholipids. In particular, the appearance of the negatively charged phosphatidylserine in the outer leaflet of the plasma membrane of platelets appears to be an essential cofactor for some steps in the coagulation cascade such as the formation of prothrombin (6, 7). Additionally, anticoagulant activities mediated by proteins of endothelial cell membranes are modulated by specific phospholipids (8, 9).

We recently observed that plasma low and high density lipoproteins (LDL, HDL) rapidly delivered PC, phosphatidylethanolamine (PE), and SM to human platelets (10). Import of these phospholipids into platelets was independent of endocytosis. Apparently, phospholipids of the monolayer that surrounds lipoproteins are transferred to the outer leaflet of the plasma membrane of platelets. It is possible that such an alteration of the phospholipid composition of the platelet plasma membrane phospholipid uptake could modulate certain

---

*This work was supported by Grants En 178/4-2 and En 178/4-3 from the Deutsche Forschungsgemeinschaft and a grant from the Friedrich-Baur-Stiftung (to B. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§To whom correspondence should be addressed. Tel.: 49-89-5996-394; Fax: 49-89-5996-378; E-mail: bernd.engelmann@med.uni-muenchen.de.

1 The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; PE, 1,2-diacyl phosphatidylethanolamine; PPE, 1-alk-1-enyl-2-acyl-phosphatidyl-ethanolamine; PC, 1,2-diacyl phosphatidylcholine; PPC, 1-alk-1-enyl-2-acyl-phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; PKC, protein kinase C; TRAP, thrombin receptor-activating peptide; py, pyrenedecanoic acid; H7, 1-(5-isouquinolinesulfonyl)-2-methylpyrazine; 18:2, linoleic acid; 20:4, arachidonic acid; OAG, 1-oleoyl-2-acetylglycerol; TXA$_2$, thromboxane A$_2$; TXB$_2$, thromboxane B$_2$; HETE, 12-hydroxyeicosatetraenoic acid; apo, apolipoprotein; cPLA$_2$, cytosolic phospholipase A$_2$; sPLA$_2$, secretory phospholipase A$_2$. 

This paper is available on line at http://www.jbc.org
steps of the coagulation process. Activation of platelets is an initial event in blood coagulation. It is accompanied by rapid changes in platelet phospholipid homeostasis (11, 12). In order to evaluate whether activation of platelets was associated with changes in phospholipid transfer from lipoproteins to platelets, we analyzed the effect of platelet agonists on the import of individual phospholipids.

### EXPERIMENTAL PROCEDURES

#### Materials

1. Palmitoyl-2-14C[arachidonoyl-sn-glycero-3-phosphorylcholine] (PC), 1-14C[arachidonoyl-sn-glycero-3-phosphorylethanolamine] (PE), and 1-14C[arachidonoyl-sn-glycero-3-phosphorylsphingomyelin] (SM) were obtained from Amersham (Braunschweig, Germany) or from ENN DuPont (Homburg, 1). Palmitoyl-2-14C-erythrocaply-sphingomyelin (CPC) was obtained from Sigma (Deisenhofen, Germany) or from Molecular Probes (Eugene, OR). 1-Arachidonoyl-2-pyreneconayl-sn-3-glycerophosphocholine (pyr-PC), 1-palmitoyl-2-pyreneconayl-sn-3-glycerophosphorylcholine (pyr-PC), and 1-arachidonoyl-2-pyreneconayl-sn-3-glycerophosphorylcholine (pyr-PCC) were synthesized as described previously (10). Briefly, 14C-labeled phospholipids were incorporated into LDL particles according to a previously published protocol (12). Very slowly, 14C-labeled LDL, pyrene-labeled LDL, pyrene-labeled HDL, or vesicle suspensions were centrifuged at 37 °C. For the preparation of LDL, 200 μg of egg phosphatidylcholine and 5 μg of py-PC, py-PCC, py-PE, or py-SM were dissolved in 200 μl of ethanol and thereafter slowly injected under argon into 2 ml of a buffer containing (in mM): 138 NaCl, 3 KCl, 1 MgCl2, 15 Hepes, 9 citrate, 5 EDTA, 5 glucose (pH 7.4, “incubation buffer”). 30 μl of the incubation buffer was used for 197 ml of incubation buffer for the incubation with washed platelets. In the case of pyrene-labeled donors, the fluorescence was monitored directly in the suspensions every 30 s (on-line conditions). After incubation with 14C-labeled LDL, platelets were separated from the donors by centrifugation, washed once, and the platelet-associated radioactivity determined. In some cases, following incubation of platelets with pyrene-labeled donors, fluorescence intensities were also determined after separation of donors and acceptors by centrifugation (of-line experiments). Monomer and excimer fluorescence of the suspensions were determined at emission wavelengths of 380 nm and 480 nm, respectively, with excitation at 340 nm (excitation and emission slits of 5 and 10 nm). The incorporation of pyrene-labeled phospholipids into the suspension was followed by measurement of the increase in fluorescence intensity after addition of platelets to the donors. Fluorescence measurements were carried out using a Shimadzu RF-5001-PC spectrophotometer (Shimadzu Germany, Duisburg, Germany).
Platelet Agonists Stimulate PE Import

**FIG. 1. Influence of thrombin on platelet-associated radioactivity as determined following incubation of platelets in the presence of LDL labeled with [14C]-phospholipids or [125I]-apo B.** Platelets (1 × 10^9/0.25 ml of incubation buffer) were incubated for 1 min in the absence or presence of thrombin (0.5 unit/ml) at 37 °C with LDL (0.2 mg of protein) containing either [14C]-phospholipids or [125I]-apo B. After some experiments, after incubation of platelets with lipoproteins, the suspensions were subsequently treated for 45 min at 37 °C with a 40-fold excess of unlabeled LDL. In order to determine the localization of [14C]-LDL within the donor lipoproteins before the start of incubation with platelets, lipids were extracted from the lipoproteins (16). The lower (organic) phase was resuspended in CHCl₃/CH₃OH (2:1), dried and subjected to 1-dimensional TLC in CHCl₃/CH₃OH/H₂O/NH₃ (90/54/5.5/5.5). More than 95% of total [14C]-peptidic fraction originated labeled. After incubation of platelets with [14C]-LDL (see above), platelets were also subjected to lipid extraction and neutral lipids as well as phospholipids separated by TLC as described (10). The percentages of platelet-associated [14C] in the same phospholipid fraction as originally labeled was >92% (absence of thrombin) and >85% (presence of thrombin). Empty columns, without thrombin. Filled columns, with thrombin. +, addition of unlabeled LDL. Figure shows mean values of experiments on platelets of six to nine (upper panel) and four donors (lower panel).

chromogenic substrate S-2238 were added (final concentration 0.26 mg/ml). The increase in absorption at 405 nm was measured and compared with standard curves obtained with different concentrations of thrombin.

**Miscellaneous Procedures**—For assessment of changes in platelet morphology, samples of platelet suspensions were transferred into aggregometer cuvettes and incubated at 37 °C while stirring (1,100 rpm). Platelet shape change and aggregation were monitored by measuring light transmission in an aggregometer (Fresenius, Bad Homburg, Germany). All mean values are given ± S. D.

**RESULTS**

Specific Enhancement of Import of Ethanolamine Phospholipids by Platelet Stimuli—LDL particles enriched with [14C]IPC, [14C]SM, or [14C]PE were incubated in the absence or presence of thrombin (0.5 unit/ml) with platelets for 1 min at 37 °C. In order to prevent platelet aggregation, which might lead to trapping of labeled lipoproteins, platelets were pretreated for 20 min with 2 mM EGTA before adding thrombin. In platelets that had been incubated with LDL containing either [14C]IPC or [14C]SM, platelet-associated [14C] was associated by thrombin (Fig. 1, upper panel). In contrast, with LDL donor particles containing [14C]PE, thrombin increased platelet-associated [14C]PE by 2.6-fold. Similarly, in platelets that had been incubated with lipoproteins enriched with [14C]-20:4-PE instead of [14C]-20:4-PE, thrombin augmented platelet-associated [14C]PE by 2.5-fold (Fig. 1, upper panel).

Thrombin did not increase platelet binding of LDL labeled in its major apoprotein component (125I-apo B) (Fig. 1, lower panel). Platelet-bound 125I-apo B was reduced by 88% (absence of thrombin) and by 86% (presence of thrombin) after addition of a 40-fold excess of unlabeled LDL. When unlabeled LDL was added to platelets previously incubated for 1 min with [14C]IPC, the amount of platelet-linked [14C]PE was reduced from 1,050 to 530 (absence of thrombin) and from 2,690 to 2,010 (cpm/10^8 platelets, presence of thrombin, upper panel of Fig. 1). As extracellularly bound LDL particles are nearly completely removed by this treatment, the quantity of residual platelet-bound [14C]PE predominantly reflects [14C]PE incorporated into platelet membranes. Incorporation of [14C]PE was thus increased by about 4-fold by thrombin. In order to estimate the amount of PE transferred from LDL to the activated platelets, the platelet-bound [14C]PE was incubated with 2 × 10^8 platelets at 37 °C with thrombin (0.5 unit/ml). 0.95% of the [14C]PE was transferred to the activated platelets (as determined following addition of unlabeled LDL), which is equivalent to 0.46% of total platelet PE (mean of two separate experiments).

During the 45-min incubation in the presence of unlabeled LDL, the particles could serve as acceptor for [14C]PE originally present in platelet membranes. Control experiments performed in the absence of thrombin indicated, however, that platelets had lost less than 3% of [14C]PE after 45 min of incubation with unlabeled LDL at 37 °C (data not shown). The time dependence of the effect of thrombin on platelet-associated [14C]PE after incubation of platelets with [14C]PE-labeled LDL is shown in Fig. 2 (upper panel). The presence of thrombin augmented platelet-associated [14C] by 2.5-, 2.7-, and 2.6-fold after 30, 60, and 120 s, respectively. Iloprost pretreatment nearly completely prevented the increase in platelet-associated [14C] as induced by thrombin within the 120-s period (upper panel of Fig. 2). Transfer of phospholipids from plasma lipoproteins to platelets can be directly monitored by using donor lipoproteins containing py-labeled phospholipids (10). LDL particles were enriched in py-labeled choline phospholipids (PC, its plasmalogel analog plasmylecholine (PPC), or SM) as well as in py-labeled ethanolamine phospholipids (py-PE, py-PPE). Platelets were incubated with LDL containing py-labeled phospholipids. For all phospholipids tested, the intensity of pyrene monomer fluorescence continuously increased within this time interval as measured under on-line conditions. In the same time period, excimer fluorescence decreased, thus indicating incorporation of py-phospholipids into the acceptor membrane. Thrombin (0.5 unit/ml) did not significantly alter the incorporation of the pyrene-labeled choline phospholipids PC, PPC, and SM (data not shown). In contrast, transfer of py-PE from LDL to platelets was increased by thrombin (Fig. 2, lower panel). A maximal 4.1-fold increase was observed after 1 min. Comparable results were obtained when the transfer of py-PE occurring within 1 min was determined under off-line conditions (from 2.9 ± 0.7 (−thrombin) to 12.4 ± 3.6 (+thrombin) ng of pyrene incorporated/3 × 10^8 platelets, n = 4). Thrombin also enhanced incorporation of py-PPE by 3.8-fold, as assessed after a 1-min incubation of py-PPE-labeled LDL with platelets (from 4.8 ± 0.6 to 18.2 ± 3.8 ng of pyrene incorporated/3 × 10^8 platelets, n = 4). In platelets pretreated for 5 min with 100 nM iloprost thrombin only slightly increased import of py-PE (Fig. 2, lower panel).

Changes in platelet morphology induced by thrombin were evaluated under identical conditions as for the experiments with suspensions of py-labeled lipoproteins. To this purpose, light transmission was measured in an aggregometer while
stirring the platelet suspension (1,100 rpm). After 10–20 s, thrombin elicited a maximal reduction in light transmission indicating shape change. In contrast, in platelets pretreated for 5 min with 100 nM iloprost, the maximal decrease in light transmission induced by thrombin was reduced by 76% and 83% as compared with untreated platelets (values of two experiments). Thus, iloprost pretreatment inhibits both stimulation of uptake of ethanolamine phospholipids and platelet shape change.

In further experiments, it was investigated whether the increase in PE import elicited by thrombin was restricted to LDL as donor. HDL particles containing either py-PE or py-PPE were incubated for 1 min at 37 °C with platelets. Thrombin increased the transfer of py-PE and py-PPE from HDL to platelets by 3.6- and 3.8-fold, respectively (Table I). Transfer of the two ethanolamine phospholipids was also enhanced by thrombin when liposomes containing PC or PE and either py-PE or py-PPE were employed as donors (Table I). Thrombin stimulated transfer of py-PE and py-PPE from vesicles to platelets by 4.2- and 4.4-fold, respectively. When platelets were incubated with HDL or lipid vesicles enriched in py-PE and subsequently platelet-associated pyrene contents assessed (offline experiments), the results obtained were essentially similar to those obtained in on-line experiments (data not shown). The effect of the thrombin inhibitors hirudin (2.5 units/ml) and danasyarginine (0.1 μM) on transfer of py-labeled ethanolamine phospholipids from LDL or vesicles to platelets was investigated. Hirudin binds to thrombin, thereby impeding interaction of thrombin with the thrombin receptor (20), while danasyarginine blocks the proteolytic activity of thrombin (21). Both agents rather completely prevented the stimulation of py-PE and py-PPE transfer to platelets as elicited by thrombin (Table I).

In order to analyze whether other platelet agonists also affected transfer of py-PE from lipid vesicles to platelets, platelets were activated with either collagen (10 μg/ml) or ADP (10 μM). The incorporation of py-PC and py-SM into platelets was not influenced by the two agonists (Fig. 3). However, transfer of py-PE was stimulated by 2.5- and 2.8-fold upon addition of collagen and ADP, respectively. When collagen plus thrombin were present together, the stimulation increased from 3.9-fold (thrombin alone) to 6.3-fold (thrombin + collagen). In the presence of ADP plus thrombin, the activation of py-PE import was 6.1-fold (thrombin + ADP) (Fig. 3). Accordingly, both ADP and collagen additively activate the thrombin-induced transfer of py-PE from lipid vesicles to platelets.

As thrombin thus emerged as most potent stimulator of platelet PE uptake, the effect of thrombin was analyzed in more detail. Fig. 4 shows data on the concentration dependence of the action of thrombin on platelet-associated 14C or pyrene as assessed after incubation of platelets with either LDL particles (labeled with 14C-phospholipids) or egg PC vesicles (containing py-phospholipids). In platelets incubated with [14C]PE-labeled LDLs, platelet-associated [14C]PE steeply increased when elevating the thrombin concentration from 0.005 to 0.1 unit/ml (Fig. 4, upper panel). At concentrations >0.1 unit/ml, no further enhancement was noted. Half-maximal stimulation was observed between 0.01 and 0.05 unit/ml. A comparable concentration dependence was observed when the effect of thrombin on transfer of py-PE from vesicles to platelets was analyzed (Fig. 4, lower panel). Half-maximal stimulation was noted at 0.02 unit of thrombin/ml. At all concentrations investigated, thrombin did not affect platelet [14C]PC levels in platelets as determined after incubation with LDL labeled in [14C]PC (Fig. 4, upper panel). The platelet agonist did also not affect transfer of py-PC (Fig. 4, lower panel).

Platelet Activation Mechanisms Involved in the Enhanced Import of Ethanolamine Phospholipids—In order to evaluate whether the platelet thrombin receptor mediated the thrombin-induced stimulation of import of ethanolamine phospholipids, the effect of TRAP was investigated. TRAP directly activates the thrombin receptor without displaying the proteolytic activity of thrombin (12). TRAP (15 μM) increased transfer of py-PE and py-PPE from vesicles to platelets by 3.6- and 4.2-fold, respectively. In platelets preincubated for 5 min with 100 nM TRAP, TRAP did not enhance the import of py-labeled ethanolamine phospholipids into platelets. Furthermore, incorporation of py-labeled choline phospholipids (PC, PPC, and SM) was unaffected by TRAP (Fig. 5, upper panel). With LDL as donor (30 μg of LDL protein containing 0.7 μg of py-PE), TRAP (15 μM) increased the import of py-PE into platelets from 2.7 ± 0.6 (without TRAP) to 10.5 ± 3.4 ng of py-PE incorporated/8 × 108 platelets within 1 min (presence of TRAP, n = 3). TRAP also augmented the amount of platelet [14C]PE determined following a 1-min incubation of platelets with [14C]-20:4-PE-labeled LDL (data not shown). The concentration dependence of the effect of TRAP on transfer of py-PE from vesicles to platelets is shown in the lower panel of Fig. 5. The lowest concentration causing maximal stimulation was 15 μM. A half-maximal effect was noted at a concentration of 4 μM TRAP.
Platelet Agonists Stimulate PE Import

| Amount of pyrene incorporated | LDL | HDL | Lipid vesicles |
|-------------------------------|-----|-----|---------------|
|                               | py-PE | py-PPE | py-PE | py-PPE |
| - Thrombin                    | 2.9 ± 0.8 | 4.6 ± 1.3 | 4.1 ± 1.7 | 6.3 ± 2.0 |
| + Thrombin                    | 12.2 ± 2.4 | 17.5 ± 3.9 | 14.9 ± 2.4 | 23.8 ± 4.7 |
| + Hirudin + thrombin          | 3.5 ± 1.7 | 4.8 ± 1.5 | 4.8 ± 2.3 | 7.2 ± 1.6 |
| Dansylarginine + thrombin     | 3.1 ± 0.8 | 5.2 ± 1.4 | ND | ND |

Fig. 3. Effect of different platelet agonists on transfer of py-labeled phospholipids from lipid vesicles to platelets. Platelets (3 × 10^7/2 ml) were incubated at 37 °C for 1 min with vesicles containing 3.8 μg of egg PC and 0.08 μg of py-PC, py-SM, or py-PE. The excimer/monomer ratios of the vesicles ranged between 0.3 and 0.6. The increase in monomer fluorescence between 0 and 1 min of incubation of platelets with labeled particles as measured under on-line conditions was taken as incorporation of py-labeled ethanolamine phospholipids into platelets. Results are from experiments on platelets of four to six different donors. ND, not determined.

Earlier work indicates that the inhibition of cyclooxygenase induced by weak platelet agonists such as ADP and epinephrine prevents the secretory response of the platelets while their capacity to induce shape change and aggregation is retained (22, 23). Accordingly, the effect of ADP on the platelet uptake of py-labeled ethanolamine phospholipids was analyzed after pretreatment of the platelets with aspirin. The transfer was registered in the presence of apyrase (0.6 unit/ml, in order to avoid desensitization of the platelet ADP receptor by ADP; Ref. 24). In untreated platelets, ADP stimulated the uptake of py-PE and py-PPE by 3.2- and 4.3-fold, respectively (Table III). In aspirin-pretreated platelets, ADP only marginally increased platelet phospholipid uptake. Control experiments using the

†TRAP thus mimicked the stimulation of transfer of ethanolamine phospholipids from different donors to platelets as induced by thrombin. This indicates that the effect of the latter agonist is mediated by the thrombin receptor.

Part of the downstream signaling of the platelet thrombin receptor is the activation of protein kinase C (12). The 2.5-fold increase in platelet [14C]PE elicited by thrombin (0.5 unit/ml) was reduced by 48% and 51% in the presence of the PKC inhibitors Ro 31-8220 and staurosporine, respectively (Table II). When a 40-fold excess of unlabeled LDL was added to the platelet suspension after incubation with [14C]PE-labeled LDL in order to remove extracellularly bound [14C]PE (cf. Fig. 1), the elevation of platelet [14C]PE induced by thrombin was reduced by 52% by both inhibitors (Table II). Among several PKC inhibitors tested (Ro 31-8220, staurosporine, calphostin C, bisindolylmaleimide, and 1-(5-isouquinolylsulfonyl)-2-methyl-piperazine (H7)), only the water-soluble H7 did not perturb the excimer and monomer characteristics of pyrene-enriched egg PC vesicles and lipoproteins. Platelets (3 × 10^7/2 ml) were pretreated for 1 min with H7 (50 μM) and thereafter incubated with LDL (30 mg of protein) containing 0.5 μg of py-PE. The stimulation of py-PE transfer from LDL to platelets as induced by thrombin (0.5 unit/ml) was reduced by 53% in the presence of H7 (2.6 ± 0.6 (– thrombin), 11.2 ± 3.8 (+ thrombin), 7.6 ± 1.3 ng of py/3 × 10^8 platelets (+ H7, + thrombin)).

When platelet PKC activity was directly stimulated with 12-O-tetradecanoylphorbol-13-acetate, platelet-associated [14C] was increased by 2.3-fold following incubation of platelets with [14C]PE-labeled LDL (Table II). OAG, another direct activator of PKC, induced a 2.0-fold elevation of platelet-associated [14C]. The effects of the two stimulators were prevented by preincubation with staurosporine (Table II). The increase in platelet-associated [14C] induced by 12-O-tetradecanoylphorbol-13-acetate and OAG persisted when bound [14C]-LDL was removed by excess of unlabeled LDL (data not shown). Thus, enhancement of PE import by thrombin is partially blocked by PKC inhibitors and PKC activators stimulate the incorporation of PE suggesting involvement of PKC in the effect of the agonist on phospholipid uptake.
Platelets (3 from lipid vesicles to platelets. 37 °C for 1 min with vesicles containing 3.8

Lower panel

end to Fig. 1.

vesicles containing 3.8

bated in the absence or presence of TRAP at 37 °C for 1 min with

measured under on-line conditions.

1-min incubation of platelets with lipid vesicles containing py-PE. Figure

into platelets as induced by different concentrations of TRAP during a

Lower panel

platelets pretreated with iloprost.

of TRAP (15 m

and aggregation.

Effect of TRAP on transfer of py-labeled phospholipids

Concentration dependence of the effect of thrombin on

FIG.4 .

Platelet Agonists Stimulate PE Import

27805

Platelets were pretreated for 1 min at 37 °C with either Ro 31–8220 (10 µM), staurosporine (1 µM), TPA (200 nM), or OAG (25 µg/ml) and thereafter incubated for 1 min with LDL containing 14C-20:4-PE. In some cases, the suspensions were subsequently treated for 45 min with a 40-fold excess of unlabeled LDL. Results are from experiments on platelets of three to six different donors. TPA, 12-O-tetradecanoylphor

bol-13-acetate.

Table II

Effect of inhibitors and activators of PKC on platelet-associated

Platelet-associat ed 14C

Control + Thrombin - + +

+ unlabeled LDL 360 ± 450 430 ± 380 310 ± 200 450 ± 300 + unlabeled LDL 360 ± 450 430 ± 380 310 ± 200 450 ± 300 Staur osporine + unlabeled LDL 360 ± 450 430 ± 380 310 ± 200 450 ± 300 TPA + staur osporine 360 ± 450 430 ± 380 310 ± 200 450 ± 300 OAG 360 ± 450 430 ± 380 310 ± 200 450 ± 300

Table III

Influence of pretreatment with aspirin on ADP-induced stimulation of

platelet uptake of py-labeled ethanolamine phospholipids

To the platelet-rich plasma 1 mM aspirin was added and the suspension incubated for 15 min at 37 °C. Platelets (3 × 10^8/2 ml) were suspended in the incubation buffer (supplemented with 0.6 unit of apyrase/ml) and incubated at 37 °C for 3 min with vesicles containing 3.8 µg of egg PC and 0.08 µg of either py-PC (●) or py-PE (▲). The incorporation of the fluorophore was measured under on-line conditions.

On the basis of these experiments, we tested the hypothesis whether the extracellular medium recovered from suspensions of activated platelets affected the transfer of py-PE between two types of lipid vesicles in vitro. Platelets were activated for 5 min with either ADP (10 µM) or thrombin (0.5 unit/ml), the extracellular medium isolated by centrifugation and added to a suspension consisting of donor vesicles (containing py-PE) and acceptor vesicles. The transfer of py-PE to the acceptor vesicles was unaffected by the presence of the extracellular medium obtained from untreated platelets (Fig. 6). The supernatant recovered from ADP- and thrombin-activated platelets, in contrast, stimulated py-PE transfer by 10.4- and 11.0-fold, respectively. The increase in py-PE transfer elicited by the supernatant isolated from ADP-activated platelets was reduced by 85% when the platelets had been pretreated with aspirin (Fig. 6). In order to remove microvesicles shed from the activated platelets, the extracellular medium from thrombin-stimulated platelets was isolated and thereafter centrifuged at 12,000 × g for 20 min. The supernatant thereby obtained stimulated py-PE transfer to an extent similar to that for the original extracellular medium (data not shown).

Table III

Amount of pyrene incorporated

- Aspirin + Aspirin

py-PE Control 0.48 0.34 ADP 1.54 0.53 py-PPE Control 0.76 ± 0.22 0.68 ± 0.15 ADP 3.23 ± 0.94 0.94 ± 0.37

In further experiments, we evaluated whether proteins were involved in the stimulating effects of the extracellular media on py-PE transfer. After dialyzing the supernatants recovered

aggregometer indicated that, under the same experimental conditions, ADP was still able to induce platelet shape change and aggregation.

Fig. 5. Effect of TRAP on transfer of py-labeled phospholipids from lipid vesicles to platelets. Platelets (3 × 10^7/2 ml) were incubated in the absence or presence of TRAP at 37 °C for 1 min with vesicles containing 3.8 µg of egg PC and 0.08 µg of py-phospholipids. The incorporation of py-PE was measured under on-line conditions. Upper panel, empty columns, absence of TRAP; filled columns, presence of TRAP (15 µM); columns with vertical lines, presence of TRAP (15 µM), platelets pretreated with iloprost. Lower panel, incorporation of py-PE into platelets as induced by different concentrations of TRAP during a 1-min incubation of platelets with lipid vesicles containing py-PE. Figure shows mean values from experiments on platelets of five different donors.

Fig. 4. Concentration dependence of the effect of thrombin on platelet-associated 14C or pyrene incorporation as determined following incubation of platelets with labeled donors. Upper panel, platelets (1 × 10^7/0.25 ml) were incubated for 1 min in presence of the indicated concentrations of thrombin at 37 °C with LDL (0.2 mg of protein) containing either 14C-20:4-PE (filled columns) or 14C-20:4-PC (columns with horizontal lines). For further information, see legend to Fig. 1. Lower panel, platelets (3 × 10^7/2 ml) were incubated at 37 °C for 1 min with vesicles containing 3.8 µg of egg PC and 0.08 µg of either py-PC (●) or py-PE (▲). The incorporation of the fluorophore was measured under on-line conditions.
Platelet Agonists Stimulate PE Import

![Graph](image)

**FIG. 6.** py-PE transfer between donor and acceptor vesicles in the presence of extracellular media obtained from suspensions of activated platelets. Platelets (3 x 10^9/ml) were activated with either thrombin (0.5 unit/ml) or ADP (10 μM; in the presence of 0.6 unit/ml apyrase) for 5 min. After centrifugation, the supernatant was isolated and added to a mixture of donor vesicles (enriched with py-PE) and acceptor vesicles (see "Experimental Procedures"). Following a 30-min incubation period at 37 °C, the acceptor vesicles were isolated and their pyrene contents determined. Columns with vertical and horizontal bars denote the presence of extracellular media from suspensions of ADP- and thrombin-activated platelets, respectively. Co, absence of extracellular media. In the other samples extracellular media from suspensions of either untreated or activated platelets were added. Boiling of the supernatant lasted 10–15 min. The concentration of ammonium sulfate used to precipitate the supernatant proteins was 3.3 M. Figure shows mean values of experiments on three to five different preparations.

from suspensions of platelets activated with either ADP or thrombin, the transfer of py-PE between donor and acceptor vesicles was still enhanced (98% and 52% of the stimulation obtained without dialysis; Fig. 6). The presence of either thrombin (0.5 unit/ml) or of apyrase alone (0.6 unit/ml) did not affect py-PE transfer between the two types of lipid vesicles (data not shown). When the supernatants obtained from ADP- or thrombin-activated platelets were briefly boiled, the stimulatory capacity was lowered by more than 80% (Fig. 6). In addition, after precipitation of the supernatant proteins with ammonium sulfate (3.3 M), the accelerating effect of the extracellular medium recovered from ADP-activated platelets on py-PE transfer was reduced by 74%.

The extracellular medium from thrombin-activated platelets was isolated, dialyzed (in order to remove ADP) and thereafter added to a suspension consisting of untreated platelets (3 x 10^9/2 ml) and egg PC vesicles (3.8 μg of phospholipid) enriched in py-PE (0.08 μg). In order to prevent stimulation of platelet py-PE uptake by thrombin itself, dazoxuline (0.1 mM; cf. Table I) was added. After a 3-min incubation at 37 °C, platelet import of py-PE was measured under on-line conditions. Under these conditions, platelet py-PE uptake was increased by 6.4-fold compared with the py-PE incorporation determined in the presence of an extracellular medium isolated from untreated platelets (0.46 ± 0.18 (supernatant from untreated platelets) versus 2.93 ± 0.55 ng of pyrene/3 x 10^9 platelets (supernatant from thrombin-activated platelets), n = 3). The enhancing effect was strongly reduced when the (dialyzed) extracellular medium recovered from the thrombin-activated platelets was briefly boiled or its proteins removed by precipitation with ammonium sulfate (data not shown). Together, these data suggest that proteinaceous products secreted by the platelets mediate the increased platelet uptake of py-PE after activation with ADP or thrombin.

**Eicosanoid Production and Platelet Procoagulant Activity during Stimulation of PE Import**—Upon activation with thrombin, stimulation of phospholipases A₄ induces liberation of arachidonic acid from platelet membrane phospholipids which, in turn, can be further metabolized to eicosanoids such as thromboxane A₂ (TXA₂) or 12-hydroxyeicosatetraenoic acid (HETE). After incubation of platelets with LDL particles containing 14C-20:4-PE, the amount of 14C associated with arachidonic acid, [14C]TXB₂ (the stable hydrolysis product of TXA₂), and [14C]HETE was determined (Table IV). As compared with control platelets, the amount of 14C present in these substances increased by 4–7-fold in platelets activated by thrombin (0.5 unit/ml) and by 6–11-fold after stimulation with thrombin plus collagen (10 μg/ml). The proportion of 14C associated with TXB₂ and HETE was higher in activated platelets while the percentage of arachidonic acid was lower (Table IV). In separate experiments, LDL was enriched with 14C-18:2-PE (instead of 14C-20:4-PE) and incubated with untreated and thrombin-activated platelets under the same experimental conditions as those described in Table IV. [14C]Linoleic acid was determined after the end of incubation by the same TLC separation as for [14C]arachidonic acid (see "Experimental Procedures"). The amount of 14C-18:2 determined was found to be increased by 1.3-fold in thrombin-activated compared with control platelets (mean of two separate experiments). Together, the data indicate that arachidonic acid-containing PE species imported into activated platelets are partially utilized for liberation of arachidonic acid and subsequent production of eicosanoids.

The procoagulant activity of platelets is supposed to be mainly due to an increase in phosphatidylserine (PS) content of the outer monolayer of the platelet plasma membrane (6, 7). However, recent data indicate that the presence of PE may also play an important role (25). It was thus tested whether the presence of a lipoprotein donor containing PE (such as LDL) affected platelet prothrombinase activity. Control experiments indicated that LDL particles did not contain any measurable PS (data not shown). Platelets were incubated for 5 min with thrombin (0.5 unit/ml), a time-interval yielding maximal stimulation of the platelet procoagulant activity under the experimental conditions applied (data not shown). Thrombin as well as thrombin plus collagen (10 mg/ml) increased the platelet procoagulant activity by 4.5- and 10.4-fold (Fig. 7). The presence of LDL further enhanced the stimulation induced by the platelet agonists resulting in a 10.5-fold (thrombin) and 18.3-fold elevation against control (Table IV). In order to assess whether the phospholipid components of LDL could be responsible for the effect of the particles on platelet procoagulant activity, thrombin-activated platelets were coincubated with pure PC vesicles. The prothrombinase activity of thrombin-activated platelets remained unaffected (Fig. 7). When 4.8 mol % of the PC vesicles was substituted by either PE or PPE, the prothrombinase activity of thrombin-activated platelets was increased by 2.5- and 3.0-fold, respectively. The procoagulant activity of platelets stimulated with thrombin plus collagen was increased by 2.3-fold with PC/PE
incubated with factor II (0.43 mLDL and phospholipid vesicles. Platelets (1 x 10^7/ml) were incubated without or with thrombin (0.5 unit/ml) for 5 min at 37 °C. In some samples either LDL (50 μg/ml) or vesicles containing egg PC (63 nmol/ml) or egg PC (60 nmol/ml) plus ethanolamine phospholipids (PE and PPE; 3 nmol/ml) were present (as indicated). Subsequently, the suspension was first incubated with 1.1 nm factor Va and 0.52 nm factor Xa (in the presence of CaCl₂; see “Experimental Procedures”) and thereafter incubated with factor II (0.43 μM). Prothrombinase activity was measured by using the chromogenic substrate S-2238 (0.26 mM). Lower panel, concentration dependence of the effect of thrombin on prothrombinase activity in the presence of PC/PPE vesicles. Figure shows mean values of experiments on platelets of four to six different donors.

Platelet Agonists Stimulate PE Import

We observed in the present study that the platelet stimuli thrombin, collagen, and ADP specifically enhance the import of ethanolamine phospholipids into platelets. Platelets possess specific binding sites for LDL on their plasma membranes, which include glycoproteins Ib/IIIa (26). Import of phospholipids into platelets may in principle be facilitated by increased binding of lipoprotein particles, as this might enhance the physical interaction between donor and acceptor monolayers. However, if so, increased binding of LDL to platelets would also be expected to stimulate incorporation of choline phospholipids into platelets (PC, PPC, and SM). This was not the case (Figs. 1 and 4). Furthermore, thrombin barely affected binding of 125I-LDL to the platelets (Fig. 1). In addition, as in both untreated and thrombin-activated platelets nearly 90% of bound 125I-apo B could be removed by an excess of unlabeled LDL, it is unlikely that under the experimental conditions employed the platelet agonist induced endocytosis of LDL particles. Thus, the stimulatory action of thrombin on transfer of ethanolamine phospholipids to platelets is apparently not mediated by increased platelet binding of LDL or by endocytosis of the lipoproteins.

In order to gain insight into the mechanism(s) responsible for the stimulatory effect of thrombin on transfer of ethanolamine phospholipids, the involvement of donor lipoproteins was evaluated. Transfer of py-labeled ethanolamine phospholipids was accelerated to a similar extent using HDL or egg PC vesicles compared with LDL as donors (Table I). The concentration dependence of the thrombin effect was also comparable using either LDL (labeled with [14C]PE) or lipid vesicles (labeled with py-PE) as donors (Fig. 4). We conclude that LDL or HDL apoproteins are not involved in the thrombin-induced stimulation of PE transfer to platelets.

In a second set of experiments, the role of the platelet acceptors for the effect of thrombin on PE transfer was evaluated. Thrombin cleaves the platelet thrombin receptor, thereby creating a new N-terminal portion of the receptor that functions as a tethered ligand to activate the receptor (12). Both proteolysis and binding to the receptor are necessary to activate intracellular signaling pathways in platelets. Inhibition of the proteolytic function of thrombin by danylarginine prevented the stimulatory action of thrombin on PE import (Table I). On the other hand, addition of TRAP, which activates the thrombin receptor, induced a 4-fold stimulation of import of ethanolamine phospholipids (Fig. 5). Inhibitors of PKC partially reduced the thrombin-induced stimulation of transfer of labeled PE into platelets while direct activators of PKC enhanced PE import (Table II). PKC is part of the signaling cascade coupled downstream to the platelet thrombin receptor. Together, the data indicate that the effect of thrombin on incorporation of ethanolamine phospholipids is mediated by activation of the thrombin receptor and requires signaling through PKC.

ADP and collagen were also found to stimulate platelet uptake of ethanolamine phospholipids (Fig. 3). When the secretory response of ADP-activated platelets was prevented by aspirin pretreatment (22), the stimulation of uptake of py-labeled ethanolamine phospholipids was reduced (Table III). We therefore tested the hypothesis that the enhanced uptake of the ethanolamine phospholipids by activated platelets might be mediated by platelet secretory products. The extracellular media obtained from suspensions of either ADP- or thrombin-activated platelets markedly accelerated the transfer of py-PE between two types of lipid vesicles (Fig. 6). In the same in vitro system of lipid vesicles, we attempted to gain some insight into the nature of the factor(s) involved in the enhancement of py-PE transfer. Different experimental approaches indicated that the products secreted by the platelets responsible for the augmented transfer of the ethanolamine phospholipids were likely to be proteins. In future work, we plan to isolate and identify these proteins.

The ethanolamine phospholipids of plasma lipoproteins are particularly enriched in arachidonic acid (27). The results of the present study indicate that the transfer of PE-bound [14C]arachidonic acid from lipoproteins to platelets is increased by thrombin. Previous studies imply that the arachidonic acid esterified to platelet membrane ethanolamine phospholipids can be released by stimulation of phospholipase A₂ activity elicited by thrombin (28–30). We observed increased production of [14C]arachidonic acid and its metabolites [14C]thromboxane B₂ and [14C]HETE in thrombin- and collagen-activated platelets after incubation with LDL containing [14C]-20:4-PE (see “Results”). The free arachidonic acid is most probably generated by hydrolysis of platelet [14C]:20:4:PE mediated by platelet cytosolic phospholipase A₂ (cPLA₂). Subsequently, the polyunsaturated fatty acid is metabolized to the eicosanoids.

Secretory phospholipase A₂ (sPLA₂), which is released by activated platelets, could additionally liberate the arachidonic acid
from extracellular 14C-20:4-PE present in LDL (or microvesicles shed from platelets). However, EDTA, which inactivates sPLA2 but not cPLA2 (31), was present in the incubation buffer, thus arguing against involvement of sPLA2. Furthermore, platelet cPLA2 preferentially hydrolyzes phospholipid species with arachidonic acid at sn2 while sPLA2 shows no fatty acid specificity for the sn2 position (31). Enhanced import of 14C-20:4-PE into activated platelets was accompanied by a severalfold increase in release of 14C-20:4 (Table IV). A comparable elevation in the incorporation of 14C-18:2-PE (Fig. 1) barely altered production of 14C-18:2 (see “Results”). This preferential liberation of 20:4 over 18:2 is again in support of the view that platelet cPLA2 is most likely responsible for the release of [14C]arachidonic acid under the experimental conditions employed.

The appearance of PS in the outer monolayer of platelet plasma membranes is thought to be responsible for the procoagulatory effect of activated platelets. The magnitude of increase in aminophospholipid content depends on the strength and type of stimulation. The platelet agonists thrombin or collagen, when present alone, elicit only minor increases (7). Recent data indicate that, apart from PS, PE may also play a decisive role (25). Under physiological conditions, platelets are surrounded by HDL and LDL. When shape change, the earliest functional response on platelet activation, was nearly completely prevented by preincubation with iloprost, stimulation of import of ethanolamine phospholipids into platelets by thrombin was greatly reduced (Figs. 2 and 5). Interestingly, a previous study indicates that in platelets suspended in platelet-rich plasma (containing the lipoproteins), prostacyclin inhibits platelet procoagulant activity (32), in line with the above mentioned hypothesis.

We observed in the present investigation that LDL enhanced the procoagulant activity of platelets stimulated with thrombin alone or with thrombin plus collagen (Fig. 7). The effect elicited by LDL was mimicked by egg PC vesicles containing ethanolamine phospholipids. Pure PC vesicles were without effect. Therefore, under the experimental conditions employed, enhancement of platelet procoagulant activity in platelets activated by thrombin and collagen requires donors containing ethanolamine phospholipids. The concentration dependence of the thrombin-elicited stimulation of py-PE import (Fig. 4) and the one of thrombin-induced procoagulant activity in the presence of PC/PPE vesicles (Fig. 7) were comparable. Together, our data indicate that the acceleration of import of ethanolamine phospholipids is associated with an increased procoagulatory activity of platelets.

In conclusion, by activating the platelet thrombin receptor and signaling through PKC thrombin specifically stimulates the transfer of ethanolamine phospholipids from donor lipoproteins to platelet acceptors. The enhanced transfer of ethanolamine phospholipids is independent of the apoprotein component of lipoproteins and does not involve the endocytosis of LDL. The platelets stimulate ADP and collagen mimic the effect of thrombin. The stimulation of platelet import of ethanolamine phospholipids is apparently mediated by proteins secreted from the activated platelets. Thus, during platelet activation, import of phospholipids from extracellular donors to platelets is specifically regulated.

Acknowledgment—We gratefully acknowledged the excellent technical assistance of Susanne Zieseniss. We also thank Robert Bräutigam, who participated in the initial phase of the experiments.

REFERENCES

1. Tall, A. (1995) Annu. Rev. Biochem. 64, 235–257
2. Diaz, C., and Schroit, A. J. (1996) J. Membr. Biol. 151, 1–9
3. Wirtz, K. W. A. (1991) Annu. Rev. Biochem. 6, 73–89
4. Spector, A. A., Hoak, J. C., and Warner, E. D. (1970) J. Clin. Invest. 49, 1489–1496
5. Habenicht, A. J., Salbach, P., Georgi, M., Zeh, W., Janssen-Timmen, U., Blattner, C., King, W. C., and Glomset, J. A. (1990) Nature 345, 634–636
6. Bevers, E. M., Comfurius, P., van Rijn, J. L. M. L., Hemker, H. C., and Zwaal, R. F. A. (1982) Eur. J. Biochem. 122, 429–436
7. Bevers, E. M., Comfurius, P., and Zwaal, R. F. A. (1983) Biochim. Biophys. Acta 736, 57–66
8. Freysinet, J. M., Gauchy, J., and Cazenave, J. P. (1986) Biochem. J. 238, 151–157
9. Galvin, J. R., Kurowska, S., Moore, K., Esmon, C. T., and Esmon, N. L. (1987) J. Biol. Chem 262, 2199–2205
10. Engelmann, B., Kög, C., Kuleschar, R., and Schaiß, B. (1996) Biochem. J. 313, 781–789
11. Nozawa, Y., Nakashima, S., and Nagata, K. (1991) Biochim. Biophys. Acta 1082, 219–238
12. Grand, R. J. A., Turnbull, A. S., and Graham, P. W. (1996) Biochem. J. 313, 353–368
13. Páltauf, F., and Hermetter, A. (1991) Methods Enzymol. 197, 134–149
14. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) J. Clin. Invest. 34, 1345–1353
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
16. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 912–917
17. Thurnhofer, H., and Hauser, H. (1990) Biochim. Biophys. Acta 1024, 249–262
18. Yamashita, T., Shimizu, T., and Ogata, E. (1991) J. Biol. Chem. 26, 3888–3892
19. Goodwin, C. A., Wheeler-Jones, C. P., Namaranian, S., Bokkala, S., Kakkar, V. V., Authi, K. S., and Scully, M. F. (1995) Biochem. J. 308, 15–21
20. Fenton, J. W. (1989) Semin. Thromb. Hemostas 15, 265–268
21. Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) Biochemistry 18, 996–1003
22. Charn, I. F., Feinman, R. D., and Detwiler, T. C. (1977) J. Clin. Invest. 60, 866–873
23. Siess, W. (1989) Physiol. Rev. 69, 58–178
24. Mustard, J. F., Perry, D. W., Ardlie, N. G., and Packham, M. A. (1972) Br. J. Haematol. 23, 193–204
25. Smeets, E. F., Comfurius, P., Bevers, E. M., and Zwaal, R. F. A. (1986) Thromb. Res. 31, 419–426
26. Koller, E., Koller, F., and Binder, B. R. (1989) J. Biol. Chem. 264, 12412–12418
27. Myher, J. J., Kuksis, A., and Find, S. (1989) Lipids 24, 408–418
28. Purdon, A. D., Patelunas, D., and Smith, J. B. (1987) Biochem. Biophys. Acta 920, 205–214
29. Takamura, H., Narita, H., Park, H. J., Tanaka, K., Matsuura, T., and Kito, M. (1987) J. Biol. Chem. 262, 2262–2269
30. Colard, O., Breton, M., Pepin, D., Chevy, F., Bereziat, G., and Polonovski, J. (1989) Biochem. J. 259, 333–339
31. Mayer, R. J., and Marshall, L. A. (1993) FASEB J. 7, 339–348
32. Ehrman, M. L., and Jaffe, E. A. (1980) Prostaglandins 20, 1103–1116