Inhibition of Platelet Phospholipid Methylation During Platelet Secretion

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A pathway for the synthesis of membrane phosphatidylcholine involving the N-methylation of phosphatidylethanolamine has been detected in several types of mammalian cells. Furthermore, it has been implicated in the coupling of agonist binding to cell response. We examined whether human platelets exhibit this synthetic pathway and whether platelet agonists influence its activity. When washed platelets were incubated with 0.15 μM [methyl-3H]methionine at 37°C, they incorporated methyl-3H into their phospholipids linearly at the rate of 1 pmole/10⁹ platelets/hr. When incubated with 20 μM radiolabeled methionine, they incorporated about 15 pmole/10⁹ platelets/hr. The radioactivity found was primarily in phosphatidyl-N,N-dimethylethanolamine, phosphatidyl-N,N-dimethylethanolamine, and phosphatidylcholine. Thrombin caused an immediate (within 15 sec) and sustained (up to 30 min) decrease in the rate and extent of N-methylation of platelet phospholipids. This was accounted for by a decrease in synthesis of methylated phospholipids rather than an increase in their degradation. This thrombin effect correlated with serotonin release and could be dissociated from platelet aggregation and prostaglandin synthesis. Thrombin also decreased the synthesis of phosphatidylethanolamine when choline was used as the radiolabeled substrate. Other agonists such as epinephrine, adenosine diphosphate (ADP), or A23187 also decreased phospholipid methylation under conditions in which they stimulated serotonin release. These data demonstrate that platelets are capable of synthesizing phosphatidylethanolamine from phosphatidylethanolamine by N-methylation and that agonists perturb this pathway as they induce platelet secretion. The precise role of phospholipid methylation in either resting or stimulated platelets remains to be established.

Platelet aggregation and secretion are initiated upon the exposure of the platelet surface membrane to agonists such as thrombin, epinephrine, and ADP. It is not clear how the binding of these agonists is coupled to the final platelet responses. This coupling, or signal transduction, may involve biochemical and/or biophysical changes of membrane lipids or proteins.

Recently, a pathway for the synthesis of the major phospholipid, phosphatidylcholine, has been documented in a variety of mammalian cell types, including human red blood cells. This pathway involves the successive N-methylation by two membrane-associated methyltransferases of the nitrogenous base of phosphatidylethanolamine to form phosphatidylcholine. The first enzyme requires magnesium and catalyzes the transfer of a single methyl group from S-adenosylmethionine to phosphatidylethanolamine to form phosphatidyl-N-monomethylethanolamine. The second enzyme catalyzes the transfer of two methyl groups to form phosphatidyl N,N-dimethylethanolamine and finally phosphatidylcholine. The involvement of this methylation pathway in signal transduction has been suggested by the fact that activation or suppression of this pathway accompanies activation of several cell types by their agonists.

The predominant lipid of platelet membranes is phospholipid. Although the precise architecture of platelet membranes is unknown, the bulk of membrane phospholipid is believed to be arranged as a fluid, amphipathic, bimolecular layer capable of interacting with the aqueous exterior and interior of the cell, the hydrophilic portions of membrane (glyco)proteins, as well as the hydrophobic portions of membrane proteins and cholesterol. The arachidonic acid residing on the second glycerol carbon of some membrane phospholipids serves as substrate for the synthesis of the platelet stimulator, thromboxane A₂. Thus, there is reason to suggest that membrane phospholipids participate in signal transduction in this cell. Therefore, we examined whether the methylation of membrane phospholipids occurs in human platelets and whether it plays a role in the activation of these cells by agonists.

Materials and Methods

Materials

1-[methyl-3H]methionine (12-15 Ci/mole) and [methyl-14C]-choline chloride (49.5 mCi/m mole) were from New England Nuclear, Boston, Mass.; 1-methionine, 1-epinephrine, ADP, and dibutyryl cyclic AMP (Sigma Chemical Co., St. Louis, Mo.); bovine thrombin (Park-Davis, Detroit, Mich.); aspirin, from Aldrich Chemical Co., Milwaukee, Wisc., was recrystallized using ether and petroleum ether; prostaglandin E₁ (PGE₁) (Upjohn Co., Kalamazoo, Mich.); fibrinogen (Kabi AB, Kabi Blood Products Div., Stockholm, Sweden); all phospholipid standards were from Supelco.
Incubation of Platelets With Agonists and Antagonists

Venous blood was obtained from normal volunteers taking no medications, collected in plastic syringes, and anticoagulated with 1/10 vol of 0.13 M trisodium citrate. Platelet-rich plasma was obtained by differential centrifugation. In some experiments, platelets in plasma were labeled with 4C-serotonin, and imipramine (1 μM) was then added to inhibit serotonin reuptake. In experiments with thrombin or A23187, platelets were washed twice and resuspended to 3 x 10^8 platelets/ml in an incubation buffer containing 0.1 M NaCl, 0.002 M MgCl2, 0.001 M EDTA, 0.0055 M dextrose, and 0.05 M Tris-HCl, pH 7.4. White blood cell contamination was < 1 white blood cell/5 x 10^7 platelets. Platelets in buffer were added in 0.5-ml aliquots to polypyrrole tubes and incubated without stirring in a 37°C water bath. After 5 min, reactions were started by adding 1 μCi L-[methyl-3H]methionine or [methyl-4C]choline per 0.5 ml of platelets. Unless indicated otherwise, platelets were incubated for 30 min at 37°C to allow incorporation of labeled methionine or choline into the cells. The extent of incorporation of radiolabel into intact cells was assessed by separating cells from unincorporated label by filtration on Gelman A/E glass-fibre filters and washing filters with 3 x 5 ml of incubation buffer. At 30 min, platelets were activated by adding 10 μl of thrombin or A23187 to each tube with gentle mixing (final concentration of thrombin, 0.005–0.5 μl/mL; A23187, 1 μM). Ten microliters of water were added to control tubes. In some experiments, platelets were incubated with aspirin (10 μM) or dibutyl cyclic adenosine monophosphate (AMP) (3 mM) for 30 and 5 min, respectively, prior to the addition of agonist. At various times, the incubation of tubes containing serotonin-labeled platelets was stopped with 100 mM formaldehyde and 5 mM EDTA, and 4C-serotonin release and incorporation of methyl-3H into platelet lipids was measured. Incubations with radiolabeled methionine or choline were stopped with 0.5 ml of 10% trichloroacetic acid.

In experiments with epinephrine or ADP, platelet-rich plasma was gel-filtered in Tyrode’s buffer and the platelet count adjusted when necessary to 3 x 10^8/ml. Platelets were incubated with radiolabeled methionine as described above for washed platelets. At 30 min, 0.45 ml platelets were added to 1.5-ml capacity square-bottomed, glass aggregation cuvettes containing 50 μl of purified fibrinogen (final fibrinogen concentration 200 μg/ml). Platelets were stirred at 1000 rpm at 37°C and activated by the addition of 5 μl epinephrine (final concentration 25 μM) or ADP (10 μM). Reactions were stopped as for washed platelets for subsequent analyses of 4C-serotonin release and incorporation of methyl-3H into platelet lipids.

Analysis of Platelet Phospholipids

One hour after the addition of trichloroacetic acid, platelet precipitates were extracted for 8 hr with 3 ml chloroform:methanol (2:1). Extracts were washed 3 times with 1.5 ml of 0.1 M KCl in 50% methanol. The yield of platelet phospholipids from trichloroacetic acid precipitates was identical to that which we have obtained previously by extraction of washed platelets directly with either chloroform:methanol or isopropanol:chloroform. To assess radioactivity in total lipids, 0.5 ml of washed extract was added in triplicate to scintillation vials, dried down, and counted for radioactivity in 10 ml of scintillation cocktail (ScintiVerse, Fischer Scientific Co., Fair Lawn, N.J.). Quenching was assessed by the "H-number" method and was found minimal and constant among samples. Efficiency of counting was 49% for 4H and 90% for 14C. As a control, platelets were boiled for 10 min before incubation with radiolabeled methionine. Only 50–60 cpm/0.5 ml platelet lipid extract were found in boiled samples. This was negligible compared to the 500–2000 cpm/0.5 ml lipid extract in a typical experiment and no corrections for this were made.

To assess the quantity of radioactivity associated with the phosphorous content of individual phospholipids, the anti-oxidant 2,3-diter-butyl, 4-methyl phenol was added to the extracts, which were then taken to dryness under nitrogen and resuspended in 100–300 μl chloroform containing 10% ethanol. All solvents used in thin-layer chromatography were of high-performance thin-layer chromatography grade. Thin-layer plates were prewashed with chloroform:ethanol (1:1) and activated for 10 min at 100°C immediately before use. Phospholipids were separated from cholesterol, cholesterol esters, and triglycerides on Whatman LK-5D silica gel G plates. The first mobile phase (hexane:ether:acetic acid, 60:40:1) was allowed to run two-thirds of the way up the plate. After drying, the second mobile phase (hexane:ether:acetic acid, 90:10:1) was allowed to run to within 1 inch of the top of the plate. In this system, phospholipids stayed at the origin, whereas the other lipids migrated from the origin. The various phospholipid classes were separated from each other using two different systems. The first, chloroform:ether:water:triethylamine (30:34:8:35) effected consistent and complete separation of phosphatidylethanolamine, phosphatidyl-N,N-dimethylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysosphosphatidylcholine. However, phosphatidyl-N-monomethylethanolamine migrated with phosphatidylethanolamine in this system. The second system, chloroform:propionic acid:1-propanol:water (20:20:30:10) effected complete separation of phosphatidylethanolamine from phosphatidyl-N-monomethylethanolamine as well as from the other phospholipids. The recovery of radioactivity was 80–100%, depending on the amount of lipid applied to the thin-layer plate. The recovery of lipid phosphorus was >96%. The location and identification of each platelet phospholipid was ascertained either by charring adjacent lanes containing lipid standards or by staining with iodine vapor. To count radioactivity, individual phospholipids were scraped into scintillation vials containing 10 ml scintillation cocktail and counted in a scintillation counter. The phosphorus content of each phospholipid fraction was quantitated. As a control, blank lanes of the thin-layer plate were also scraped and submitted to the same analyses.

Carboxymethylation of platelet proteins was assessed by the method of O’Dea et al.

Statistics

Mean values are expressed ± 1 SEM. The difference between means was examined by Student’s t test for paired data.

RESULTS

Phospholipid Methylation in Platelets

When washed platelets were incubated at 37°C with 0.15 μM 1-[methyl-3H]methionine, they took up 15–20% of the radiolabel after 30 min, at which time the rate of uptake plateaued. Radioactivity was incorporated into platelet lipids in a linear fashion over 90 min. Approximately 1 pmol of methyl-3H was incorporated per 10^6 platelets/hr (Fig. 1). When the concentration of radiolabeled methionine was increased to 20 μM, platelets incorporated into their
lipids about 15 pmole/10⁹ platelets/hr. Essentially all of the radioactivity (>98%) was recovered in the platelet phospholipid fraction, with less than 1% each in the cholesterol and triglyceride fractions. Thin layer chromatography of chloroform-methanol extracts of platelets demonstrated that after a 60-min incubation, radioactivity was found predominantly in those phospholipid fractions that cochromatographed with pure phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine, and phosphatidylcholine (Fig. 2, open bars). Another peak of unidentified radioactivity migrated with the solvent front. Little radioactivity was found in all other phospholipid fractions. These experiments strongly suggest that human platelets are capable of converting phosphatidylethanolamine to phosphatidylcholine via the N-methylation pathway.

**Effect of Thrombin on Phospholipid Methylation**

The incorporation of methyl-³H into platelet lipids from L-[methyl-³H]methionine was then examined in lipids about 15 pmole/10⁹ platelets/hr. Essentially all of the radioactivity (>98%) was recovered in the platelet phospholipid fraction, with less than 1% each in the cholesterol and triglyceride fractions. Thin layer chromatography of chloroform-methanol extracts of platelets demonstrated that after a 60-min incubation, radioactivity was found predominantly in those phospholipid fractions that cochromatographed with pure phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine, and phosphatidylcholine (Fig. 2, open bars). Another peak of unidentified radioactivity migrated with the solvent front. Little radioactivity was found in all other phospholipid fractions. These experiments strongly suggest that human platelets are capable of converting phosphatidylethanolamine to phosphatidylcholine via the N-methylation pathway.
unstirred platelets stimulated with thrombin. In the absence of stirring, thrombin induced secretion by washed platelets but did not induce macroscopic platelet aggregation. Thrombin (1 U/ml) decreased the incorporation of methyl-3H into platelet phospholipids. This effect was detectable as early as 15 sec after the addition of thrombin, and it continued for up to 30 min (Fig. 3). It was observed either when thrombin was added to platelets preincubated for 30 min with radiolabeled methionine (Fig. 3) or when thrombin was added simultaneously with radiolabeled methionine (data not shown). Thrombin decreased the incorporation of methyl-3H into all three reaction products of the methylation pathway (Fig. 2, closed bars). At 30 min, thrombin had decreased the amount of radioactivity incorporated into phosphatidylcholine by 34% ± 2% (SEM) when expressed in terms of cpmm/2 × 10^8 platelets and by 31% ± 2% when expressed in terms of cpmm/μg phosphatidylcholine phosphorous. In contrast with agonist stimulation of some other mammalian cells, no increase in phospholipid methylation relative to control platelets was seen at any time after thrombin addition, even when incorporation of radiolabel was examined as early as 5 sec.

We confirmed the observation of O’Dea et al. that carboxymethylation of proteins occurs in platelets. After the addition of L-[methyl-3H]-methionine (0.4 μM) to intact platelets, carboxymethylation occurred at a rate of 0.13 pmole/10^9 platelets/min and was linear for up to 15 min. However, unlike O’Dea et al., we could detect no effect of thrombin (0.2–2 U/ml) on either the rate or extent of carboxymethylation of platelet proteins.

Several experiments were carried out to characterize further the effect of thrombin on platelet phospholipid methylation. First, thrombin did not impair the uptake of L-[methyl-3H]methionine into platelets (data not shown). Second, platelet lipids were pulse-labeled with methyl-3H for 45 min, after which platelets were washed. Platelets were resuspended in buffer containing a 357-fold excess of unlabeled methionine and then stimulated with thrombin (open circles). Control platelets (closed circles) were handled identically but not stimulated with thrombin. Data represent the means of three experiments.

Third, the synthesis of platelet phosphatidylycerol from [methyl-14C]-choline was examined. In 2 experiments, platelets were incubated with 20 μM radiolabeled methionine or choline, each with the same specific activity (49.5 mCi/mole). After 90 min, platelets had incorporated 13-fold more 14C-choline (357 pmole/10^9 platelets) than methyl-3H (27 pmole/10^9 platelets) into their phospholipids. Ninety percent of the 14C-choline incorporated into platelet lipids was found in the phosphatidylycerol fraction. In further experiments, when platelets were preincubated with the radiolabeled choline for 30 min, thrombin (1 U/ml) decreased the subsequent rate of incorporation of choline into phospholipids (Table 1). Thus, the depression of phosphatidylycerol synthesis by throm-
inhibited by >99% (1 U/ml) thrombin nor prevented the decrease in methylation by thrombin. In contrast, dibutyryl cyclic AMP (3 mM) inhibited serotonin release and thrombin’s effect on methylation for up to 30 min.

Fifth, the extent of decrease of phospholipid N-methylation induced by various concentrations of thrombin was found to correlate strongly with the extent of thrombin-induced platelet secretion (Fig. 5). Moreover, in three experiments, thrombin’s effect on methylation was observed even in the presence of 5 mM EDTA, an amount of chelator sufficient to inhibit completely even microscopic platelet aggregation without inhibiting 3H-serotonin release. Thus, thrombin’s effect on methylation correlates with platelet secretion and does not depend on platelet aggregation.

Effect of Other Agonists and Antagonists on Phospholipid Methylation

One micromolar A23187 stimulated the release of 53.8% ± 1.4% ³H-serotonin from washed, unstirred platelets. After preincubation of platelets with L-[methyl-³H]methionine for 30 min, A23187, like thrombin, decreased the rate and extent of methylation of membrane phospholipids (Table 2).

Platelet aggregation and secretion do not occur in response to the agonists, epinephrine or ADP, unless platelets are stirred in the presence of fibrinogen. When unstirred platelets were suspended in buffer without fibrinogen, epinephrine (25 µM) or ADP (10 µM) caused no platelet aggregation, serotonin release, or alteration of phospholipid N-methylation. However, gel-filtered platelets stirred in the presence of fibrinogen aggregated and released ³H-serotonin in response to epinephrine or ADP. Moreover, under these conditions, epinephrine decreased phospholipid N-methylation (Fig. 6). Similar results were obtained with ADP. The platelet inhibitor, PGE1 (0.27 µM), had no effect on phospholipid methylation when added alone to washed platelets.

DISCUSSION

These studies demonstrate that intact platelets are capable of synthesizing their predominant phospholi-
Others have also found that platelets can synthesize several hundred picomoles of phosphatidylcholine/10^9 platelets/hr via the choline pathway. In addition, Elsbach et al. found that about 3000 pmole of phosphatidylcholine/10^9 platelets/hr can be formed by acylation of exogenous lysophosphatidylcholine. Moreover, 10^9 platelets are capable of releasing several thousand picomoles of phosphatidylcholine/hr with serum lipoproteins. These comparative data, taken together with the fact that 10^9 platelets contain about 200 nmoles of phosphatidylcholine, indicate that in human platelets, the N-methylation pathway for phosphatidylcholine synthesis is a quantitatively minor one.

Although the exact requirements for renewal of phosphatidylcholine during the platelet's lifespan in vivo are unknown, the maintenance of membrane lipid composition would be expected to preserve the functional integrity of platelet membranes as well as preserve membrane fluidity. Perturbations of membrane lipid fluidity are known to affect the function of platelet membranes. It has been demonstrated that the methylated intermediate, phosphatidyl-N-monomethyl ethanolamine, is capable of increasing the fluidity of rat red blood cell membrane lipids. Whether maintenance of a trace level of this lipid might play a role in maintaining the function of platelet membranes or their fluidity within a narrow range is unknown.

In this study, thrombin caused an immediate and sustained decrease in the N-methylation of platelet phospholipids (Fig. 3). This effect appears due to a decrease in synthesis of methylated phospholipids rather than an increase in their degradation (Fig. 4). Decreased phospholipid methylation correlated strongly with serotonin release. Others have shown that platelet secretion by thrombin occurs (1) by a mechanism dependent on platelet aggregation; (2) by platelet prostaglandin synthesis; and (3) by a mechanism independent of either. The biochemical and/or biophysical events involved in these various routes for coupling agonist binding to secretion are incompletely understood. However, all of these are currently thought to eventuate in a shift of calcium from calcium-sequestering membranes into the platelet cytoplasm. In turn, this increase in cytoplasmic calcium is believed to mediate platelet secretion. In our study, inhibition of methylation and stimulation of secretion by thrombin was observed even if platelet aggregation was prevented by EDTA. In addition, thrombin’s effects were observed when platelet cyclooxygenase and prostaglandin endoperoxide synthesis were inhibited by aspirin. These data suggest that if perturbation of the methylation pathway parti-

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**Fig. 6. The effect of epinephrine, 25 μM, on the methylation of platelet phospholipids.** At 5 min, epinephrine had stimulated 30.6% ± 0.9% ^14_C-serotonin release compared to 1.7% ± 0.4% release for the controls. Closed circles depict control platelets, open circles depict platelets incubated with epinephrine. Data represent the means ± SEM of four experiments.

Unstimulated platelets are capable of renewing their pool of phosphatidylcholine by at least three routes: (1) de novo synthesis from glycerol, free fatty acids, and choline; (2) reacylation of lysophosphatidylcholine; and (3) methylation of phosphatidylethanolamine. In addition, a one-for-one exchange of phosphatidylcholine between platelets and serum lipoproteins occurs.

In the present study, when platelets were incubated with 0.15 μM radiolabeled methionine, about 1 pmole of methyl-^3_H was incorporated into the phospholipids of 10^9 platelets after 1 hr (Fig. 1). With 20 μM methionine, about 15 pmole methyl-^3_H were incorporated/10^9 platelets/hr. In comparison, under identical conditions, the incorporation of ^14_C-choline into platelet phospholipids was an order of magnitude greater.
PLATELET PHOSPHOLIPID METHYLATION

Depleted the methylation and decreased the methylation in response to throm-...calcium,38 inhibited both serotonin release and the decreased methylation in response to thrombin.

The fact that epinephrine, ADP, or A23187 also decreased the methylation of phospholipids indicates that thrombin’s effect is not unique. Indeed, just as with thrombin, these compounds decreased methylation under conditions that led to serotonin release. Stimulation of some cell types by agonists has resulted in a transient increase in the incorporation of methyl-3H into membrane phospholipids.4,7,8,10 In contrast, the decrease in methylation induced by platelet agonists is similar to that reported in guinea pig peritoneal macrophages following stimulation of chemotaxis. It should be emphasized that although our experiments suggest some relationship between phospholipid methylation and platelet secretion, there is no direct demonstration that specific perturbation of the methylation pathway actually causes or facilitates secretion. Moreover, the fact that thrombin decreases phosphatidylcholine synthesis as measured by the incorporation of radiolabeled glycerol,26 fatty acids,32 or choline (Table 1) indicates a generalized depression of phosphatidylcholine synthesis during platelet secretion. It is entirely possible that decreased phospholipid methylation results from the process of secretion and does not contribute to its initiation.

Recently, Kannagi et al. found in rabbit platelets that the phosphatidylcholine synthesized via the methylation pathway was relatively rich in arachidonic acid compared to that synthesized via the choline pathway. Current evidence favors hydrolysis of phosphatidylinositol as the main source of arachidonic acid for prostaglandin synthesis in human platelets; however, some evidence also suggests a contribution of phosphatidylcholine. Thus, phosphatidylcholine formed by N-methylation could conceivably be a source of arachidonic acid for protaglandin synthesis, either directly by hydrolysis of phosphatidylcholine by phospholipase A2 or indirectly by supplying arachidonic acid for the resynthesis of phosphatidylinositol after its hydrolysis by phospholipase C and diglyceride lipase.40,43

The present studies do not clarify how agonists decrease the N-methylation of platelet phospholipids. We did not measure the platelet levels of S-adenosyl-L-methionine, the proximate methyl donor for lipid methylation. Although unlikely, the effect of agonists could be explained by a sudden change in pool size affecting the relative amounts of labeled and unlabeled S-adenosyl-L-methionine. We did exclude an effect of thrombin on both the rate of uptake of radiolabeled methionine by the platelet and on the rate at which the methyl donor was used for carboxymethylation of platelet proteins. Phospholipid methylation is also dependent, in part, on the availability of the initial substrate, phosphatidylethanolamine. The content of phosphatidylethanolamine in platelets changes minimally after thrombin stimulation. However, we cannot exclude the possibility that thrombin affects the membrane in such a way as to limit the phosphatidylethanolamine available to the methyl transferase. Alternatively, the decrease of N-methylation by agonists could result from inhibition of methyl transferases within the platelet membrane. Further studies are required to clarify the mechanism of the agonist effect as well as the significance of phospholipid N-methylation in platelet physiology.

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