The Role of Platelet-activating Factor-dependent Transacetylase in the Biosynthesis of 1-Acyl-2-acetyl-sn-glycero-3-phosphocholine by Stimulated Endothelial Cells*

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Acyl analogs of platelet-activating factor (PAF) (1-acyl-2-acetyl-sn-glycero-3-phosphocholine, acylacetyl-GPC) are the predominant products synthesized during thrombin or ionophore A23187-mediated activation of endothelial cells. However, the biosynthetic pathway responsible for the production of acylacetyl-GPC is not well understood. In the present investigation, we have demonstrated that the acyl analogs of PAF are also the major products from calf pulmonary artery endothelial cells in response to a time-dependent stimulation of ATP (10−3 m), bradykinin (10−8 m), or ionophore A23187 (2 μm). In addition, we have found that the CoA-independent PAF:acyllyso-GPC transacetylase recently identified by us is concurrently and transiently induced with maximal 4-fold enhancement at 5 min and returned to near basal level by 10 min treatment of endothelial cells with ATP. Acid phosphatase reduces the increased PAF:acyllyso-GPC transacetylase activity from the homogenates of ATP-activated endothelial cells. Reduced PAF:acyllyso-GPC transacetylase activity can be restored by incubating the acid phosphatase-treated homogenates with ATP (5 mM) and Mg2+ (10 mM). Furthermore, okadaic acid, a protein phosphatase 1 and 2A inhibitor, incubated with endothelial cells in a dose-dependent manner (1–100 nM) for 10-min potentiates and sustained the stimulation of PAF:acyllyso-GPC transacetylase activity by ATP. On the other hand, genistein, tyrphostin-25 (inhibitors of tyrosine-specific protein kinase), and calphostin C (an inhibitor of protein kinase C) block the activation of PAF:acyllyso-GPC transacetylase by ATP. These results are consistent with the notion that ATP regulates the transacetylase activity by reversible activation and inactivation via the phosphorylation and dephosphorylation cycle. ATP also augments the activities of alkyllyso-GPC/acyllyso-GPC:acetyl-CoA acetyltransferase. However, the activation of the acetylttransferases precedes that of the transacetylase with peak activation occurring at 1–2 min of the ATP treatment. In addition, sodium vanadate, also an inhibitor of protein phosphatase, stimulates the increase in the incorporation of [3H]acetate into acyl[3H]acetate-GPC of the ATP-treated endothelial cells. Collectively, our data show that both acetylttransferases and transacetylase particpate in and contribute to the biosynthesis of acyl analogs of PAF in a coordinate fashion in endothelial cells.

Platelet-activating factor (PAF)1 is a potent lipid mediator with diverse pathophysiological responses such as inflammation, allergic reactions, and reproduction (see reviews Refs. 1–3). Different cell types from various species activated by specific stimuli are able to produce PAF (4). The chemical structure of PAF is originally elucidated as 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkylacyl-GPC, 5–7). However, it recently becomes apparent that acylacetyl-GPC can be the predominant product in certain cell types. For instance, the major compound produced by human umbilical vein endothelial cells activated with thrombin and ionophore A23187 (8–11) or bovine pulmonary artery endothelial cells stimulated by ionophore A23187 (12) is acylacetyl-GPC. Also, depending on the agonist used, either alkylacyl-GPC or acylacyl-GPC can be synthesized within the same cell type. For example, the human basophils generate mainly acylacetyl-GPC in response to anti-IgE and mostly alkylacyl-GPC in response to ionophore A23187 (13).

Although the biological activities of acylacetyl-GPC are >500-fold less potent than alkylacyl-GPC in lowering the blood pressure of spontaneous hypertensive rats, in releasing serotonin from rabbit platelets (14), in increasing the intracellular Ca2+ concentrations, and in activating microtubule-associated protein-2 kinase activity (15), however, acylacyl-GPC can act as a specific noncompetitive inhibitor of alkylacyl-GPC-induced activation of the human neutrophils and leukotriene C4 release from the human leukocytes (10, 16). Furthermore, 1-acyl analogs of PAF at nM concentrations can prime polymorphonuclear leukocytes for enhanced O2 production after stimulation with fMet-Leu-Phe or human recombinant C5a (17). Additionally, acylacetyl-GPC has the ability to decrease the susceptibility of the low density lipoprotein particles to oxidative modification mediated by copper ions, monocytes, or endothelial cells, whereas PAF has no effect (18).

The pathways leading to the accumulation of acylacyl-GPC are not completely understood. It is suggested that the remodeling pathway (3) is responsible for the biosynthesis of alkylacyl-GPC/acylacyl-GPC during short term stimulation by agonists in endothelial cells (19). The first step of the remodeling pathway involved the conversion of alkylarachidonoyl-PC/acylarylachidonoyl-GPC to alkyllyso-GPC/acyllyso-GPC by a putative phospholipase A2 or/and a CoA-independent transacylase.

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1 The abbreviations used are: PAF, platelet activating factor; GPC, glycero-3-phosphocholine; PLA2, phospholipase A2; HBSS, Hank’s balanced salt solutions; Me2SO, dimethyl sulfoxide; DRB, 5,6-dichloro-1-h-d-ribofuranosylbenzimidazole.

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Transacylase and Biosynthesis of Acyl Analogs of PAF

Phospholipid metabolism in stimulated cells is a complex process involving the de novo synthesis of acyl analogs of PAF. The biosynthesis of acyl analogs of PAF in agonist-activated endothelial cells initiates the production and controls the level of acyl analogs of PAF. Based on the temporal relationships that exist between the biosynthesis of acyl analogs of PAF in agonist-activated endothelial cells and the level of acyl analogs of PAF, peritoneal macrophages that preferentially synthesize acyl analogs of PAF contain only one-sixth of the lysophospholipase activity in comparison to that of alveolar macrophages which primarily make PAF. Besides, the amounts of acylacyl-GPC produced within a specific cell type can be increased by blocking the phenylmethylsulfonyl fluoride-sensitive phospholipase A₄ or lysophospholipase activity.

We have previously identified a novel CoA-independent and PAF-dependent transacylase that transfers the acetic group from PAF to a variety of lysophospholipids; acylacyl-GPC is the most active acceptor that converts to acylacyl-GPC by the transacylase (36). In the present studies, we investigate the possibility that PAF:acyllyso-GPC transacylase is induced severelfold during agonist-coupled activation of the endothelial cells and is most likely to be regulated via covalent modification of the enzyme through phosphorylation and dephosphorylation. Based on the temporal relationships that exist between the synthesis of acylacyl-GPC and induction of the transacylase activity in stimulus-activated cells, our findings suggest that the PAF-dependent transacylase participates in the production and controls the level of acylacyl-GPC in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Hexadecyl-2-[3H]acetyl-GPC (7.1 Ci/mmol), [3H]acetate (1.9 Ci/mmol), and [3H]acyethyl-CoA (1.54 Ci/mmol) were purchased from NEN Life Science Products. Hexadecylacetyl-GPC, hexadecyllyso-GPC, palmitoyllyso-GPC, acetyl-CoA, phospholipase C C type V from Bacillus cereus, acid phosphatase from potato, ionophore A23187, thrombin, bradykinin, ATP, AGT, genistein, and tyrophostin 25 were the products of Sigma. Sodium salt of okadaic acid, calphostin C, KT5720, H-89 dihydrochloride, and 5,6-dichloro-1-ß-d-ribofuranosylbenzimidazole (DRB) were from Calbiochem. Alkylacyl-GPC and oleoylacyl-GPC were obtained from Avanti Polar Lipids Inc. All cell culture reagents were from Life Technologies, Inc.

**Cell Culture**—Calf pulmonary artery endothelial cells (CCL-209) were obtained from the American Type Culture Collection and grown in Eagle’s minimum essential medium with 20% fetal bovine serum. Cells were cultured in 75-cm² flasks, and only the confluent monolayers between passages 20 and 25 were used for experiments. Unless indicated otherwise, the cells were washed twice with 10 ml of Hank’s balanced salt solutions (HBSS) before starting the experiments.

**Determination of the Rate of Incorporation of [3H]Acetate into Radyl[3H]acetyl-GPC and Its Subclasses**—Washed monolayers of calf pulmonary artery endothelial cells were incubated with 25 μCi of [3H]acetate in the presence of 10 ml of HBSS, 10 mM Hepes (pH 7.4), and with or without agonist, thrombin (2 units/ml), bradykinin (10⁻⁸ M), ATP (10⁻⁸ M), or ionophore A23187 (2 μM) at 37°C for various times as indicated in the figures. At the end of incubations, the media were removed, and the cells were rinsed twice with 5 ml of HBSS, 10 mM Hepes (pH 7.4) before scraping into 3 ml of methanol. The thin layer chromatography solvent system of chloroform/methanol/acetic acid/H₂O (50:35:8.6, v/v/v/v) was used to isolate the radyl[3H]acetyl-GPC fraction. Radioactivity in thin layers separated by thin layer chromatography (TLC) was determined by area or zonal scraping of the silica gel into vials for liquid scintillation counting.

The purity of the isolated radyl[3H]acetyl-GPC was further confirmed by using a TLC solvent system of chloroform/methanol/concentrated NH₄OH/H₂O (60:35:8.2, v/v/v/v). The purified samples of radyl[3H]acetyl-GPC that were treated with phospholipase C to form radyl[3H]acetylglycerols were derivatized with benzoic anhydride. The benzoyl derivatives of radyl[3H]acetylglycerols were separated into acylacyl-, alkylacetyl-, and alk-1-enacylacylglycerol benzoates on silica G TLC plates as described (38). The radioactivities in each subclass were determined by liquid scintillation spectrometry.

**Enzyme Assays**—PAF:acylacyl-GPC transacylase and PAF:lyso-plasmalogen transacylase were determined as reported earlier by us (36) with minor modifications. Briefly, standard incubations consisted of 50 μM PAF (0.5 μCi in 50 μl of 0.1% bovine serum albumin/saline), 300 μM palmitoyllyso-GPC or lysosphalomalogen (in 50 μl of 0.1% bovine serum albumin/saline), 5 mM EDTA, 1 mM sodium acetate, 100 mM Tris-HCl (pH 7.4), and 100 μg of homogenate protein in a final volume of 0.50 ml. The incubations were carried out at 37°C for 15 and 30 min to ensure that the enzyme activity is measured at quasi-steady state. The cell homogenates were prepared by scraping agonist-treated or nontreated cells from the surface of the flasks into 2 ml of homogenizing buffer (0.25 M sucrose, 100 mM Tris-HCl (pH 7.3), 1 mM MgCl₂, 1 mM dithiothreitol, 50 mM NaF). The cell suspensions were homogenized by using an ultrasonic cell disruptor (Microson™) at 30% power output for 15 s × 5 times. The extracted lipid product (>95% as radyl[3H]acetyl-GPC) was treated with phospholipase C. The resulting radyl[3H]acetylglycerols were converted to their respective benzoyl derivatives and analyzed by a combination of TLC and liquid scintillation counting as described in the above section.

Alkylacyl-GPC/acyethyl-CoA acetyltransferase and PAF acetylhydrolase activities were measured according to the methods we previously described (39, 40) except that the cell homogenates were used as the enzyme sources in most of the experiments. The protein content of the enzyme preparations was determined by the method of Lowry et al. (41).

**RESULTS**

Incorporation of [3H]Acetate into Radyl[3H]acetyl-GPC and Its Subclasses in Agonist-activated Endothelial Cells—To study the possible involvement of PAF-dependent transacylase in the biosynthesis of acylacyl-GPC of endothelial cells, it was necessary to establish in our laboratory that the stimulus-treated endothelial cells produced acyl analogs of PAF as the predominant product. We chose bovine pulmonary artery endothelial cells as a model system for the convenience. Data in Fig. 1A showed that the incorporation of [3H]acetate into radyl[3H]acetyl-GPC was increased in a time-dependent fashion with noticeable rise at 1 min and approaching plateau at 10 min, when the endothelial cells were incubated with ATP (10⁻³ M). Treatment of endothelial cells with bradykinin (10⁻⁸ M) or ionophore A23187 (2 μM) yielded similar time course responses (data not shown). However, the increase in radylacylacyl-GPC synthesis was minimal in thrombin-treated (2 units/ml) endothelial cells (data not shown). These results were consistent with the findings demonstrated by Garcia et al. (12). They showed that 1 μM bradykinin or 10 μM ionophore A23187 enhanced the [3H]acetate incorporation into radylacyl-GPC in bovine pulmonary artery endothelial cells. Besides, our data were similar to that reported by Whatley et al. (42) in that cultured endothelial cells from various bovine blood vessels responded to ATP, bradykinin, or ionophore A23187 but not to thrombin stimulation in generating an increase in PAF production.
Minary artery endothelial cells were incubated with ATP and 25 Ci of ATP-stimulated endothelial cells (data not shown). The kinetics of the time-dependent incorporation of [3H]acetate into radylacetyl-GPC were similar among the three agonists tested. On the other hand, the kinetic of the time-dependent incorporation of [3H]acetate into the subclasses of radylacyethyl-GPC from experiments in A were converted to benzoylated derivatives, and the radioactivities in each subclasses were analyzed as described under “Experimental Procedures.” A duplicate experiment showed similar results.

Garcia et al. (12) described that when calf pulmonary artery endothelial cells were treated with ionophore A23187 (10 nM), the primary product formed under the conditions was acyl analogs of PAF rather than PAF. We found that acyl analogs of PAF were also the major product when calf pulmonary artery endothelial cells were stimulated with ATP (approximately 85% as acylacetyl-GPC) (Fig. 1B). Bradykinin (about 67% as acylacetyl-GPC), or ionophore A23187 (nearly over 80% as acylacetyl-GPC) (data not shown). The kinetics of the time-dependent incorporation of [3H]acetate into the subclasses of radylacyethyl-GPC were similar among the three agonists tested. On the other hand, the kinetic of the time-dependent incorporation of [3H]acetate into the subclasses of radylacyethyl-GPC treated with thrombin was different from that treated with ATP, bradykinin, or ionophore A23187. It is possible that the kinetics of the time-dependent incorporation of [3H]acetate in thrombin-treated endothelial cells reflected the turnover of the endogenous subclasses of radylacyethyl-GPC, since bovine pulmonary artery endothelial cells did not respond to thrombin activation.

Induction of PAF:acyllyso-GPC Transacylase Activity by ATP and Its Mechanism.—Since ATP caused the highest stimulation on the incorporation of [3H]acetate into radyl[3H]acyethyl-GPC among the physiological agonists we tested, ATP was chosen as the agonist in the subsequent experiments. Fig. 2 depicted that ATP induced a rapid and transient increase in PAF:acyllyso-GPC transacylase activity with maximal activation at 5 min. The activity of PAF:acyllyso-GPC transacylase returned to near basal levels after 10 min of ATP treatment. The increase in transacylase activity at 5 min incubation of ATP with endothelial cells was about 4-fold over that in the untreated cells.

It was unlikely that the increase in PAF:acyllyso-GPC transacylase activity by ATP was due to de novo protein synthesis, because the induction of the transacylase activity by ATP was a rapid process (within 2 min). Therefore, we investigated the possibility that the activity of PAF:acyllyso-GPC transacylase may be regulated by ATP through covalent modifications, specifically through phosphorylation and dephosphorylation. When the homogenates of ATP-stimulated endothelial cells were treated with an acid phosphatase from potato for 15 min, a decrease of 61% in transacylase activity was observed (Fig. 3, 2nd column versus 1st column). The percent of decrease in transacylase activity depended on the concentrations of acid phosphatase used and the lengths of the preincubation times of the homogenates with acid phosphatase (data not shown). Furthermore, preincubation of the homogenates with boiled acid phosphatase (at 100 °C for 10 min) had no effect on the transacylase activity (Fig. 3, 3rd column versus 1st column). On the other hand, addition of ATP (5 mM) plus MgCl2 (10 mM) to the homogenates of ATP-stimulated endothelial cells (Fig. 3, 4th column versus 1st column) or to the homogenates of ATP-stimulated endothelial cells that were pretreated with acid phosphatase (Fig. 3, 5th column versus 2nd column) could further potentiate the transacylase activity. These data from in vitro experiments suggested that the transacylase activity is activated/inactivated through phosphorylation/dephosphorylation.

To further substantiate the fact that the transacylase activity is regulated through phosphorylation and dephosphorylation, several following experiments using whole cell systems were performed.

Endothelial cells were preincubated with various concentrations of okadaic acid (1 to 100 nM) for 30 min and then incubated with ATP (10 nM) for an additional 10 min. The activity of transacylase was closed to near basal levels when endothelial cells were treated with ATP for 10 min (see Fig. 2). Okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A (43), induced a 2-fold increase in PAF:acyllyso-GPC transacylase activity over that of the 10-min ATP-treated control (Fig. 4). Optimal concentrations of okadaic acid that potentiated the ATP-augmented transacylase activity occurred between 10 and 100 nM (Fig. 4). Therefore, these results were consistent with the notion that by blocking the dephosphorylation of the proteins with okadaic acid, the transacylase activity is amplified. A different protein phosphatase inhibitor, sodium orthovanadate (1 mM), likewise sustained and
prolonged the activation of transacetylase in calf pulmonary artery endothelial cells treated with ATP for 10 min (data not shown).

When endothelial cells were preincubated with a selective inhibitor of tyrosine-specific protein kinase, tyrphostin-25 (44, 45) for 10 min, and incubated with ATP for additional 5 min, tyrphostin-25 blocked the induction of PAF:acyllyso-GPC transacetylase activity by ATP in a dose-dependent manner with close to total inhibition at 50 μM (Fig. 5). Similar results were obtained when another inhibitor of tyrosine-specific protein kinase, genistein (46), was used (data not shown). These data provided additional support that PAF:acyllyso-GPC transacetylase activity is controlled by reversible activation and inactivation through phosphorylation and dephosphorylation.

To gain insight as to which of the protein kinase(s) may be involved in the activation of PAF:acyllyso-GPC transacetylase besides tyrosine-specific protein kinase, we tested the effects of inhibitors for three different kinds of protein kinases, namely protein kinase C, protein kinase A, and casein kinase II, on both PAF:lysoplasmalogen transacetylase and PAF:acyllyso-GPC transacetylase activities (Table I). Based on the results we obtained previously from the mixed substrate experiments, the transfer of the acetate group from PAF to either lysoplasmalogen or acyllyso-GPC is catalyzed by a single enzyme (47). Therefore, it is not surprising that the effects of various protein kinase inhibitors are similar for both PAF:lysoplasmalogen transacetylase and PAF:acyllyso-GPC transacetylase activities (Table I). Only calphostin C, a specific inhibitor for protein kinase C, exerted inhibitory effect on both transacetylase activities, whereas the inhibitors for protein kinase A (KT 5720 or H-89) and casein kinase II (DRB) did not block neither ATP-induced transacetylase activities. Thus, our data suggest that tyrosine protein kinase and protein kinase C are directly or indirectly involved in the activation of the transacetylase activity through protein phosphorylation.

To determine which of the amino acids (i.e. tyrosine, serine, and/or threonine) in the transacetylase is/are phosphorylated by the protein kinase(s), we carried out several experiments similar to that described by Whitman et al. (48) to show the phosphorylation of tyrosine in the phosphatidylinositol 3-kinase. When ATP-treated or control untreated endothelial cell lysates were prepared and immunoprecipitated with polyclonal antibody against either phosphotyrosine, phosphoserine, or phosphothreonine (Zymed Laboratories Inc.) using a combined method specified by Whitman et al. (48), Domin et al. (49), and manufacturer’s instructions, our preliminary data showed that the PAF:lysoplasmalogen transacetylase activity in ATP-treated endothelial cell lysates was enriched in part in the immunocomplex precipitated by anti-phosphotyrosine antibody in comparison to that of the untreated control endothelial cell lysates. Similarly, the PAF:lysoplasmalogen transacetylase activity was higher in anti-phosphotyrosine-treated immunoprecipitate of the ATP-stimulated endothelial cell lysates than that in antigen affinity purified IgG antibody precipitated immunocomplex of the same cell lysates (a kind gift from Dr. Steve Kennel, Oak Ridge National Laboratory). These results suggest that PAF:lysoplasmalogen transacetylase is phosphorylated by a tyrosine kinase. However, we are currently attempting to purify the transacetylase and prepare the antibody against the purified protein to conclusively address the questions concerning the specific protein kinase(s) and the phosphorylation site of the amino acids involved in the activation of the transacetylase.

A Protein Phosphatase Inhibitor, Sodium Vanadate, Potenti-
Alkyllyso-GPC/Acyllyso-GPC:Acetyl-CoA Acetyltransferases in Activated Endothelial Cells—To determine the role of alkyllyso-GPC/acyllyso-GPC:acytely-CoA acetyltransferases in the synthesis of acylacetyl-GPC, we measured the $K_m$ and $V_{max}$ values, and the time course of induction by ATP for the acetyltransferases in the membrane fraction of the calf pulmonary artery endothelial cells. The $V_{max}$ was only slightly less when palmitoyllyso-GPC was the substrate in comparison with that when hexadecyllyso-GPC was the substrate (7.6 nmol/min · mg protein versus 9.2 nmol/min · mg protein). However, the acetyltransferase had a slightly higher substrate affinity for the palmitoyllyso-GPC than that of hexadecyllyso-GPC (8.3 versus 9.1 μM). Additionally, both alkyllyso-GPC:acytely-CoA acetyltransferase and acyllyso-GPC:acytely-CoA acetyltransferase in the homogenates of calf pulmonary artery endothelial cells responded to a time-dependent ATP activation with maximal increase within 1–2 min after the addition of ATP (Fig. 6).

Effect of ATP on the PAF Acetylhydrolase Activity in Endothelial Cells—Since the incubations of $[^3H]$acetate and ATP with endothelial cells were short durations (1–10 min), the rates of incorporation of $[^3H]$acetate into radyl$[^3H]$acytely-GPC that we measured should reflect mostly the rates of synthesis of radyl$[^3H]$acytely-GPC. Nevertheless, we did assess the possible effects ATP may have on the PAF acetylhydrolase activity. Results (data not shown) indicated that ATP had minimal effect on the PAF acetylhydrolase activity.

**DISCUSSION**

We have established that ATP (10$^{-3}$ M), bradykinin (10$^{-8}$ M), and ionophore A23187 (2 μM) stimulate the incorporation of $[^3H]$acetate into radyl$[^3H]$acytely-GPC.
We and others (19, 23, 24, 58) have shown that alkyllyso-GPC:acyt-CoA acyltransferase can use acyllyso-GPC as a substrate, but at a reduced rate (50%), to generate acylacetyl-GPC. This is consistent with the findings reported in this work except that the differences in V_{max} between alkyllyso-GPC:acyt-CoA acyltransferase and acyllyso-GPC:acyt-CoA acyltransferase are less in calf pulmonary artery endothelial cells than in other cell systems previously observed. Further studies are needed to discern the possibility for the existence of two isoforms of acyltransferase. Importantly, based on the kinetic patterns of [3H]acetate incorporation into acyl[3H]acyclic-GPC and the inductions of acetylated transferases and transacylase activities by ATP, and the potentiation effect of sodium vanadate on the increased synthesis of acylacyl-GPC by ATP (Figs. 1, 2, and 6 and Table II), our studies establish that activations of alkyllyso-GPC:acyt-CoA acyltransferase, acyllyso-GPC:acyt-CoA acyltransferase, and PAF:acylacyl-GPC transacylase by ATP contribute to the stimulated synthesis of acylacyl-GPC in calf pulmonary artery endothelial cells.

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