The Bioactive Phospholipid, Lysophosphatidylcholine, Induces Cellular Effects via G-Protein-dependent Activation of Adenylyl Cyclase*

(Received for publication, January 5, 1996, and in revised form, August 2, 1996)

Yuping Yuan‡§, Simone M. Schoenwaelder‡, Hatem H. Salem‡, and Shaun P. Jackson‡¶
From the ‡Department of Medicine, Monash Medical School, Box Hill Hospital and the ¶Department of Pathology, Box Hill Hospital, Victoria, Australia 3128

The naturally occurring phospholipid, lysophosphatidylcholine (lyso-PC), regulates a broad range of cell processes, including gene transcription, mitogenesis, monocyte chemotaxis, smooth muscle relaxation, and platelet activation. Despite the growing list of cellular effects attributable to lyso-PC, the mechanism(s) by which it alters cell function have not been elucidated. In this report, we have examined the effects of exogenous lyso-PC on signal transduction processes within a variety of lyso-PC-responsive cells, including human platelets, monocyte-like THP-1 cells, and the megakaryoblastic cell line, MEG-01. Pretreatment of each of these cells with increasing concentrations of lyso-PC (25–150 μg/ml) was associated with a progressive increase in the cytosolic concentration of cAMP. The accumulation of cAMP in platelets correlated closely with the ability of lyso-PC to inhibit multiple platelet processes, including platelet aggregation, agonist-induced protein kinase C activation, thromboxane A2 generation, and the tyrosine phosphorylation of platelet proteins. In each of the cell types examined, the ability of lyso-PC to increase the cellular levels of cAMP was synergistically enhanced by pretreating the cells with the cAMP phosphodiesterase inhibitor, theophylline (5 mM), and specifically inhibited by the P-site inhibitor of adenyl cyclase, 2,5-dideoxyadenosine. A role for the stimulatory G-protein, Gs, in the lyso-PC-induced activation of adenylyl cyclase was suggested by the ability of the GTPase inhibitor, guanyl-5’-thiophosphate (0.2 mM), to inhibit the lyso-PC-stimulated increase in cAMP, and also by the ability of cholera toxin to inhibit increases in membrane GTPase activity in response to lyso-PC. The functional significance of lyso-PC-induced activation of adenylyl cyclase was investigated in MEG-01 cells. Treatment of these cells with either lyso-PC or dibutyryl cAMP for 36–40 h resulted in a 3–5-fold increase in the surface expression of the natural anticoagulant protein, thrombomodulin (TM). The ability of lyso-PC to increase TM expression was abolished by pretreating these cells with the adenyl cyclase inhibitor, 2,5-dideoxyadenosine, whereas the dibutyryl cAMP-induced increase in TM remained insensitive to adenyl cyclase inhibition. These studies define an important role for the adenylyl cyclase signaling system in mediating cellular effects induced by lyso-PC.

* This work was funded by a grant from the National Health and Medical Research Council of Australia. 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: Dept. of Medicine, Monash Medical School, Box Hill Hospital, Box Hill, Victoria, Australia 3128. Tel.: 61-3-9895-0311; Fax: 61-3-9895-0332.

The hydrolysis of phospholipids by phospholipase A2 (PLA2) generates free fatty acids and lysophospholipids. While free fatty acids act as potential second messengers (1, 2) and as precursors for biologically active eicosanoids (3), the role of lysophospholipids in cellular physiology remains unclear. The natural phospholipid, lysophosphatidylcholine (lyso-PC), has recently emerged as an important mediator of the atherogenic effects of oxidized low density lipoprotein (4, 5). This lysophospholipid constitutes as much as 40% of the total lipid content of oxidized low density lipoprotein (6) and has been detected in elevated levels in atherosclerotic lesions from animals fed an atherosclerotic diet (7). In vitro, lyso-PC has multiple atherogenic effects. It is mitogenic for murine macrophages and has been postulated to play a critical role in oxidized low density lipoprotein-induced foam cell formation (8). Lyso-PC also induces endothelial cell gene expression of multiple growth factors involved in atherogenesis and inflammation (9). Furthermore, it can up-regulate the surface expression of endothelial cell adhesion molecules involved in the recruitment of mononuclear leukocytes into the arterial intima (4).

In addition to its atherogenic effects, there is growing evidence that in certain clinical settings the extracellular generation of lyso-PC by secretory PLA2 may promote tissue inflammation and induce hemostatic disturbances (10–12). Lyso-PC’s proinflammatory effects include its ability to alter the adhesive properties of endothelial cells, induce chemotaxis of monocytes (5) and T-lymphocytes (13), and stimulate T-cell (14) and macrophage activation, leading to enhanced Fc-mediated phagocytosis (15, 16). Lyso-PC also has vasoactive properties, stimulating endothelium-dependent vascular smooth muscle relaxation (17) and impairing endothelial-regulated vasomotor control (18). Furthermore, this lipid has potent platelet inhibitory effects and is essential for the inhibition of platelet aggregation by a subset of secretory PLA2 (12).

A major outstanding issue regarding lyso-PC pathophysiology is the mechanism(s) by which this polar phospholipid alters cell function. Other naturally occurring phospholipids, such as lysophosphatidic acid (lyso-PA), appear to evoke cellular responses (19), through specific G protein-coupled receptor(s) linked to the activation of phospholipase C and inhibition of adenylyl cyclase (20, 21). Adenylyl cyclase is a ubiquitous signaling system that is activated by a variety of hormones, neurotransmitters, cyclic AMP (cAMP), and G proteins. Both cAMP and the G proteins are involved in atherogenic processes, including gene transcription, mitogenesis, monocyte chemotaxis, smooth muscle relaxation, and platelet activation. Despite the growing list of cellular effects attributable to lyso-PC, the mechanism(s) by which it alters cell function have not been elucidated. In this report, we have examined the effects of exogenous lyso-PC on signal transduction processes within a variety of lyso-PC-responsive cells, including human platelets, monocyte-like THP-1 cells, and the megakaryoblastic cell line, MEG-01. Pretreatment of each of these cells with increasing concentrations of lyso-PC (25–150 μg/ml) was associated with a progressive increase in the cytosolic concentration of cAMP. The accumulation of cAMP in platelets correlated closely with the ability of lyso-PC to inhibit multiple platelet processes, including platelet aggregation, agonist-induced protein kinase C activation, thromboxane A2 generation, and the tyrosine phosphorylation of platelet proteins. In each of the cell types examined, the ability of lyso-PC to increase the cellular levels of cAMP was synergistically enhanced by pretreating the cells with the cAMP phosphodiesterase inhibitor, theophylline (5 mM), and specifically inhibited by the P-site inhibitor of adenyl cyclase, 2,5-dideoxyadenosine. A role for the stimulatory G-protein, Gs, in the lyso-PC-induced activation of adenylyl cyclase was suggested by the ability of the GTPase inhibitor, guanyl-5’-thiophosphate (0.2 mM), to inhibit the lyso-PC-stimulated increase in cAMP, and also by the ability of cholera toxin to inhibit increases in membrane GTPase activity in response to lyso-PC. The functional significance of lyso-PC-induced activation of adenylyl cyclase was investigated in MEG-01 cells. Treatment of these cells with either lyso-PC or dibutyryl cAMP for 36–40 h resulted in a 3–5-fold increase in the surface expression of the natural anticoagulant protein, thrombomodulin (TM). The ability of lyso-PC to increase TM expression was abolished by pretreating these cells with the adenyl cyclase inhibitor, 2,5-dideoxyadenosine, whereas the dibutyryl cAMP-induced increase in TM remained insensitive to adenyl cyclase inhibition. These studies define an important role for the adenylyl cyclase signaling system in mediating cellular effects induced by lyso-PC.

† The abbreviations used are: PLA2, phospholipase A2; lyso-PC, lysophosphatidylcholine; lyso-PA, lysophosphatidic acid; BSA, bovine serum albumin; PGE2, prostaglandin E2; TXA2, thromboxane A2; TXB2, thromboxane B2; G protein, GTP-binding protein; TM, thrombomodulin; GDPβS, guanyl-5’-yl thiophosphate; PKC, phosphate kinase C; MOPS, 4-morpholinopropanesulfonic acid; FACS, fluorescence-activated cell sorter.
Signal Transduction Induced by Lyso phosphatidylcholine

27091

Lyso-PC has been demonstrated to regulate the activity of a number of intracellular signaling enzymes, including protein kinase C, as a necessary step for T-cell activation and possibly monocyte chemotaxis (14, 31). Lyso-PC directly stimulates guanylate cyclase enzyme activity and inhibits adenyl cyclase in 3T3 mouse fibroblast membrane preparations (32), indicating a potential role for this lysophospholipid in the co-ordinated regulation of cyclic nucleotide second messengers. In other studies, thrombin stimulation of the prostaglandin synthesis in mouse fibroblasts and seminal vesicle microsomes was attenuated by exogenous lyso-PC, suggesting a role for this lysophospholipid in the regulation of prostaglandin synthesis (33). Although these studies suggest that the generation of lyso-PC within the cell can directly regulate the activity of a variety of signaling enzymes, they provide limited information on the signal transduction processes stimulated by extracellular lyso-PC.

In this study, we have examined the signaling events induced by lyso-PC in a range of lyso-PC-responsive cells. Our results indicate that lyso-PC specifically stimulates the activation of adenyl cyclase in human platelets, THP-1 and MEG-01 cell lines, leading to the production of cAMP. The increase in cellular cAMP correlated with the lyso-PC-mediated inhibition of platelet activation, and increased surface expression of thrombomodulin on MEG-01 cells. These studies suggest that the cellular effects induced by extracellular lyso-PC may be mediated, at least in part, by the adenyl cyclase signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—All lysophospholipids, phosphatidylcholine, GTP, GDP, GDP, GTP, and phosphatidylcholine E (PGE), were purchased from Sigma. Collagen was from Chrono-Log Corp., Havertown, PA, and bovine thrombin was from Parke-Davis Co., Morris Plains, NJ. (γ-32P)GTP, [32P]GTP, and [32P]phosphatidylcholine were from DuPont NEN, Wilmington, DE. Choler toxin was from ICN Biomedicals, Costa, Mesa, CA. Anti-phosphotyrosine monoclonal antibody (mAb 4G10) was from Upstate Biotechnology Inc. Anti-phosphotyrosine monoclonal antibody (mAb PY20) was supplied by ICN Biomedicals Inc.

Preparation of Washed Platelets—Platelets were collected from healthy volunteers who had not taken anti-platelet medication for 2 weeks and washed according to a modified method of Baenziger and Majerus (34). Blood was collected in acid-citrate-dextrose, 6.1 (v/v), containing 90 mM sodium citrate, 7 mM citric acid, pH 4.8, 140 mM dextrose, supplemented with 70 mM theophylline. Platelet-rich plasma was centrifuged by 1,200 g for 20 min, and the platelet-rich plasma was centrifuged by 1,200 g for 20 min, and the platelet-rich plasma was subjected to 12.5% SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The gel was fixed, dried, and autoradiographed.

Anti-phosphotyrosine Immunoblots—Washed platelets were stained in the presence of 0.05 unit/ml thrombin or platelet resuspension buffer for 5 min. The cells were lysed with SDS-reducing buffer and immediately boiled for 10 min. After electrophoresis and transfer to polyvinylidene difluoride membranes, immunoblots of the platelet extracts were performed using the anti-phosphotyrosine antibody.
RESULTS

To investigate the mechanism(s) by which lyso-PC regulates cell function, we examined signal transduction processes in a number of lyso-PC-responsive cells, including human platelets, monocyte-like THP-1 cells, and the megakaryoblastic cell line, MEG-01. Platelets are a particularly useful cell model for studying signal transduction processes, as these cells undergo rapid functional and morphological changes in response to stimulation, and the signaling processes regulating these cell processes have been investigated in considerable detail. Furthermore, these cells are responsive to several bioactive lipids, including platelet activating factor (37), lyso-PA (38), and lyso-PC (12). Both platelet activating factor and lyso-PA stimulate platelet aggregation via receptor-linked signaling pathways (19, 20, 37), while lyso-PC inhibits platelet function by an as yet undefined mechanism (12). In our initial studies, we investigated the effects of exogenous lyso-PC on signal transduction processes in human platelets.

**Lysophosphatidylcholine Inhibits Agonist-induced Platelet Aggregation and Activation of Protein Kinase C, TXA2 Generation, and the Tyrosine Phosphorylation of Multiple Platelet Proteins**—Stimulation of platelets by a range of agonists is associated with the activation of cytosolic phospholipase A2, leading to the hydrolysis of membrane phospholipids and the subsequent generation of prostaglandin endoperoxides and thromboxane A2 (TXA2) (3). We examined the effect of lyso-PC on TXA2 synthesis, by measuring the production of its stable metabolite thromboxane B2 (TXB2) in collagen-activated platelets. In these studies, we observed a close correlation between the inhibition of collagen-induced platelet aggregation by increasing concentrations of lyso-PC (12.5–100 μM), and the inhibition of TXB2 generation (Fig. 1A, lanes 3–6). Increasing the concentration of collagen (5–20 μg/ml), while maintaining a constant concentration of lyso-PC (100 μM), overcame the platelet inhibitory effects of lyso-PC and lead to increased production of TXB2 (Fig. 1A, lanes 7–10). Qualitatively similar results were obtained in thrombin-stimulated platelets (data not shown).

Although lyso-PC has well described toxic effects on cells at increased concentrations of lyso-PC required to inhibit platelet aggregation when higher concentrations of agonist were used. C, effect of lyso-PC on the thrombin-stimulated increase in protein tyrosine phosphorylation. Washed platelets (3 × 10^9/ml) were incubated for 5 min at 37°C with: 1) Tyrode’s buffer; 2) 1 unit/ml thrombin alone; 3) 0.65 unit/ml thrombin, or in the presence of increasing concentrations of lyso-PC; 4) 10; 5) 20; 6) 40; 7) 60; or 8) 100 μg/ml. Lane 9 represents platelets incubated with 100 μg/ml lyso-PC in the absence of thrombin. At the end of the incubation period, platelets were freeze-thawed then boiled in SDS-reducing buffer. The platelet proteins were then separated using 12.5% SDS-polyacrylamide gel electrophoresis, and the 32P-labeled proteins identified by autoradiography. These results are from one experiment, representative of three. It should be noted that in this particular experiment 40 μg/ml lyso-PC did not abolish p47 phosphorylation nor platelet aggregation, which contrasts with collagen-stimulated platelets (1 μg/ml). This difference is a reflection of the
high concentrations (39) due to its natural detergent properties, several lines of evidence suggest that its inhibitory effects on platelet function are unlikely to be due to membrane lysis. First, the extracellular concentration of lactose dehydrogenase did not increase following pretreatment of the cells with lyso-PC (12.5–150 μg/ml). Second, the morphology of lyso-PC-treated platelets, as observed with phase-contrast microscopy, remained indistinguishable from untreated cells. Finally, the ability of high concentrations of collagen (20 μg/ml) and thrombin (0.2 unit/ml) to overcome the platelet inhibitory effects of lyso-PC, confirms the functional integrity of these cells.

Previous studies have reported a role for free fatty acids and lyso-PC in the activation of protein kinase C (PKC) (1, 2, 14, 31). We therefore examined the effect of lyso-PC on PKC activation in resting and thrombin-stimulated platelets, by measuring the phosphorylation of the PKC substrate, p47. Pretreatment of platelets with lyso-PC alone (100 μg/ml) had minimal effect on the phosphorylation status of p47 (Fig. 1B, lane 9). In contrast, doses of thrombin (0.05 unit/ml) which can activate PKC independent of TXA2 production (40, 41), induced prominent p47 phosphorylation (Fig. 1B, lane 3). Concentrations of lyso-PC which inhibited thrombin-induced aggregation (60–100 μg/ml) abolished p47 phosphorylation (Fig. 1B, lanes 7 and 8). In all experiments there was a close correlation between the inhibition of p47 phosphorylation and the abolition of thrombin-induced platelet aggregation. Furthermore, as with TXB2 generation, the inhibitory effects of lyso-PC on PKC activation could be completely reversed by increasing the concentration of thrombin (data not shown). These studies indicate that the platelet inhibitory effects of lyso-PC are not confined to the inhibition of TXA2 generation.

In addition to activating serine/threonine kinases, thrombin stimulation of platelets also leads to the activation of multiple non-receptor tyrosine kinases, leading to the tyrosine phosphorylation of multiple platelet proteins. These phosphorylation events are considered to play an important role in regulating platelet functional responses (42). We therefore examined the effects of lyso-PC on the level of protein tyrosine phosphorylation in human platelets. Immunoblot analysis of resting platelet lysates using anti-phosphotyrosine antibodies, revealed the presence of tyrosine-phosphorylated proteins of 109, 75, 67, and 60 kDa (Fig. 1C, lane 1). Pretreatment of platelets with lyso-PC (10–100 μg/ml) alone did not induce the tyrosine phosphorylation of platelet proteins (data not shown). In contrast, thrombin stimulation (0.05 unit/ml) of platelets led to an increase in the level of phosphorylation of the 109, 75, 67, and 60 kDa proteins, along with the appearance of additional minor bands at 124, 118, 112, 100, 87, 57, and 46 kDa (Fig. 1C, lane 2). Concentrations of lyso-PC (50 or 100 μg/ml) (Fig. 1C, lanes 4 and 5) or PGE1 (200 ng/ml) (Fig. 1C, lane 3), which negated platelet aggregation, inhibited the thrombin-stimulated tyrosine phosphorylation of these platelet proteins. These same concentrations of lyso-PC also abolished the thrombin-induced aggregation (data not shown). Collectively, these studies indicate that lyso-PC treatment of platelets leads to a global and reversible down-regulation of multiple signal transduction pathways required for platelet activation.

Effect of Lyso-PC on the Platelet Cytosolic Concentration of cAMP—Activation of adenyl cyclase is associated with the enzymatic production of the soluble second messenger, cAMP, from cellular ATP. cAMP in turn regulates a myriad of cell processes by modulating the catalytic activity of protein kinase A (23, 43). This serine-threonine kinase phosphorylates a range of cellular proteins and is responsible for attenuating all signaling events required for platelet activation (26, 44). We therefore investigated whether the inhibition of platelet function by lyso-PC may be due to the cellular accumulation of cAMP. Consistent with this possibility, treatment of platelets with lyso-PC caused a dose-dependent increase in cAMP (Fig. 2A), which correlated closely with its ability to inhibit platelet aggregation (data not shown). The maximal increase in the cytosolic cAMP concentration was obtained using 150 μg/ml lyso-PC, which resulted in a 5.5-fold increase in cAMP levels from 1.2 to 6.7 pmol/10^8 platelets. This level of increase in the intracellular concentration of cAMP was specific to lyso-PC, as similar concentrations of phosphatidylcholine, lysophosphatidic acid, lysophosphatidylglycerol, lysophosphatidylethanolamine, and lysophosphatidylserine had only a minor effect on cAMP levels (Fig. 2A). Furthermore, this effect was not selec-
tive for platelets, as a similar dose-dependent increase in cAMP was observed in two other lyso-PC-responsive cell lines, the monocyte-like cell line, THP-1, and the megakaryoblastic cell line, MEG-01 (Fig. 2B).

**Lyso-PC Increases the Intracellular Concentration of cAMP by Activating Adenylyl Cyclase**—The intracellular level of cAMP is dynamically regulated by adenylyl cyclase and cAMP phosphodiesterase (22, 26, 44). To determine whether the cellular accumulation of cAMP in response to lyso-PC was due to increased synthesis or reduced metabolism of the second messenger, we examined the effects of the phosphodiesterase inhibitor, theophylline, or the P-site inhibitor of adenylyl cyclase, 2,5-dideoxyadenosine, on the lyso-PC-induced increase in cAMP. Inhibition of cAMP phosphodiesterase by saturating concentrations of theophylline (10 mM) led to a modest increase in the cellular level of cAMP (Fig. 3A) (30). In contrast, when platelets were stimulated with PGE1 (200 ng/ml), in the presence of theophylline (10 mM), there was a dramatic increase in the cellular concentration of cAMP. Similarly, the lyso-PC induced increase in cytosolic cAMP in human platelets, THP-1, and MEG-01 cells, was synergistically enhanced by theophylline, suggesting activation of adenylyl cyclase as the primary mechanism by which lyso-PC increases intracellular cAMP. Further evidence supporting this hypothesis stems from the observation that when near saturating concentrations of PGE1 (200 ng/ml) and lyso-PC (100 μg/ml) were combined, the cytosolic concentration of cAMP was not elevated above that achieved with PGE1 alone.

To confirm that the cellular accumulation of cAMP was due to increased generation, rather than decreased metabolism of cAMP, we pretreated MEG-01 cells and human platelets with 2,5-dideoxyadenosine, prior to exposing the cells to lyso-PC. This inhibitor reduced the lyso-PC- and PGE1-stimulated increase in cAMP by 60–70% in both platelets and MEG-01 cells (data not shown). It is likely that the inability of 2,5-dideoxyadenosine to completely abolish the increase in cAMP in intact cells was due to its incomplete access to the intracellular pool of adenylyl cyclase, as the pretreatment of isolated platelet membranes with 2,5-dideoxyadenosine, completely inhibited the lyso-PC-stimulated increase in cAMP (Fig. 3B).

**A Role for the Stimulatory G-protein, Gs, in the Lyso-PC-induced Activation of Adenylyl Cyclase**—Adenylyl cyclase can be activated directly, by agents such as forskolin (45, 46), or via the receptor-linked stimulatory G-protein, Gs or βγ (22, 47). We investigated the possibility that Gs may be required for the lyso-PC-induced activation of adenylyl cyclase. For these studies, we examined the ability of the specific GTPase inhibitor, GDPβS, to block the lyso-PC-stimulated production of cAMP from isolated platelet membranes. Platelet membranes were exposed to either lyso-PC or PGE1, in the presence of theophylline (10 mM), and the level of cAMP determined, as described under “Experimental Procedures.” As shown in Fig. 4A, in the presence of theophylline (10 mM), both PGE1 (200 ng/ml) and lyso-PC (100 μg/ml) increased the generation of cAMP from intact platelet membranes approximately 5-fold. GDPβS (0.2 mM) alone had minimal effect on cAMP levels. However, the same concentration of GDPβS dramatically reduced the PGE1 and lyso-PC-stimulated production of cAMP. In control experiments, concentrations of GDPβS up to 1.2 mM had no inhibitory effect on forskolin’s ability to stimulate adenylyl cyclase.

To strengthen our hypothesis further that Gs plays a critical role in inducing the activation of adenylyl cyclase by lyso-PC, we examined the ability of this lysosolipid to stimulate platelet mem-
theophylline for 10 min at 37°C in the presence of buffer (500 mlyso-PC-induced activation of adenylyl cyclase. Platelet membranes (500 μg/ml) containing 5 mg/ml BSA, were incubated with 10 mM theophylline for 10 min at 37°C in the presence of buffer (Cont),

**FIG. 4.** Role of the stimulatory G-protein, Gs, in the lyso-PC-induced activation of adenylyl cyclase. A, effect of GDPβS on the lyso-PC-induced activation of adenylyl cyclase. Platelet membranes (500 μg/ml) containing 5 mg/ml BSA, were incubated with 10 mM theophylline for 10 min at 37°C in the presence of buffer (Cont),

GDPβS (0.2 mM) (GDPβS), PGE1 (200 ng/ml), PGE2 (200 ng/ml) and GDPβS (0.2 mM) (PGE1 + GDPβS), lyso-PC (100 μg/ml) (LPC), lyso-PC (100 μg/ml) and GDPβS (0.2 mM) (LPC + GDPβS), forskolin (1 μm) (Forsk) or forskolin (1 μm) and GDPβS (1.2 mM) (Forsk + GDPβS). The membranes were sedimented by centrifugation and the supernatant assayed for cAMP, as described under "Experimental Procedures." Results represent the mean ± S.E. from four experiments. B, effect of lyso-PC on platelet membrane GTPase activity. Platelet membranes (500 μg/ml) were incubated with the indicated concentrations of lyso-PC (●) or Triton X-100 (○) for 30 min at 37°C in the presence of [γ-32P]GTP. The non-hydrolyzed GTP was extracted with activated charcoal and released 32P quantitated as described under "Experimental Procedures." GTPase activity was expressed as a % of the activity of non-stimulated platelet membranes. Results were from one experiment, representative of four. C, effect of cholera toxin on the lyso-PC-induced activation of membrane GTPase activity. Platelet membranes were treated with or without activated cholera toxin as described under "Experimental Procedures." GTPase assays were performed on the treated membranes in the presence of lyso-PC (50 μg/ml), PGE1 (200 ng/ml), or Triton X-100 (0.4%). Results represent the mean ± S.E. of four experiments.
Results represent the mean ± S.E. from four experiments. Incubation of these cells with lyso-PC (20–40 μg/ml) for 36–40 h resulted in a 3–5-fold increase in the surface expression of TM. Pretreatment of the cells with 2,5-dideoxyadenosine inhibited the lyso-PC-stimulated increase in cAMP by approximately 75% and dramatically reduced the up-regulation of TM expression (Fig. 6). It is unlikely that the effects of 2,5-dideoxyadenosine on TM expression were due to the inhibition of cell growth or survival, as the total number of viable cells was no different in control or 2,5-dideoxyadenosine-pre- treated cells (data not shown). Furthermore, in control studies, the addition of 2,5-dideoxyadenosine failed to inhibit the up-regulation of TM by dibutyryl-cAMP, confirming that the cellular effects of 2,5-dideoxyadenosine are likely to be due to the inhibition of adenyl cyclase.

**DISCUSSION**

While there is growing evidence that lyso-PC plays an important role in mediating the atherogenic effects of oxidized low density lipoprotein and the proinflammatory effects of secretory phospholipase A₂, the mechanism by which this natural lysolipid alters cell function has remained elusive. The studies reported in this article indicate that non-toxic concentrations of lyso-PC stimulate the cytosolic accumulation of cAMP in a number of lyso-PC-responsive cells. Studies with specific inhibitors of adenyl cyclase and cAMP phosphodiesterase clearly demonstrate that the lyso-PC-stimulated increase in intracellular cAMP involves the activation of adenyl cyclase, rather than the inhibition of cAMP phosphodiesterase.

Several lines of evidence indicate that the synthesis of cAMP is important for the induction of specific cellular effects by lyso-PC. First, the ability of lyso-PC to reversibly inhibit all platelet responses, including shape change, serotonin release, and platelet aggregation, is consistent with the intracellular effects of elevated cAMP. Furthermore, cAMP accumulation causes a down-regulation of all signaling processes linked to platelet activation, including PKC activation, protein tyrosine phosphorylation, and thromboxane A₂ generation. Second, the similar dose-response curves for inhibition of platelet aggregation by lyso-PC and PGE₁ indicate that the stimulation of adenyl cyclase is likely to be the predominant mechanism by which this lysolipid inhibits platelet function. Third, the increased surface expression of thrombomodulin on MEG-01 cells in response to lyso-PC was specifically inhibited by the adenyl cyclase inhibitor, 2,5-dideoxyadenosine. While these studies do not exclude a role for other signaling pathways in mediating the cellular effects of lyso-PC, they demonstrate that activation of adenyl cyclase is at least one means by which this polar phospholipid regulates cell function. In this regard, PKC activation has previously been suggested as a mechanism by which lyso-PC may mediate its cellular effects (14, 31). Although PKC is a well characterized activator of adenyl cyclase in certain cell types, it is unlikely that this mechanism operates in platelets, as lyso-PC appears to down-regulate PKC activity in these cells. In endothelial cells, signaling pathways uncoupled from PKC activation appear to be important for the lyso-PC-induced up-regulation of cell adhesion receptors (4) and for the enhanced gene expression of nitric oxide synthase (30). It is...
tempting to speculate that the adenyl cyclase signaling system may be involved in modulating these cellular events, as cAMP is a well characterized regulator of gene transcription, and has previously been demonstrated to regulate the transcription of several lyso-PC-responsive genes, including thrombomodulin and cyclo-oxygenase II (29, 50).

The mechanism by which non-toxic concentrations of lyso-PC activate adenyl cyclase is clearly an important issue for future study. There are conflicting reports in the literature regarding the effects of lyso-PC on the enzyme activity of adenyl cyclase. Lysoospholipids have recently been shown to directly activate yeast adenyl cyclase in the absence of other membrane components (28). In contrast to our results, this study found that the stimulation of yeast adenyl cyclase only occurred in the presence of very high concentrations of lyso-phospholipids. Using multiple cell types, we have demonstrated that low concentrations of lyso-PC (12.5 to 50 μg/ml) can activate mammalian adenyl cyclase leading to the cellular accumulation of cAMP. The actual concentration of free lyso-PC in our experiments is likely to be much lower than 25 mg/ml as each experiment was performed in the presence of 5 mg/ml albumin which has a high affinity for lyso phospholipids (51). Direct comparison of yeast and mammalian adenyl cyclase is complicated by the fact that there are several major differences between these two enzymes. The yeast enzyme is not an integral membrane protein and is structurally dissimilar from mammalian adenyl cyclase (23, 28). Furthermore, the yeast enzyme is associated with the membrane via its interaction with the GTPase-activating protein IRA1 and can be readily solubilized with high concentrations of salt, whereas the solubilization of mammalian adenyl cyclase requires detergent treatment of membranes. Extraction of adenyl cyclase from its lipid component by these conditions completely abolished its catalytic activity, precluding any direct examination of the effects of lyso-PC on mammalian adenyl cyclase. Despite this, two independent lines of evidence suggest that the effects of lyso-PC on mammalian adenyl cyclase is unlikely to be due to a direct effect of this lipid on the enzyme but rather an indirect effect through the activation of Gs. First, the lyso-PC-stimulated increase in adenyl cyclase activity could be abolished with the G-protein inhibitor, GDPβS, and second, pretreatment of platelet membranes with lyso-PC was able to stimulate an increase in membrane GTPase activity. This increase is likely to involve activation of Gs, as cholera toxin was able to substantially reduce this increase in membrane GTPase activity. An additional study by Shier et al. (32) demonstrated that lyso-PC at concentrations greater than 1 mg/ml markedly inhibited sodium fluoride-stimulated adenyl cyclase activity in 3T3 mouse fibroblast membranes, whereas concentrations less than 100 μg/ml had a minimal effect on adenyl cyclase. The inhibitory effects of high concentrations of lyso-PC on mammalian adenyl cyclase are not surprising given the detergent properties of this lysolipid. The reason why lower concentrations of lyso-PC did not stimulate adenyl cyclase in these studies is not immediately apparent, although it may reflect cell type specific differences.

A number of phospholipid-derived bioactive messengers have now been demonstrated to mediate cellular effects through the activation of G-proteins. The simplest natural phospholipid, lysophosphatidic acid (19–21, 38), and the phospholipid, platelet-aggregating factor (37), induce cell changes through putative G-protein coupled receptors on the cell surface, while the products of arachidonic acid metabolism, prostaglandin L2 and E1, mediate their cellular effects through specific G-protein-coupled receptors linked to the activation of adenyl cyclase (23, 47, 52). Our studies indicate that lyso-PC should be added to this list of lipid messengers which alter cell function through the activation of G-proteins. While the precise mechanism by which lyso-PC activates Gs is unclear from our studies, it is possible that lyso-PC may intercalate into the membrane lipid bilayer and directly activate Gs, in a receptor-independent manner, or alternatively, lyso-PC may bind to and activate a specific membrane surface receptor coupled to Gα. The exact mechanism by which lyso-PC stimulates intracellular cAMP synthesis remains to be defined and will ultimately require reconstitution of the response with purified components.

Acknowledgments—We thank Dr. Christina Mitchell for helpful discussions, Dr. Phil Bird for the generous donation of the anti-thrombomodulin antibody, and Shane Gray and Anna Romeo for assistance with FACS analysis.

REFERENCES

1. McPhail, L. C., Clyton, C. C., and Snyderman, R. (1984) Science 224, 622–625
2. Tetsutaro, S., Yoshinoro, A., Mashahiro, O., Kimihisa, Y., and Yasutomi, N. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5149–5153
3. Needleman, P., Turk, J., Jakshchuk, B. A., Morrison, A. R., and Lefkowith, J. B. (1986) Ann. Rev. Biochem. 55, 69–102
4. Kume, N., Cymbalyuk, M. I., and Gimbrone, M. A., Jr. (1992) J. Clin. Invest. 90, 1138–1144
5. Quinon, M. T., Parthasarathy, S., Fong, L. G., and Steinberg, D. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2995–2998
6. Parthasarathy, S., Steinbrecher, R. P., Barnett, J., Witzum, J. C., and Steinberg, D. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3000–3004
7. Portman, O. W., and Alexander, M. D. (1989) J. Lipid Res. 30, 158–165
8. Sakai, M., Miyazaki, A., Hakamata H., Sasaki, T., Uyi, S., Yamazaki, M., Shiichi, M., and Hiroshi S. (1994) J. Biol. Chem. 269, 31430–31435
9. Kume, N., and Gimbrone, M. A., Jr. (1994) J. Clin. Invest. 93, 907–911
10. Vadus, P., and Pruzanski, W. (1986) Lab. Invest. 55, 391–403
11. Schroder, T. S., Kivilaakso, E., Kinnunen, P. K. J., and Lempiinen, M. (1980) Scand. J. Gastroent. 15, 633–638
12. Yuan, Y., Jackson, S. P., Newnham, H. H., Mitchell, C. A., and Salem, H. L. (1995) Blood 86, 4166–4174
13. McMurray, H. F., Parthasarathy, S., and Steinberg, D. (1993) J. Clin. Invest. 92, 1094–1098
14. Asaoka, Y., Oka, M., Yoshida, K., Sasai, Y., and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6447–6451
15. Nygrenya, B. Z., and Yamamoto, N. (1990) Proc. E. S. M. B. 183, 118–124
16. Yamamoto, N., Homma, S., and Millman, L. (1991) J. Immunol. 147, 273–280
17. Saito, T., Wolf, A., Menon, N. K., Saeed, M., and Bing, R. J. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 8226–8230
18. Kugiyama, K., Korns, S. A., Morriset, J. D., Roberts, R., and Henry, P. (1990) Nature 344, 160–162
19. Moelenaar, W. H. (1995) J. Biol. Chem. 270, 12849–12862
20. Van Corven, E. J., Groenink, A., Janik, E., Hessel, T., and Moelenaar, W. H. (1989) Cell 59, 45–54
21. Van der Bend, R. L., Brunner, J., Jalink, K., van Corven, E. J., Moelenaar, W. H., and van Blitterswijk, W. J. (1992) EMBO J. 11, 2485–2501
22. Logothetis, D. E., Hildebrandt, J. D., and Iyengar, R. (1995) Science 268, 1166–1169
23. Brass, L. F. (1988) Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology, pp. 441–492, Alan R. Liss, Inc., New York
24. Kroll, M. H., and Schara, A. I. (1989) Blood 74, 1181–1195
25. Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanai, F., Northup, J., and Wigler, M. (1985) Cell 61, 763–769
26. Resnick, R. J., and Tomasselli, G. (1994) J. Biol. Chem. 269, 22336–22341
27. Zembowicz, A., Jones, S. L., and Wu, K. K. (1995) J. Clin. Invest. 96, 1688–1692
28. Valone, F. H. (1988) Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemistry, and Pathology, pp. 319–340, Alan R. Liss, Inc., New York
29. Benten, A. M., Gerrard, J. M., Michiel, T., and Kindom, S. E. (1982) Blood 60, 642–649
30. Weltsen, H. U. (1979) Biochim. Biophys. Acta 559, 259–287

Y. Yuan, S. M. Schoenwelder, H. H. Salem, and S. P. Jackson, unpublished observations.
40. Sweatt, J. D., Blair, I. A., Cragoe, E. J., and Limbird, L. E. (1986) J. Biol. Chem. 261, 8660–8666
41. Sweatt, J. D., Connolly, T. M., Cragoe, E. J., and Limbird, L. E. (1986) J. Biol. Chem. 261, 8667–8673
42. Golden, A., and Brugge, J. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 901–905
43. Walsh, D. A., and Van Patten, S. M. (1994) FASEB J. 8, 1227–1236
44. Holmsen, H. (1987) in Platelet Responses and Metabolism (Holmsen, H., ed) Vol. III, pp. 52–68, CRC Press, Boca Raton, FL
45. Seamon, K., and Daly, J. W. (1981) J. Biol. Chem. 256, 9799–9801
46. Seamon, K. B., Padgett, W., and Daly, J. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3363–3367
47. Insel, P. A., Stengel, D., Ferry, N., and Hanoune, J. (1982) J. Biol. Chem. 257, 7485–7490
48. Tsai, S.-C., Adamik, R. Moss, J., Vaughan, M., Manne, V., and Kung, H.-F. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8310–8314
49. Casey, P., and Gilman, A. G. (1988) J. Biol. Chem. 263, 2577–2580
50. Ito, T., Ogura, M., Morishita, Y., Takamatsu, J., Maruyama, I., Yamamoto, S., Ogawa, K., and Saito, H. (1990) Thromb. Res. 58, 615–624
51. Ulrich, K. H. (1981) Pharmacol. Rev. 33, 17–54
52. Dutta-Roy, A. K., and Sinha, A. K. (1987) J. Biol. Chem. 262, 12685–12691
The Bioactive Phospholipid, Lysophosphatidylcholine, Induces Cellular Effects via G-Protein-dependent Activation of Adenylyl Cyclase
Yuping Yuan, Simone M. Schoenwaelder, Hatem H. Salem and Shaun P. Jackson

J. Biol. Chem. 1996, 271:27090-27098.
doi: 10.1074/jbc.271.43.27090

Access the most updated version of this article at http://www.jbc.org/content/271/43/27090

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 28 of which can be accessed free at http://www.jbc.org/content/271/43/27090.full.html#ref-list-1