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A ROLE OF LYSO-PHOSPHATIDYLCHOLINE IN GM3-DEPENDENT INHIBITION OF EPIDERMAL GROWTH FACTOR RECEPTOR AUTOPHOSPHORYLATION IN A431 PLASMA MEMBRANES

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SUMMARY: EGF-dependent receptor autophosphorylation (EDRA) in A431 plasma membrane was specifically stimulated by lysophospholipids having phosphorylcholine head group (e.g., lyso-phosphatidylcholine; lyso-PC), but not other lysophospholipids, in the absence of detergent. In contrast, GM3 specifically inhibited EDRA under the same experimental conditions in which lyso-PC stimulated EDRA. This GM3-dependent inhibition was more efficient in the absence (vs. presence) of a detergent (Triton X-100). These results indicate an essential role of lyso-PC in GM3-regulated EGF receptor functions.

GM3 ganglioside (sialosyllactosylceramide; II^{3}NeuAcLacCer) is a common, ubiquitous component in most mammalian cells. A remarkable functional role of this ganglioside is its ability to inhibit EGF - or platelet-derived growth factor-dependent autophosphorylation of its receptor (1,2). Enrichment through exogenous addition of GM3 *in vivo* has been shown to inhibit cell growth, possibly through blocking of growth factor receptor function (3). EGF receptor, solubilized and immunoprecipitated with anti-receptor antibody, is associated with GM3 (3), indicating that the receptor is non-covalently but stably bound to the GM3 molecule. Thus, physical and functional association of GM3 and the EGF receptor has been increasingly clear. However, EGF-dependent receptor autophosphorylation (EDRA) *per se*, and its inhibition by GM3 *in vitro*, have only been demonstrated in the presence of detergent (e.g., Triton X-100), which promotes catalytic reactions in mixed micellar conditions. Data presented here clearly show that lysophospholipids containing phosphorylcholine head group, but not

Abbreviations used are: EDRA, EGF-dependent receptor autophosphorylation; EGF, epidermal growth factor; PAF, platelet-activating factor; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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other lysophospholipids, enhanced EDRA in a manner similar to that of Triton X-100. Interestingly, under the same conditions in which stimulation by lysophospholipids was observed, EDRA was strongly inhibited by a lower dose of GM3 than in the conditions with Triton X-100. These results suggest a novel functional role of lyso-PC as a catalytic modulator in GM3-regulated EGF receptor functions.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (4.5 Ci/μmol) was purchased from ICN (Irvine, CA). ^{125}I-EGF (178.4 μCi/μg) was purchased from NEN Research Products (Boston, MA). Human EGF (recombinant) was a generous gift of Earth Pharmacological Co. (Osaka, Japan). Triton X-100 (10% solution) was from Pierce (Rockford, IL).

Lipid preparation. Sphingosine, lyso-PI, lyso-PS, lyso-PE, lyso-PCs, PMME, PDME, lyso-PAF, and lyso-SM were purchased from Sigma. Lyso-SM was further purified by TLC using HPTLC plate (EM Science, Gibbstown, NJ) and developed in CM/acetic acid/water (50:30:8:4). Lyso-PMME and lyso-PDME were prepared by treatment of PMME and PDME with phospholipase A_2 (from bee venom; Sigma) (4). Dimethylsphingosine was prepared from commercially-obtained D-erythrosphingosine as previously described (5). Phospholipids were quantitatively determined as previously described (6), using vitamin C as a reducing agent.

EGF-dependent receptor autophosphorylation (EDRA). ^{32}P autophosphorylation of EGF receptor in purified plasma membranes was carried out essentially according to previously-described procedures (2,3,5). Briefly, plasma membranes (5 μg protein/ tube) were incubated in the reaction buffer (20 mM Hepes, pH 7.4, 1 mM $MnCl_2$, 1 mM $ZnCl_2$, 30 μM Na_3VO_4) with or without human EGF (200 nM) or Triton X-100 (0.05%) in the presence of various lysophospholipids for 20 min at 25°C in plastic conical tubes (1.5 ml, Sarstedt, Princeton, NJ). Introduction of lysophospholipids in the reaction mixture is as follows. Various amounts of lysophospholipids dissolved in CM 2:1 were pipetted into the bottom of conical tubes, evaporated gently to dryness under weak N_2 gas flow. The dried samples were dispersed in the reaction buffer with Vortex mixer and then with sonication at warm temperature for 30 min. The lipid solutions were collected to the bottom of conical tubes by slight centrifugation (minicentrifuge, Beckmann). The reaction was started by addition of 5 μM [α-^{32}P]ATP (10 μCi/tube), which was the optimum concentration in this assay system, and the mixture was incubated for 5 min at 4°C (on ice). The final reaction volume was 50 μl. The reaction was terminated by addition of Laemmli's sample buffer (x5) containing 100 mM EDTA, which was added to avoid non-enzymatic phosphorylation during heating of sample (7). The mixture was heated for 3 min at 100°C, and aliquots were subjected to 8% SDS-PAGE (8). In order to reduce serine or threonine O-phosphate, the gels were fixed and then treated with 1 M NaOH at 50°C for 1 hr as previously described (9). The treated gel was dried, and phosphorylation was visualized by autoradiography with Kodak O-Mat films. For the radioactive quantification, the region containing the EGF receptor (Mr 170 kDa) was excised from the gel and ^{32}P radioactivity was determined by liquid scintillation counter. Background values (usually $<10\%$ of sample) were subtracted from observed values.

RESULTS

Specific stimulation of EDRA in A431 plasma membranes by lysophospholipids having phosphorylcholine head group. EDRA was greatly stimulated by lyso-PC in a

dose-dependent manner in the 50-200 μM range, but not by lyso-PE, lyso-PS, or lyso-PI at concentrations up to 300 μM (Figs. 1 and 2). Lyso-SM showed maximal effect at 100 μM and less effect at higher concentrations. In contrast, lyso-PDME showed a weak

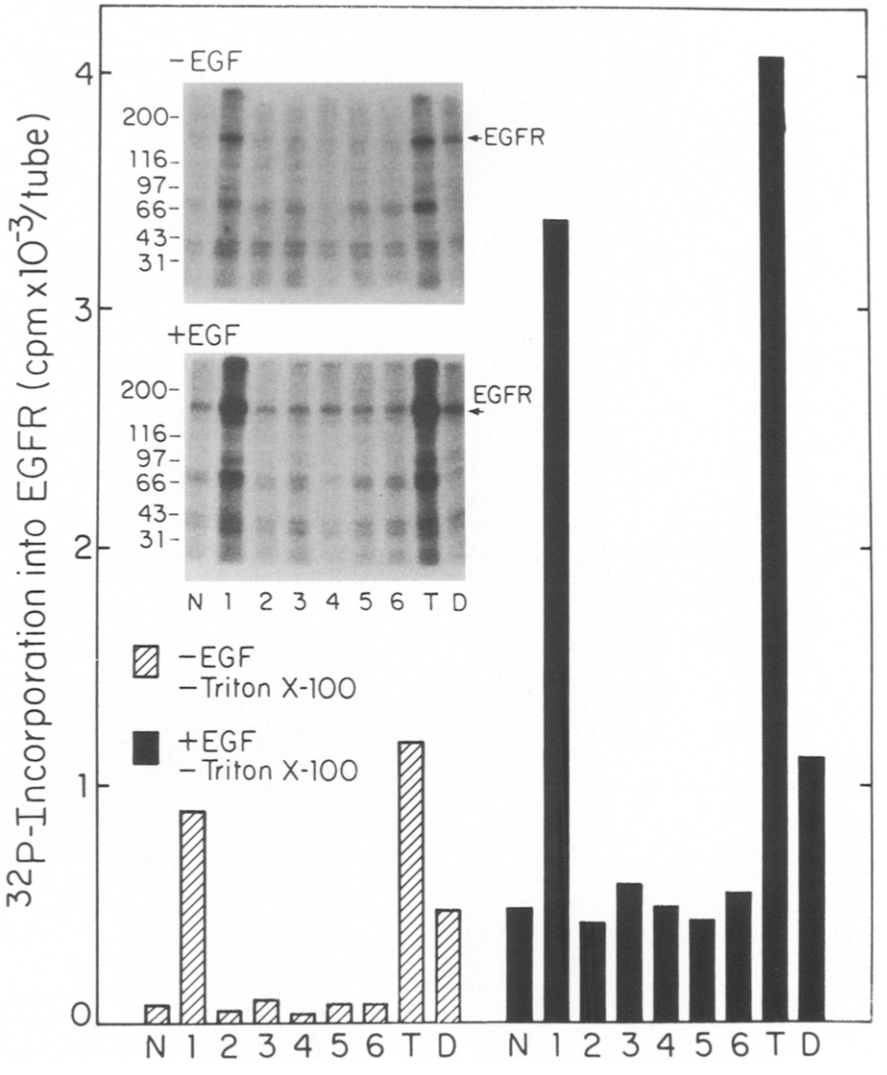

Figure 1. Effect of various phospholipids and lysophospholipids on EDRA in purified A431 plasma membranes

Plasma membranes were purified from cultured A431 cells and phosphorylation assay was performed in the absence of Triton X-100, except for lane T. Membranes were incubated in the presence or absence of human EGF (0.2 μM) for 20 min at room temperature, and then with 5 μM [γ-^{32}P]ATP (10 μCi/tube) for 5 min at 0°C in the presence or absence of lipid samples. Reaction was stopped by addition of Laemmli's sample buffer containing 20 mM EDTA. Aliquots were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gel. The gel was treated with 1 M NaOH at 50°C for 1 hr, ^{32}P incorporation was visualized by autoradiography, and the portion containing EGF receptor was excised and counted. Arrow indicates location of EGF receptor (Mr 170K). Molecular weight standards are shown x10^{-3}. Lane N, no addition (control); lane 1, lyso-PC; lane 2, lyso-PE, lane 3, lyso-PS; lane 4, lyso-PI; lane 5, PC; lane 6, PE; lane T, 0.05% Triton X-100; lane D, 200 μM dimethylsphingosine.

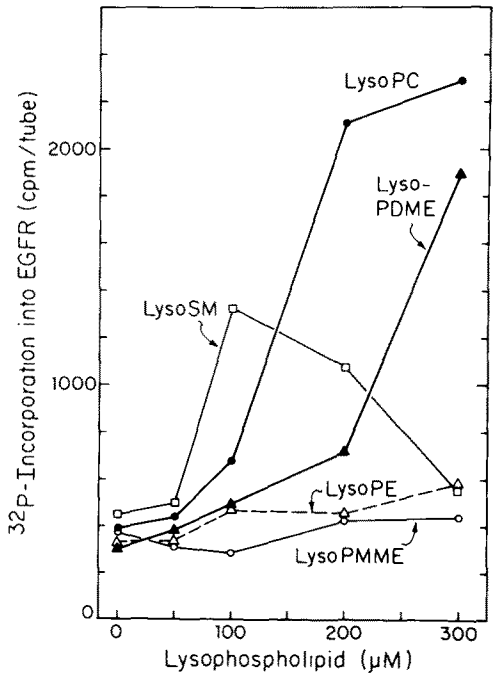

Figure 2. Dose dependency of lysophospholipid effects on EDRA

Experiments were performed as described in Materials and Methods and Fig. 1 legend, at various concentrations of lysophospholipids in the presence of 0.2 μM human EGF.

TABLE I. Effects of various lysophospholipids on EDRA in the presence and absence of EGF

Addition		Concentration (μM)	EDRA (% of control) −EGF	+EGF
None (control)			100	100
lyso-phosphatidic acid		200	110	125
lyso-PE		200	56	82
lyso-PS		200	127	110
lyso-PI		200	44	92
lyso-PC	myristate	200	475	525
	palmitate	100*	315	256
	stearate	100*	140	170
	oleate	200	250	340
lyso-PAF		50	177	323
		200	258	660
lyso-PMME		200	–	105
lyso-PDME		200	–	250
lyso-SM		100*	–	275

* optimal concentration of stimulation for these lysophospholipids was 100 μM.

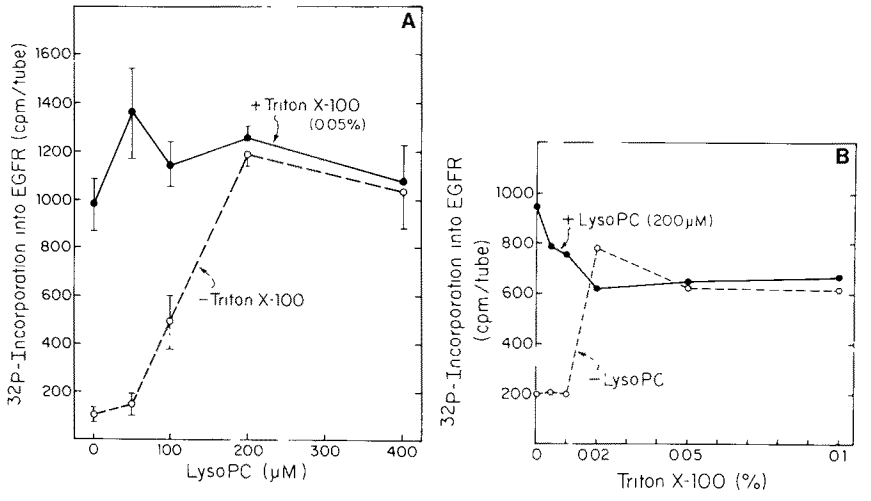

Figure 3. Relationship between effects of Triton X-100 and lyso-PC on EDRA

Experiments were performed as described in Materials and Methods and Fig. 1 legend, at various concentrations of lyso-PC (A) or Triton X-100 (B), in the presence of 0.2 μM human EGF. Values represent mean ± S.E. of three different experiments (A), or mean of two experiments (B).

stimulatory effect at 100 μM, but much greater stimulation at 300 μM. Lyso-PMME, like lyso-PE, had essentially no effect (Fig. 2). Unexpectedly, variation in fatty acid in lyso-PC made a significant difference; i.e., lyso-PC containing myristic acid had a much greater effect than lyso-PC containing palmitic or stearic acids (Table I). Interestingly,

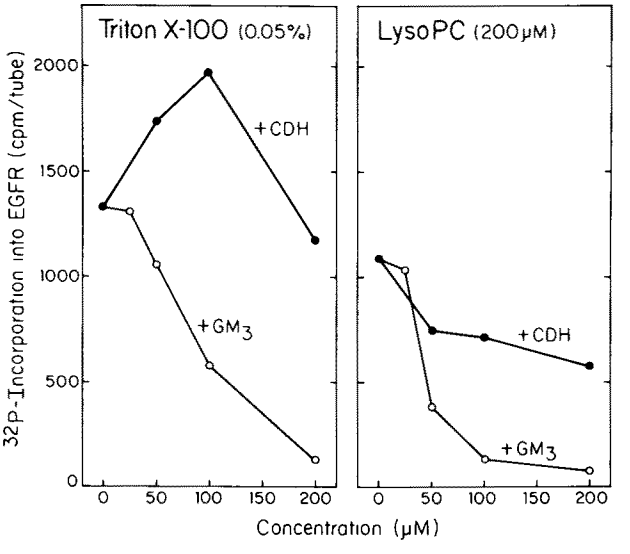

Figure 4. Inhibitory effect of GM3 on EDRA in the presence of Triton X-100 or lyso-PC

Experiments were performed as described in Materials and Methods and the Fig. 1 legend, using various concentrations of GM3 and CDH (lactosylceramide), in the presence of Triton X-100 (0.05%) or lyso-PC (200 μM). Values represent mean of two different experiments.

stimulatory effect of lyso-PAF (which also has the phosphorylcholine head group) on EDRA was also remarkable, although the physiological significance of this phenomenon is obscure (Table I). Parent phospholipids such as PC, PE, and PAF have no effect on EDRA at the same range of concentration. Various lyso-glycosphingolipids (e.g., lyso-lactosylceramide, lyso-glycosylceramide) also showed no significant stimulating activity; one exception was N,N-dimethylsphingosine (Fig. 1) (5).

The stimulatory effect of lyso-PC on EDRA was similar to that of the detergent Triton X-100. The effects of these two compounds were not additive when both were applied simultaneously (Fig. 3).

GM3-dependent inhibition of EDRA in the presence of lyso-PC. Previous observations from this laboratory (2,3) showed that GM3 (out of various glycosphingolipids containing native gangliosides) specifically inhibits EDRA. However, this inhibition was only obvious in the presence of the detergent Triton X-100 (concentration 0.02-0.1%). In the present study, we utilized lyso-PC, rather than Triton X-100, as a stimulating reagent for EDRA. As shown in Fig. 4, GM3 (but not lactosylceramide) inhibited EDRA in the presence of lyso-PC (apparent I_{50} value 40 μM) more effectively than in the presence of Triton X-100 (I_{50} value 80 μM).

DISCUSSION

The role of lysophospholipids in signal transduction has been discussed recently in terms of effect of lyso-PC on PK-C (10), lyso-phosphatidic acid on G-protein (11), and lyso-PC and lyso-PE in connection with *ras* oncoprotein function (12) (also see for reviews 13,14). The functional role of lyso-PC in modulation of transmembrane signal

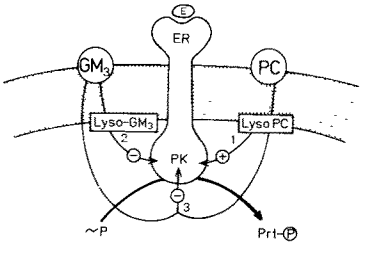

Figure 5. Negative or positive modulation of EGF receptor kinase by GM3 and PC, and their breakdown products

EGF (E), when bound to its receptor (ER), activates receptor-associated kinase (PK) by a yet-unidentified mechanism (most plausibly through di- or oligomerization of receptor) (15). EGF-dependent activation of PK is promoted by lyso-PC which is derived from PC (route 1). GM3 can inhibit PK through its degradation to lyso-form (route 2). Lyso-PC-stimulated PK can be inhibited directly by associated GM3 molecules (route 3). However, free GM3, which is not accessible to lyso-PC, is incapable of modulating PK activity.

transduction is made clearer by the data presented in the present paper; i.e., lyso-PC (but not other lysophospholipids) promotes EDRA. In our experimental system, EGF-dependent receptor phosphorylation was demonstrable only in the presence of Triton X-100, the detergent used in essentially all previous studies. Obviously, Triton X-100 does not exist *in vivo*. Lyso-PC may therefore function as a "natural detergent" occurring in cell membranes. The fact that lyso-PC containing myristic acid, compared to other acyl species of lyso-PC, showed significant enhancing effect may reflect a selective or preferential utilization of this particular species of lyso-PC for the *in vivo* detergent/carrier effect.

The inhibitory effect of GM3 on EDRA has been demonstrated repeatedly (2,3), and specific association of GM3 with EGF receptor has been reported (3). Since GM3 is a normal, ubiquitous component of plasma membranes, a specific population of GM3 molecules must be exerting this inhibitory effect. We postulate that some modulator molecule mediates transformation of non-functional GM3 into functional GM3 capable of interacting with and inhibiting EGF receptor kinase. The present study strongly suggests that lyso-PC, and possibly lyso-SM, functions as a mediator in a GM3-regulated EGF receptor activation system (Fig. 5). The ultimate mechanism of GM3-regulated EGF receptor function, however, remains to be elucidated. There is evidence that GM3 inhibits EGF-dependent di- or oligomerization (Igarashi Y, Kitamura K, Hakomori S, in preparation).

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