Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6274–6278, July 1994
Biochemistry

Regulation of phospholipase A_2 in human leukemia cell lines: Its implication for intracellular signaling

(protein kinase C/diacylglycerol/phorbol ester/tyrosine phosphorylation/G protein)

YOSUKE TSUJISHITA*, YOSHINORI ASAOKA†, AND YASUTOMI NISHIZUKA*†

*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan; and †Biosignal Research Center, Kobe University, Kobe 657, Japan

Contributed by Yasutomi Nishizuka, March 14, 1994

ABSTRACT **Permeabilized human leukemia HL-60 and U-937 cells suspended in an acidic or alkaline medium release various unsaturated fatty acids, most abundantly oleic and arachidonic acids. Concomitant production of lysophospholipids suggests that phospholipases A_2 play a major role in this fatty acid release reaction. The fatty acid release at acidic conditions depends on the intracellular Ca^{2+} concentrations at the 10^{-8}–10^{-7} M range and is enhanced by membrane-permeant diacylglycerols, although this enhancement seems independent of protein kinase C activation. On the other hand, the fatty acid release at alkaline conditions is potentiated by vanadate, and this potentiation is counteracted by genistein, suggesting a role of tyrosine phosphorylation in this release reaction. GTP[γS], an activator of G proteins, greatly enhances the fatty acid release. Aluminum fluoride, another activator of heterotrimeric G proteins, also greatly potentiates this release reaction. Phorbol ester increases the fatty acid release at alkaline conditions, to some extent, whereas it counteracts the vanadate-induced potentiation of fatty acid release. The results imply that several phospholipases A_2 are coupled to receptors for their activation, thereby functioning in the transmembrane control of cellular events.**

Phospholipid degradation initiated by receptor stimulation produces various lipid mediators that relay information from extracellular signals to intracellular events (for a review, see ref. 1). It is now accepted that phosphatidylinositol hydrolysis by phospholipase C as well as phosphatidylcholine (PtdCho) hydrolysis by phospholipase D generate diacylglycerol (DAG) that is essential to protein kinase C (PKC) activation (for reviews, see refs. 1–5). We have previously reported (6–9) that the primary products of PtdCho hydrolysis by phospholipase A_2, cis-unsaturated fatty acid and 2-lysophosphatidylcholine (lysoPtdCho), both appear to serve as enhancer molecules for DAG-dependent PKC activation in cell-free enzymatic reaction and for cellular responses. Dynamic change of various lipids in the membrane, therefore, appears to be critical for the regulation of intracellular events. The qualitative and quantitative analysis of this spatio-temporal aspect of lipid constituents after cell stimulation, however, is still insufficient, and elucidation of the regulatory mechanism of various phospholipases is possibly a key step to understanding the mechanism of transmembrane signaling.

Phospholipase A_2 is ubiquitously present in mammalian tissues, and receptor-mediated activation of this enzyme has been proposed (10). It is also known that phospholipase A_2 is composed of heterogenous isoforms with distinctly different enzymological properties, including substrate specificity and Ca^{2+} requirement for their activation (for a review, see ref. 11). The regulatory mechanism of a cytosolic 85-kDa phospholipase A_2 that cleaves preferentially arachidonic acid has recently been elucidated (12–15), while the cytosolic phospholipases A_2 responsible for nonselective release of various cis-unsaturated fatty acids remain to be explored. The studies presented herein were undertaken to determine quantitatively various fatty acids that are released from permeabilized human leukemia HL-60 and U-937 cells and to understand potential regulatory mechanisms of the enzyme responsible for this fatty acid release reaction, possibly phospholipase A_2.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. HL-60 cells were donated by J. Minowada (Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama, Japan) and U-937 cells were donated by T. Okazaki (Osaka Dental University). Cells were maintained at a cell density between 0.1 and 1.0 × 10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (Flow Laboratories) at 37°C in a humidified 5% CO_2 atmosphere.

Chemicals. A membrane-permeant DAG, 1,2-dioctanoylglycerol (1,2-DiC_8), was obtained from Nacalai Tesque (Kyoto). This preparation consists of approximately 95% DL-1,2-DiC_8 and 5% 1,3-DiC_8 as estimated by thin-layer chromatography. 1,3-DiC_8 and 1-oleoyl-2-acetylglycerol were purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA) was a product of LC Services (Woburn, MA). LysoPtdCho, 2-lysophosphatidylethanolamine (lysoPtdEtn), standard fatty acids, GTP[γS] and adenosine 5′-[β,γ-imido]triphosphate were products of Sigma. GDP[βS] was obtained from Boehringer Mannheim. 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi (Tokyo), dissolved in acetone at a concentration of 2.5% (wt/vol), and stored at −20°C in the dark. Acetoxymethyl ester of fura 2, a product of Dojindo Laboratories (Kumamoto, Japan), was dissolved in dimethyl sulfoxide at a concentration of 1 mM and kept at −20°C in the dark. [*methyl*-^{14}C]Choline chloride (55.1 mCi/mmol; 1 Ci = 37 GBq) was a product of Amersham. [1,2-^{14}C]Ethanolamine (3.0 mCi/mmol) and *myo*-[^{14}C]inositol (200 mCi/mmol) were obtained from New England Nuclear. Sodium vanadate was purchased from Fisher Scientific, and genistein was from Funakoshi.

Electropermeabilization. The cells (2 × 10^7 cells) were washed and suspended in 600 μl of ice-cold electropermeabilization buffer (EPB), which contained 120 mM potassium glutamate, 20 mM potassium acetate, 3 mM NaCl, 5 mM glucose, 3 mg of bovine serum albumin per ml, 1 mM EGTA, and 1 mM $CaCl_2$. The pH of EPB was adjusted to obtain an adequate buffer solution with 20 mM glycine, Mes, Hepes, or Tris. The Ca^{2+} concentration was arranged with various

Abbreviations: PtdCho, phosphatidylcholine; DAG, diacylglycerol; PKC, protein kinase C; lysoPtdCho, 2-lysophosphatidylcholine; DiC_8, dioctanoylglycerol; PMA, phorbol 12-myristate 13-acetate; lysoPtdEtn, 2-lysophosphatidylethanolamine; ADAM, 9-anthryldiazomethane; EPB, electropermeabilization buffer.

concentrations of EGTA (1–25 mM) in the presence of 1 mM $CaCl_2$. Cells were electropermeabilized with a Gene Pulser (Bio-Rad) by subjecting the cell suspension to six successive discharges of an electric field (1.0 kV/cm) using a 3-μF capacitator to provide a time constant of ≈200 μsec (16). The suspension was mixed after every three discharges to ensure uniform permeabilization.

Fatty Acid Analysis. Fatty acids produced by the electropermeabilized cells were extracted as described by Dole and Meinertz (17) and analyzed with an HPLC system using ADAM as a fluorescent probe (18). Briefly, the reaction was terminated after incubation for 1 hr at 37°C by the addition of 1.25 ml of Dole's reagent [isopropanol/*n*-heptane/0.5 M H_2SO_4, 78:20:2 (vol/vol)]. Following the addition of 0.75 ml of *n*-heptane, 0.5 ml of water, and 5 nmol of tridecanoic acid as an internal standard, the sample was mixed thoroughly and centrifuged at 1000 × *g* for 10 min to extract fatty acids. The upper phase (0.8 ml) was collected followed by the addition of 0.75 ml of *n*-heptane and 100 mg of silica gel G (type 60, Merck). The sample was mixed thoroughly and centrifuged at 1000 × *g* for 10 min. The supernatant was collected and dried using a vacuum evaporator. After evaporation, the sample was dissolved with 100 μl of methanol/ethylacetate, 50:50 (vol/vol), and derivatized with ADAM (0.1% at the final concentration of ADAM). The ADAM derivatives of fatty acids were separated on a Capcell Pak C_8 AG120 column (4.6 mm × 250 mm, Shiseido, Tokyo) and detected by a fluorescent HPLC monitor (RF-535; Shimadzu) at the emission of 412 nm with the excitation at 365 nm. The solvent system was methanol/water, 90:10 (vol/vol), at a flow rate of 1.2 ml/min.

Measurement of Intracellular Ca^{2+} Concentration. The intracellular Ca^{2+} concentration was measured as described by Poenie *et al.* (19) with a slight modification. To a cell suspension (2 × 10^6 cells per 600 μl) in RPMI 1640 medium containing 10% fetal bovine serum, a solution of acetoxymethyl ester of fura 2 in dimethyl sulfoxide was added to give a final concentration of 1 μM of this Ca^{2+} indicator. The cell suspension was incubated for 30 min at 37°C and washed twice with and resuspended in EPB (pH 5.2) at a density of 2 × 10^7 cells per 600 μl. The cells were stimulated as specified. The fluorescent intensities at 340 nm and 380 nm were measured by using a CAF-100 calcium analyzer (Japan Spectroscopic, Tokyo).

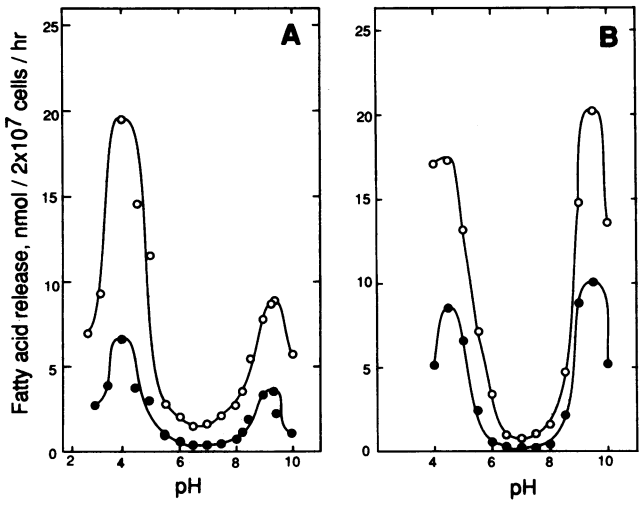

FIG. 1. Fatty acid release at various pH conditions. HL-60 cells (*A*) or U-937 cells (*B*) suspended in EPB at various pH conditions (2 × 10^7 cells per 600 μl) were electropermeabilized. After the incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM. ●, Arachidonic acid; ○, oleic acid.

Table 1. Release of various fatty acids from permeabilized U-937 cells

Fatty acid	Fatty acid released, nmol per 2 × 10^7 cells per hr		
	pH 5.0	pH 7.0	pH 9.0
Palmitic acid	7.98	ND	0.34
Palmitoleic acid	2.55	0.11	2.50
Stearic acid	2.05	ND	3.13
Oleic acid	13.2	0.78	14.8
Linoleic acid	3.23	0.15	2.94
Linolenic acid	2.95	0.73	2.62
Arachidonic acid	6.62	0.20	8.86

U-937 cells (2 × 10^7 cells per 600 μl) were suspended in EPB at the pH conditions indicated and electropermeabilized. After incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM. ND, not detected.

Calibration of intracellular Ca^{2+} concentration was carried out as described by Habara and Kanno (20).

Lysophospholipid Formation. Cells (1 × 10^7 cells per ml) in RPMI 1640 medium containing 10% fetal bovine serum were labeled with [^{14}C]choline (0.1 μCi/ml; 1 Ci = 37 GBq) or [^{14}C]ethanolamine (0.02 μCi/ml) for 12 hr. After incubation, the cells were washed and suspended in EPB at the pH conditions indicated (2 × 10^7 cells per 600 μl) and stimulated with electropermeabilization. After incubation, lipids were extracted by the method of Bligh and Dyer (21) and separated by TLC with chloroform/methanol/water, 65:25:4 (vol/vol), as the solvent system (8). The radioactivity of the spot corresponding to the respective lysophospholipid was determined with a BAS-2000 Bioimage analyzer (Fuji).

RESULTS

Release of Fatty Acids. When cells (HL-60 and U-937) were permeabilized, various fatty acids were produced during the subsequent incubation in a buffer medium. This release reaction was apparent at both acidic and alkaline conditions and proceeded linearly with time for at least 1 hr (Fig. 1). At neutral conditions, however, the release of fatty acids was negligible or very slow. Quantitative analysis of fatty acids indicated that large amounts of unsaturated fatty acids, particularly oleic and arachidonic acids, were released at both acidic and alkaline conditions (Table 1). These fatty acids are normally esterified at the *sn*-2 position of phospholipids. At acidic conditions, a significant amount of palmitic acid was produced, suggesting that several phospholipases and other lipolytic enzymes may be involved in this fatty acid release reaction.

To clarify the role of phospholipase A_2 in this reaction, lysoPtdCho and lysoPtdEtn were determined. In this experiment, radioactive choline and ethanolamine were employed as tracers. The results obtained could not be directly com-

Table 2. Release of lysophospholipids from permeabilized U-937 cells

Lysophospholipid	Lysophospholipid formation, fold increase		
	pH 5.0	pH 7.0	pH 9.0
LysoPtdCho	4.27	1.00	1.13
LysoPtdEtn	4.56	1.00	1.10

U-937 cells were labeled with [^{14}C]choline or [^{14}C]ethanolamine. Labeled cells (2 × 10^7 cells per 600 μl) were suspended in EPB at the pH conditions indicated and electropermeabilized. After incubation was terminated, lipids were extracted and each lysophospholipid was determined. The intracellular Ca^{2+} concentration was 200 nM. Values give the fold increase of each lysophospholipid formation at pH 7.0 as 1.00.

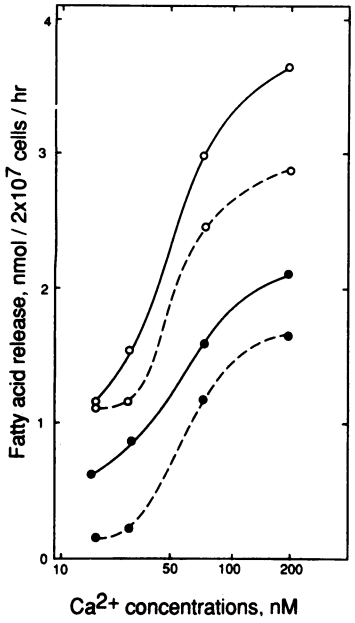

FIG. 2. Fatty acid release at various Ca^{2+} concentrations. HL-60 cells (2×10^7 cells per 600 μl) suspended in EPB (pH 5.2) at various concentrations of EGTA were electropermeabilized. After the incubation was terminated, fatty acids were extracted and determined. 1,2-DiC_8 (100 μM) was added twice (at time zero and at time 30 min) during the incubation. The intracellular Ca^{2+} concentrations were determined with the fura 2 procedure. ●, Arachidonic acid; ○, oleic acid; ——, in the presence of 1,2-DiC_8; – – –, in the absence of 1,2-DiC_8.

pared quantitatively with the fatty acids released, but it was clear that at both acidic and alkaline conditions lysophospholipids were produced in significant quantities (Table 2). The amounts of lysophospholipids at alkaline conditions were relatively small, presumably due to their rapid conversion to other lipids. The results suggest that various unsaturated fatty acids, if not all, are released directly from membrane phospholipids by the action of an arachidonic acid-nonselective type of phospholipase A_2. Although other pathways for the fatty acid release may not be ruled out, subsequent studies were undertaken to characterize the nature and possible linking of the fatty acid release reactions to receptor functions.

Ca^{2+} as a Potential Regulator. The fatty acid release at an acidic condition (pH 5.2) appeared to depend on the intracellular Ca^{2+} concentration at the 10^{-8}–10^{-7} M range (Fig. 2). This fatty acid release was accompanied with lysophospholipid production. The fatty acid release reaction was potentiated by the addition of a membrane-permeant DAG such as 1,2-DiC_8 or 1-oleoyl-2-acetylglycerol. PMA was ineffective, indicating that PKC does not appear to play a role in this fatty

Table 3. Effect of Ca^{2+} on fatty acid release

Cells	$[Ca^{2+}]_i$, nM	Fatty acid released, nmol per 2×10^7 cells per hr: Arachidonic acid	Oleic acid
HL-60, at pH 5.2	17	0.15	1.12
	200	1.65	2.88
U-937, at pH 8.5	17	1.17	4.26
	200	1.13	4.15

HL-60 cells and U-937 cells were suspended in EPB at the pH conditions indicated in the presence of various concentrations of EGTA. Cells were electropermeabilized. After incubation was terminated, fatty acids were extracted and determined. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined as described.

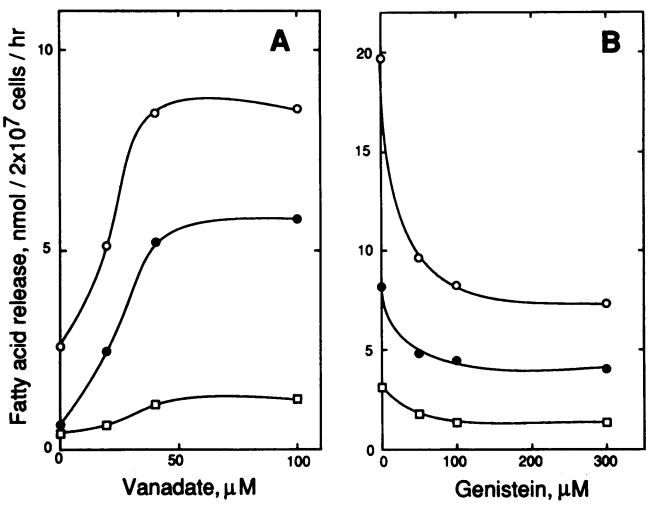

FIG. 3. Effect of vanadate and genistein on fatty acid release. U-937 cells (2×10^7 cells per 600 μl) were suspended in EPB (pH 8.4 in *A*, pH 8.5 in *B*) containing 6 mM $MgCl_2$ and 250 μM ATP in the presence of various concentrations of vanadate (*A*) or in the presence of various concentrations of genistein plus 40 μM vanadate (*B*). Cells were electropermeabilized. After the incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM. ●, Arachidonic acid; ○, oleic acid; □, linoleic acid.

acid release reaction. Presumably, DAG perturbs the membrane lipid bilayer, thereby facilitating the hydrolysis of phospholipids. In contrast, the fatty acid release in alkaline conditions was not affected by the concentration of Ca^{2+} (Table 3).

Possible Role of Protein Phosphorylation. The fatty acid release at alkaline conditions was greatly enhanced by the addition of vanadate, a tyrosine phosphatase inhibitor (Fig. 3 and Table 4). This potentiation by vanadate was counteracted by genistein, a tyrosine kinase inhibitor. The formation of lysophospholipids, lysoPtdCho and lysoPtdEtn, was also enhanced by vanadate. ATP added exogenously enhanced the fatty acid release reaction by 30%. This enhancement did not appear to be due to the stimulation of the purinergic receptors, since a nonhydrolyzable ATP analogue, adenosine 5′-[β,γ-imido]- triphosphate, was ineffective. Cyclic AMP showed no effect. The fatty acid release at acidic conditions was not sensitive to vanadate (Table 4).

Involvement of Heterotrimeric G Protein. GTP[γS], a nonhydrolyzable analogue of GTP that activates both heterotrimeric and monomeric G proteins, enhanced the fatty acid release at alkaline conditions. This enhancement was particularly significant in the presence of vanadate and was blocked by the addition of GDP[βS] (Fig. 4). Aluminum fluoride, an activator of heterotrimeric G protein but not of monomeric G

Table 4. Effect of vanadate on fatty acid release

Cells	Vanadate	Fatty acid released, nmol per 2×10^7 cells per hr: Arachidonic acid	Oleic acid
HL-60, at pH 5.2	–	2.67	7.51
	+	1.80	5.89
U-937, at pH 8.4	–	0.63	2.59
	+	5.19	8.43

HL-60 cells and U-937 cells were suspended in EPB containing 6 mM $MgCl_2$ and 250 μM ATP at the pH conditions indicated in the presence or absence of 40 μM vanadate. Cells were electropermeabilized. After incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM.

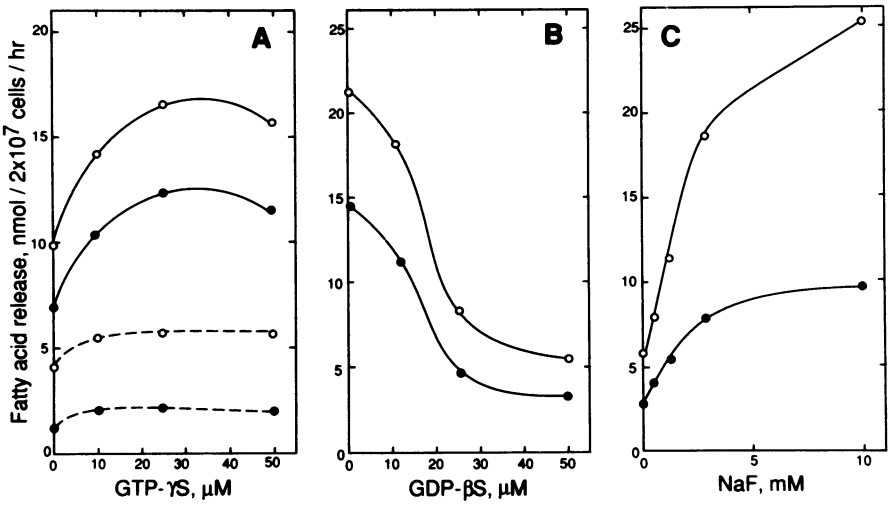

FIG. 4. Effect of GTP[γS] and GDP[βS] on fatty acid release. U-937 cells (2×10^7 cells per 600 μl) were suspended in EPB (pH 8.5) containing 6 mM $MgCl_2$ and 250 μM ATP in the presence of various concentrations of GTP[γS] with or without 40 μM vanadate (*A*), in the presence of various concentrations of GDP[βS] with 40 μM vanadate plus 25 μM GTP[γS] (*B*), or in the presence of various concentrations of NaF plus 20 μM $AlCl_3$ (*C*). Cells were electropermeabilized. After the incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM. ●, Arachidonic acid; ○, oleic acid; ——, with vanadate; – – –, without vanadate.

protein, also greatly enhanced the fatty acid release reactions at alkaline conditions. The results suggest that heterotrimeric G protein may play a role in the enhancement of fatty acid release, which is possibly catalyzed by phospholipase A_2. There was no indication that G protein is involved in the fatty acid release at acidic conditions.

Dual Action of PKC. In the absence of GTP[γS] and vanadate, PMA slightly stimulated fatty acid release at alkaline conditions (Fig. 5). However, the vanadate-induced enhancement of fatty acid release was counteracted by PMA. Similar results were obtained when PMA was replaced by 1,2-DiC_8 but not by 1,3-DiC_8. Such a dual action of PMA and DiC_8 was not observed for the fatty acid release at acidic conditions.

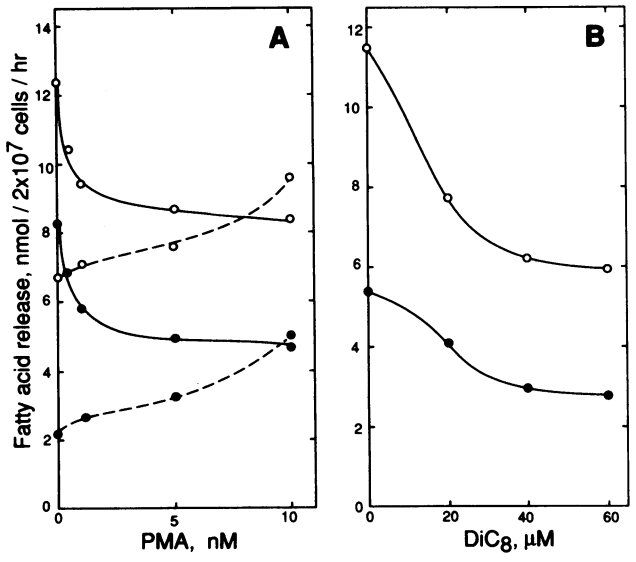

FIG. 5. Effect of PMA and DiC_8 on fatty acid release. U-937 cells (2×10^7 cells per 600 μl) were suspended in EPB (pH 8.5) containing 6 mM $MgCl_2$ and 250 μM ATP in the presence of various concentrations of PMA (*A*) or 1,2-DiC_8 (*B*) with or without 40 μM vanadate. Cells were electropermeabilized. 1,2-DiC_8 (indicated concentrations) was added six times with an interval of 10 min during the incubation. After the incubation was terminated, fatty acids were extracted and determined. The intracellular Ca^{2+} concentration was 200 nM. ●, Arachidonic acid; ○, oleic acid; ——, with vanadate; – – –, without vanadate.

DISCUSSION

The enzyme termed phospholipase A_2, which liberates fatty acids from the *sn*-2 position of phospholipids, is widely distributed in mammalian tissues, and several soluble enzymes, secretory and cytosolic, have been identified. The secretory enzymes require millimolar concentrations of Ca^{2+} and are nonselective toward acyl moieties to be cleaved (11). The cytosolic enzymes, on the other hand, appear to be heterogenous with the molecular size ranging from 30 kDa to 110 kDa (11). The cytosolic enzymes are divided into two groups, arachidonic acid selective and nonselective types. Thus far, much attention has been paid to the arachidonic acid-selective type of enzymes, because this fatty acid is the rate-limiting precursor to prostaglandins and leukotrienes (10), and most studies on the phospholipase A_2 activation have been made with the cells prelabeled with radioactive arachidonic acid. We have previously reported (7–9) that the primary products of phospholipase A_2 reaction, various unsaturated fatty acids and lysophospholipids, particularly lysoPtdCho, both greatly enhance cellular responses that are mediated by the PKC signal pathway. Thus, it is possible that cytosolic, arachidonic acid-nonselective type of phospholipases A_2 may have a potential to play roles in transmembrane signaling (1, 6). However, little evidence has been available, to date, indicating the coupling of this type of phospholipases A_2 to receptor functions, although a cytosolic phospholipase A_2 with 85 kDa molecular size, which cleaves preferentially arachidonic acid, is activated by reversible phosphorylation by mitogen-activated protein kinase, followed by binding to membranes in a Ca^{2+}-dependent fashion (15). In fact, the release of arachidonic acid has often been shown to be coupled to receptor functions, such as G proteins and protein phosphorylation in a wide variety of cell types (for a review, see ref. 22).

The results presented in this paper show that permeabilized human leukemia cell lines release various unsaturated fatty acids that are normally esterified at the *sn*-2 position of membrane phospholipids, when suspended in acidic or alkaline medium, although the intracellular pH is not necessarily equilibrated with the extracellular pH. Obviously, various pathways may be responsible for the fatty acid release from

cellular lipids, such as those initiated by phospholipases C and D and also by phospholipase A_2 followed by lysophospholipase (23). It is likely, however, that phospholipase A_2 may play a major role in the fatty acid release described herein for the following reasons. (*i*) Lysophospholipids could be detected under the conditions where the fatty acid release was enhanced. (*ii*) The fatty acid release that was observed in the present studies could not be contributed much by the action of phospholipase C followed by DAG lipase, because the amount of phosphatidylinositol was unchanged among various pH conditions when determined with [^{14}C]inositol as a tracer. Phospholipases C thus far purified from mammalian tissues are reactive only with phosphatidylinositol but not with PtdCho (24), although the occurrence of phospholipase C that is reactive with PtdCho has often been proposed. This proposal is based primarily on the observation that phosphocholine can be detected. However, no definitive evidence has so far been presented for the occurrence of phospholipase C that is reactive with PtdCho. Phosphocholine may likely be produced by consecutive actions of phospholipase A_2, lysophospholipase, and phosphodiesterase. Phosphocholine may also be produced from choline by the action of choline kinase, which is active in mammalian tissues (25). In addition, sphingomyelinase may produce phosphocholine. (*iii*) Most molecular species of phosphatidylinositol contain arachidonic acid at the *sn*-2 position. (*iv*) Phospholipase D did not appear to be responsible much for the observed fatty acid release, because fatty acids are released consistently both in the presence and absence of ethanol, an acceptor of the transphosphatidylation reaction. Nevertheless, the results described above do not completely eliminate the possibility that some of the fatty acids are released from cellular lipids by the action of some other lipases than phospholipase A_2. The physiological picture will be clarified further by analysis in cell-free systems.

Putative phospholipase A_2 that is responsible for the fatty acid release at acidic conditions appears to be regulated finely by Ca^{2+} at concentrations less than the 10^{-7} M range. The release reaction is enhanced by membrane-permeant DAGs, but it is unlikely that these DAGs exert their action through activation of PKC since PMA is inert under comparable conditions. Presumably, DAGs perturb the lipid bilayer structure, thereby facilitating phospholipid hydrolysis (26). On the other hand, the enzyme responsible for the fatty acid release at alkaline conditions appears to be controlled by several receptor-mediated mechanisms. Secretory phospholipases A_2 are unlikely to play roles in this fatty acid release because the enzymes require the millimolar range of Ca^{2+} for their catalytic activity (11). It is possible that, analogous to phospholipase C, there exist several cytosolic arachidonic acid-nonselective phospholipases A_2, which may be regulated by heterotrimetic G proteins and by protein phosphorylation involving tyrosine kinases and PKC. PKC appears to exert a dual, positive and negative, action, and the negative feedback loop is predominant over the vanadate-stimulated reaction. Although it is premature to discuss the overall aspect of the intracellular signaling network controlling various phospholipases, the present results suggest that activation of several cytosolic phospholipases A_2, not only the 85-kDa enzyme recently described (12–15) but also arachidonic acid-nonselective type of enzymes, is tightly coupled to receptor stimulation and plays roles, in concert with phospholipases C and D, in the transmembrane control of major cellular functions.

The data are taken in part from the dissertation that will be submitted by Y.T. to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Sciences. We cordially acknowledge Dr. J. Minowada (Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama) for the generous gift of HL-60 cells and Dr. T. Okazaki (Osaka Dental University) for U-937 cells. The skillful secretarial assistance of Mrs. S. Nishiyama, Miss Y. Yamaguchi, and Miss M. Taoka is cordially acknowledged. This work was supported in part by research grants from the Special Research Fund of Ministry of Education, Science, and Culture, Japan; the Yamanouchi Foundation for Research on Metabolic Disorders; the Sankyo Foundation of Life Science; Merck Sharp & Dohme Research Laboratories; the Terumo Life Science Foundation; and the Osaka Cancer Research Fund.

1. Nishizuka, Y. (1992) *Science* **258**, 607–614.
2. Pelech, S. L. & Vance, D. E. (1989) *Trends Biochem. Sci.* **14**, 28–30.
3. Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4.
4. Billah, M. M. & Anthes, J. C. (1990) *Biochem. J.* **269**, 281–291.
5. Liscovitch, M. (1992) *Trends Biochem. Sci.* **17**, 393–399.
6. Asaoka, Y., Nakamura, S., Yoshida, K. & Nishizuka, Y. (1992) *Trends Biochem. Sci.* **17**, 414–417.
7. Yoshida, K., Asaoka, Y. & Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6443–6446.
8. Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y. & Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6447–6451.
9. Asaoka, Y., Yoshida, K., Sasaki, Y. & Nishizuka, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4917–4921.
10. Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988) *Trends NeuroSci.* **11**, 117–123.
11. Kudo, I., Murakami, M., Hara, S. & Inoue, K. (1993) *Biochim. Biophys. Acta* **117**, 217–231.
12. Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. & Knopf, J. L. (1991) *Cell* **65**, 1043–1051.
13. Lin, L.-L., Lin, A. Y. & Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6147–6151.
14. Nemenoff, R. A., Winitz, S., Quian, N.-X., Putten, V. V., Johnson, G. L. & Heasley, L. E. (1993) *J. Biol. Chem.* **268**, 1960–1964.
15. Lin, L.-L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. & Davis, R. J. (1993) *Cell* **72**, 269–278.
16. Baker, P. F. & Knight, D. E. (1978) *Nature (London)* **276**, 620–622.
17. Dole, V. P. & Meinertz, H. (1960) *J. Biol. Chem.* **235**, 2595–2599.
18. Barker, S. A., Monti, J. A., Christian, S. T., Benington, F. & Morin, R. D. (1980) *Anal. Biochem.* **107**, 116–123.
19. Poenie, M., Alderton, J., Tsien, R. Y. & Steinhardt, R. A. (1985) *Nature (London)* **315**, 147–149.
20. Habara, Y. & Kanno, T. (1991) *Cell Calcium* **12**, 533–542.
21. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
22. Cockcroft, S. (1992) *Biochim. Biophys. Acta* **1113**, 135–160.
23. Lesile, C. C. (1991) *J. Biol. Chem.* **266**, 11366–11371.
24. Dennis, E. A., Rhee, S. G., Billah, M. M. & Hannun, Y. A. (1991) *FASEB J.* **5**, 2068–2077.
25. Macara, I. G. (1989) *Mol. Cell. Biol.* **9**, 325–328.
26. Dawson, R. M. C., Hemington, N. L. & Irvine, R. F. (1983) *Biochem. Biophys. Res. Commun.* **117**, 196–201.