Enzymatic measurement of phosphatidic acid in cultured cells

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Abstract In this work, we developed a novel enzymatic method for measuring phosphatidic acid (PA) in cultured cells. The enzymatic reaction sequence of the method involves hydrolysis of PA to produce glycerol-3-phosphate (G3P), which is then oxidized by G3P oxidase to generate hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacted with Amplex Red to produce highly fluorescent resorufin. We found that lipase from *Pseudomonas* sp. can completely hydrolyze PA to G3P and FAs. The calibration curve for PA measurement was linear between 20 and 250 μM, and the detection limit was 5 μM (50 pmol in the reaction mixture). We also modified the method for the enzymatic measurement of lysophosphatidic acid. By this new method, we determined the PA content in the lipid extract from HEK293 cells. The cellular content of PA was decreased with increasing cell density but not correlated with the proliferation rate. The diacylglycerol kinase inhibitor R59949 markedly reduced the cellular PA content, suggesting the diacylglycerol kinase activity was involved in a large part of the PA production in HEK293 cells. ⬛ This novel method for PA quantification is simple, rapid, specific, sensitive, and high-throughput and will help to study the biological functions of PA and its related enzymes.— Morita, S-y., K. Ueda, and S. Kitagawa. **Enzymatic measurement of phosphatidic acid in cultured cells.** *J. Lipid Res.* **2009.** 50: **1945–1952.**

Supplementary key words lipase • Amplex Red • lysophosphatidic acid • cell density • diacylglycerol kinase • R59949

Phosphatidic acid (PA) is a lipid component of cell membranes and a central intermediate for the synthesis of membrane lipids and storage lipids (1). PA is present in cell membranes in small amounts but is involved in the regulation of diverse cellular functions, including cell growth, membrane trafficking, differentiation, and migration (2). Three different enzymes generate PA: diacylglycerol kinase (DGK), phospholipase D, and lysophosphatidic acid (LPA) acyltransferase (3). DGK produces PA through phosphorylation of diacylglycerol. PA is reconverted into diacylglycerol by PA phosphatase. Phospholipase D hydrolyzes the phosphodiester bond of phosphatidylcholine (PC) to produce PA and free choline. PA is deacylated by a phospholipase A activity to form LPA, a monoacylated form of PA, which is reconverted to PA by LPA acyltransferase. PA is tightly regulated in cells and converted to other potentially bioactive lipids, including diacylglycerol and LPA.

PA is linked to many intracellular signal transduction events (1–4). PA has been identified as a critical component of mammalian target of rapamycin (mTOR) signaling that regulates both cell cycle progression and cell growth (5–7). PA directly interacts with mTOR at the domain targeted by rapamycin, and PA generator enzymes are potential regulators of mTOR (5, 6). Lipopolysaccharide stimulation caused an increase in PA, which plays a role in cytokine release through the activation of mTOR (8). PA is needed for the activation of only type I but not type II phosphatidylinositol (PI) 4-phosphate 5-kinase (9, 10). Protein phosphatase-Iγ binds PA, which exerts an inhibitory effect on the enzymatic activity (11). Raf-1 kinase (12), sphingosine kinase 1 (13), and cAMP phosphodiesterase 4A1 (14) contain PA binding sites, and the direct interactions with PA are required for their translocations. PA also modulates the membrane localization and/or activity of protein kinase C (15, 16), the tyrosine phosphatase SHP-1 (17), phospholipase C (18), and the guanine nucleotide exchange factor Son of sevenless (19). DGK-derived

Abbreviations: DAOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline; DGK, diacylglycerol kinase; G3P, glycerol-3-phosphate; GPO, glycerol-3-phosphate oxidase; LPA, lysophosphatidic acid; MGL, monoglyceride lipase; mTOR, mammalian target of rapamycin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

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PA is necessary for interleukin-2-dependent proliferation of T-cells (20), CD4$^+$/CD8$^+$ cell survival during thymic development (21), hepatocyte growth factor- and vascular endothelial growth factor-induced migration and invasion in epithelial cells (22, 23), and the activity of hypoxia-inducible factor-1α during onset of the hypoxic response (24). PA is linked to vesicular trafficking, secretion, and endocytosis (1, 3). PA is thought to lower the activation energy required for negative curvature at the inward membrane curve and act as a fusogenic lipid (25). In plants, various PA targets have been identified, including phosphoinositide-dependent protein kinase 1, phosphoenolpyruvate carboxylase, heat-shock protein 90, and 14-3-3 proteins (26, 27). In yeast, PA binding maintains the transcriptional regulator of phospholipid synthesis, Opi1p, at the endoplasmic reticulum, preventing its nuclear localization (28).

The conventional method for measuring PA involves solvent extraction of total lipids followed by separation by TLC and by quantification of phosphate from the TLC spot (1, 29). This method is time-consuming and has low sensitivity. To assess PA newly synthesized in response to a stimulus, cellular lipids are prelabeled with radioisotopes or fluorescence lipids, and radioactive or fluorescent PA is quantified (1, 5, 30, 31). As with most labeling studies, it is an important issue whether the measurement of labeled PA is a true reflection of the unlabeled PA. The labeling extent depends on many factors, including pooling and equilibration times. MS has been used to detect and identify PA molecular species that differ in FA composition (32). However, it is difficult to determine the total PA concentration and perform quantitative comparisons between PA and other phospholipids using MS. There is no rapid and sensitive method for PA measurement. Various enzymatic assays have been reported for the quantification of other phospholipids, including PC (33, 34), phosphatidylglycerol (35), sphingomyelin (36, 37), lysophosphatidylcholine (38), and LPA (39). In the present study, we aimed to develop a convenient new PA assay that can be used routinely to analyze PA levels in cells. We described a novel enzymatic method for measuring PA level in lipid extracts from cultured cells. The method has better specificity, requires a smaller quantity of sample and shorter time than previously reported methods, and allows many samples to be processed in parallel. Here, we also explored the relationship between cell density and PA content and investigated the effect of the DGK inhibitor on the PA content in HEK293 cells.

MATERIALS AND METHODS

Materials

Lipase (lipoprotein lipase) from *Pseudomonas* sp. and choline oxidase from *Alcaligenes* sp. were provided by Wako Pure Chemical Industries (Osaka, Japan). L-Glycerol-3-phosphate (G3P) oxidase (GPO) from *Aerococcus viridians* was obtained from Roche Diagnostics (Mannheim, Germany). Amplex Red reagent was purchased from Molecular Probes (Eugene, OR). Peroxidase from horseradish roots was obtained from Oriental Yeast (Suita,

Osaka, Japan). Monoglyceride lipase (MGL) from *Bacillus* sp. was provided by Asahi Kasei Pharma (Tokyo, Japan). L-α-Palmitoyl-oleoyl-phosphatidylethanolamine and L-α-palmitoyl-oleoyl-phosphatidylserine (PS) sodium salt were purchased from Avanti Polar Lipids (Alabaster, AL). Glycerophospholipid-specific phospholipase D and L-α-PI from soybean were purchased from Biomol International (Plymouth Meeting, PA). 4-Aminoantipyrine was provided by Nacalai Tesque (Kyoto, Japan). *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS) was purchased from Dojindo Laboratories (Kumamoto, Japan). R59949 was obtained from Calbiochem (La Jolla, CA). All other chemicals used were of the highest reagent grade.

Preparation of standard PA solution

L-α-PA monosodium salt from chicken egg was purchased from Avanti Polar Lipids. PA standard solutions were dissolved in 1% Triton X-100 aqueous solution.

Assay for PA hydrolysis

PA (100 µM) in 1% Triton X-100 was diluted 5-fold with 40 mM Tris-HCl buffer (pH 7.4) containing 40 mM NaCl and incubated with 4,000 units/ml lipase from *Pseudomonas* sp. at 37°C. At the indicated time points, hydrolysis was stopped by heat inactivation at 96°C, and the denatured enzyme was removed by centrifugation (7,200 *g*, 5 min). The degree of PA hydrolysis was determined as the amount of released FA, which was measured using an enzymatic assay kit purchased from Wako. The relative activities of the lipase (4,000 units/ml) toward different phospholipids (200 µM) were determined at 37°C for 15 min.

Enzymatic measurement of PA

The 3 steps for the enzymatic measurement of PA are illustrated in **Fig. 1**: *1*) Bacterial lipase hydrolyzes PA to G3P and two FAs, 2) G3P is oxidized by GPO, which generates hydrogen peroxide and dihydroxyacetone phosphate, and *3*) in the presence of peroxidase, hydrogen peroxide reacted with Amplex Red to produce highly fluorescent resorufin that can be measured.

Measurement was performed using a three-reagent system. Reagent A1 contained 10,000 units/ml lipase from *Pseudomonas* sp., 50 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Reagent A2 contained 5 units/ml GPO, 5 units/ml peroxidase, 300 µM Amplex Red, 0.2% Triton X-100, 40 mM NaCl, and 40 mM Tris-HCl (pH 7.4). Amplex Red Stop Reagent was obtained from Molecular Probes.

Sample (20 µl) was added to 80 µl of Reagent A1 and incubated at 37°C for 1 h. After the incubation, the lipase was heat-inactivated at 96°C for 3 min, and the denatured enzyme was precipitated by centrifugation (7,200 *g*, 5 min). The supernatant (50 µl) was added to 50 µl of Reagent A2, and after 30 min of incubation at room temperature, 20 µl of Amplex Red Stop Reagent was added. The fluorescence intensity was measured using a fluorescence microplate reader (Fluoromark, Bio-Rad Laboratories,
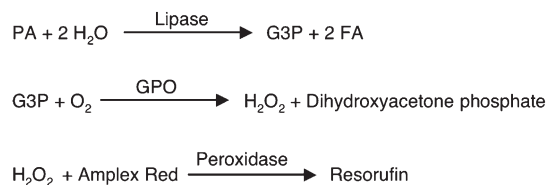
$$PA + 2\ H_2O \xrightarrow{\text{Lipase}} G3P + 2\ FA$$

$$G3P + O_2 \xrightarrow{\text{GPO}} H_2O_2 + \text{Dihydroxyacetone phosphate}$$

$$H_2O_2 + \text{Amplex Red} \xrightarrow{\text{Peroxidase}} \text{Resorufin}$$

Fig. 1. Strategy for PA measurement. Lipase catalyzes the hydrolysis of PA to G3P and two FAs. Oxidation of G3P is catalyzed by GPO, which produces hydrogen peroxide. In the presence of peroxidase, Amplex Red reacts with hydrogen peroxide to produce highly fluorescent resorufin that can be measured.

Hercules, CA), and the excitation and emission wavelength filters were set at 544 and 590 nm, respectively.

Enzymatic measurement of LPA

The LPA measurement was performed by a modified version of the method of Aoki (39). First, MGL is used to hydrolyze LPA to G3P and FA. G3P is oxidized by GPO to dihydroxyacetone phosphate and hydrogen peroxide. Finally, hydrogen peroxide, in the presence of peroxidase, reacts with Amplex Red to generate the highly fluorescent product, resorufin.

Reagent L1 contained 12.5 units/ml MGL, 50 mM NaCl, and 50 mM Tris-HCl (pH 7.4). Sample (20 µl) was added to 80 µl of Reagent L1 and incubated at 37°C for 30 min. After the incubation, MGL was heat-inactivated at 96°C for 3 min, and the denatured enzyme was precipitated by centrifugation (7,200 g, 5 min). The supernatant (50 µl) was added to 50 µl of Reagent A2, and after 30 min of incubation at room temperature, 20 µl of Amplex Red Stop Reagent was added. The fluorescence intensity was measured using a fluorescence microplate reader and the excitation and emission wavelength filters were set at 544 and 590 nm, respectively. L-α-Monooleoyl PA sodium salt was purchased from Avanti Polar Lipids, and LPA standard solutions were dissolved in 1% Triton X-100.

Enzymatic measurement of G3P

The measurement of G3P was performed with Reagent A2 for PA measurement. sn-Glycerol 3-phosphate bis(cyclohexylammonium) salt was purchased from Sigma. The detection limit for G3P was as low as 0.2 µM.

Enzymatic measurement of PC

There are three steps for the enzymatic measurement of PC. PC is hydrolyzed to choline and PA by glycerophospholipid-specific phospholipase D, which does not react with sphingomyelin or lysophosphatidylcholine. Choline is reacted with choline oxidase to generate hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide couples DAOS and 4-aminoantipyrine to yield a blue dye with an optimal absorption at 595 nm.

Measurement was performed using a one-reagent system. Reagent C contained 120 units/ml glycerophospholipid-specific phospholipase D, 4 units/ml choline oxidase, 5 units/ml peroxidase, 1 mM 4-aminoantipyrine, 1 mM DAOS, 0.2% Triton X-100, 1.1 mM CaCl$_2$, 50 mM NaCl, and 50 mM Tris-HCl (pH 7.4). PC from egg yolk was obtained from Nacalai Tesque, and PC standard solutions were dissolved in 1% Triton X-100. Sample (10 µl) was added to 200 µl of Reagent C and incubated at 37°C for 20 min. Absorption was measured at 595 nm using a microplate reader (Model 680, Bio-Rad). The linear range for the PC measurement was 0.25–2.5 mM (r = 0.9992).

Measurement of PA and PC contents in cells

HEK293 cells were grown on 10 cm dishes in DMEM supplemented with 10% heat-inactivated FBS in a humidified incubator (5% CO$_2$) at 37°C. For experiments with DGK inhibitor R59949, HEK293 cells were incubated with various concentrations of R59949 in DMEM containing 10% FBS at 37°C for 24 h. After incubation, the cells were chilled on ice, washed, and scraped with cold PBS. The cells were centrifuged (8,590 g, 10 min, 4°C), and the pellet was resuspended in PBS and sonicated. The cell protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). The cellular lipids were extracted by the method of Bligh and Dyer (31, 40) and dissolved in 1% Triton X-100. The contents of PA and PC in the lipid extract from cells were measured by enzymatic assay.

RESULTS AND DISCUSSION

PA hydrolysis by bacterial lipase

A screen was made for lipases hydrolyzing PA to generate G3P and two FAs, which was necessary to establish the enzymatic measurement of PA using the novel three-step procedure as shown in Fig. 1. Lipase from *Pseudomonas* sp. reacts with triglyceride to form glycerol and FAs (41). To examine whether PA is also hydrolyzed by lipase from *Pseudomonas* sp., PA from chicken egg was incubated with a high concentration of the lipase (4,000 units/ml) in Tris-HCl buffer containing 0.2% Triton X-100 at 37°C (**Fig. 2**). The degree of hydrolysis was determined from the amount of FA released. Under the above conditions, almost all of the egg PA molecules were hydrolyzed by lipase from *Pseudomonas* sp. after incubation for 60 min, indicating that lipase from *Pseudomonas* sp. can completely hydrolyze PA containing mixed acyl chains to yield G3P and FAs. The relative activities of *Pseudomonas* lipase toward PC, phosphatidylethanolamine (PE), PS, and PI were 17.9, 79.9, 10.5, and 15.3% of the activity toward PA, respectively. However, G3P is generated only from PA but not from other phospholipids by *Pseudomonas* lipase.

PA measurement

Recently, Amplex Red has been widely used to monitor hydrogen peroxide levels and a variety of oxidase-mediated reactions (37, 42–44). It has been reported that Amplex Red can detect hydrogen peroxide at a concentration as low as 50 nM or 5 pmol/100 µl sample in a 96-well fluorescence microplate assay (42). This new method uses Amplex Red to quantify hydrogen peroxide release following a series of enzymatic digestion of PA.

To validate the new method for PA measurement, a calibration reaction was performed using PA standard solutions. **Fig. 3A** shows that there was a linear relationship from 20 to 250 µM PA (r = 0.9995) and that a much better linear fit was obtained without forcing the curve through zero. At lower concentrations of PA, the curve was slightly nonlinear and fit a quadratic regression equation (r = 0.9993) (Fig. 3B). As little as 5 µM PA (50 pmol PA in the
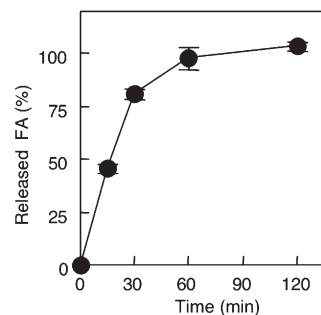

Fig. 2. PA hydrolysis by lipase from *Pseudomonas* sp. PA (100 µM) in 1% Triton X-100 was diluted 5-fold with Tris-HCl buffer (pH 7.4) and incubated with 4,000 units/ml lipase from *Pseudomonas* sp. at 37°C. At the indicated time points, PA hydrolysis was stopped by heat inactivation of lipase. Results are expressed as a percentage of FA released from PA. Each point represents the mean ± SD of three measurements.
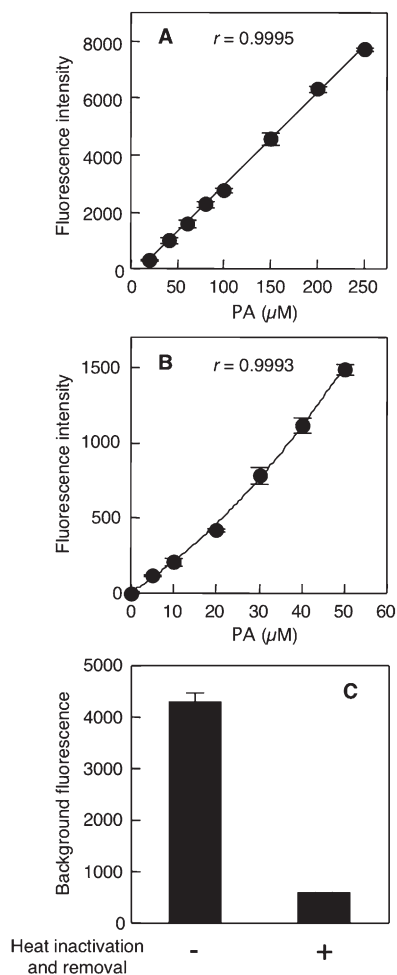
Fig. 3. Standard curves for PA measurement up to 250 µM (A) and 50 µM (B). The PA standard solution (20 µl) was added to 80 µl of Reagent A1 containing 10,000 units/ml lipase from *Pseudomonas* sp. and incubated at 37°C for 1 h. The lipase was heat-inactivated at 96°C for 3 min, and the denatured enzyme was removed by centrifugation. The supernatant (50 µl) was added to 50 µl of Reagent A2 containing 5 units/ml GPO, 5 units/ml peroxidase, 300 µM Amplex Red. After 30 min of incubation at room temperature, 20 µl of Stop Reagent was added. The fluorescence intensity (excitation 544 nm, emission 590 nm) was measured using a fluorescence microplate reader. Each point represents the mean ± SD of triplicate measurement. Background fluorescence was 585 ± 16 (A) or 529 ± 31 (B), which was subtracted from each value. The lines were obtained by linear regression analysis (A) and quadratic regression analysis (B). The correlation coefficients were $r = 0.9995$ (A) and $r = 0.9993$ (B). The background fluorescence with (+) or without (−) heat inactivation and removal of the denatured enzyme was measured (C). Each bar represents the mean ± SD of triplicate measurement. The absence of an error bar signifies an SD value smaller than graphic symbol.

reaction mixture) could be detected. The background fluorescence was dramatically decreased by the heat inactivation and removal of denatured enzyme (Fig. 3C), suggesting that the lipase and/or other contaminants caused an increase in the background fluorescence. This result indicated that the present method is well capable of measuring PA concentration.

We assessed whether this assay also detects LPA, because LPA is an intermediate product in the hydrolysis of PA by

lipase. As expected, PA and LPA increased the fluorescence to the same extent when normalized to moles (**Fig. 4**). The species of the fatty acyl chain in PA and LPA were not distinguished by this measurement. Bisphosphatidic acid, semilysobisphatidic acid, and lysobisphatidic acid will not be detected by this assay, because these phospholipids have *sn*-glycero-1-phospho-1'-*sn*-glycerol backbones (45, 46).

When Reagent A1 containing 10,000 units/ml lipase was used, the presence of 1 mM PC, 0.5 mM PE, 1 mM PS, or 1 mM PI did not affect G3P generation from 50 µM PA. However, the G3P generation was slightly suppressed in the presence of 1 mM PE (17.6% inhibition). When Reagent A1 containing 40,000 units/ml lipase was used, G3P generation was not influenced by 1 mM PE (data not shown). To measure PA in samples containing large amounts of other lipids, Reagent A1 should contain more than 40,000 units/ml lipase, and/or the samples should be diluted sufficiently.

LPA measurement

We also modified the method for the enzymatic measurement of LPA reported by Aoki et al. (39). The standard curve for LPA measurements was linear between 20 and 250 µM ($r = 0.9979$) (**Fig. 5A**), and the detection limit was 2 µM (20 pmol in the reaction mixture) (Fig. 5B). The background fluorescence without heat-inactivation and removal of the denatured enzyme was 27-fold higher than that with heat inactivation and removal (Fig. 5C). In this assay, PA led to a slight increase in fluorescence, which was about 3% of the increase in the fluorescence by LPA at the same concentration (**Fig. 6**). The enzymatic method for PA measurement quantifies the total concentration of PA
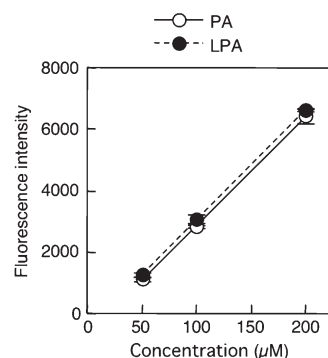

Fig. 4. Fluorescence changes in response to PA and LPA treated with lipase from *Pseudomonas* sp. The PA (open circles) or LPA (closed circles) standard solution (20 µl) was added to 80 µl of Reagent A1 containing 10,000 units/ml lipase from *Pseudomonas* sp. and incubated at 37°C for 1 h. The lipase was heat-inactivated at 96°C for 3 min, and the denatured enzyme was removed by centrifugation. The supernatant (50 µl) was added to 50 µl of Reagent A2. After 30 min of incubation at room temperature, 20 µl of Stop Reagent was added. The fluorescence intensity was measured using a fluorescence microplate reader. Each point represents the mean ± SD of triplicate measurements. Background fluorescence was 404 ± 22, which was subtracted from each value. The lines were obtained by linear regression analysis (PA, $r = 0.99992$; LPA, $r = 0.99995$).
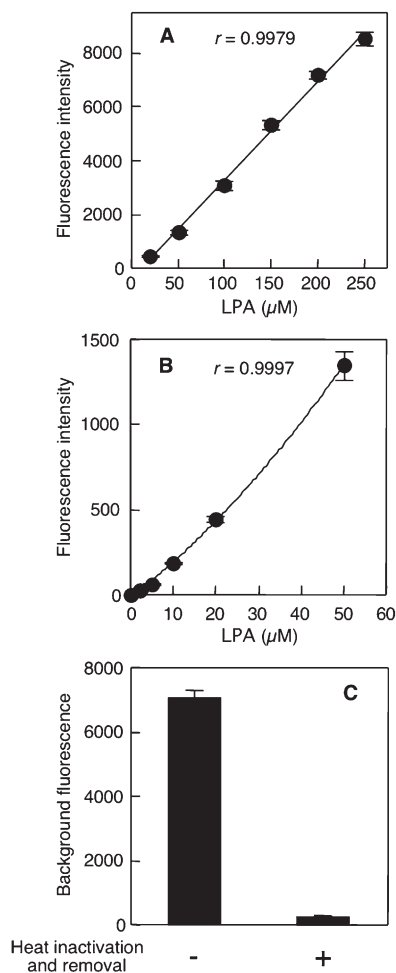
Fig. 5. Standard curves for LPA measurement up to 250 µM (A) and 50 µM (B). The LPA standard solution (20 µl) was added to 80 µl of Reagent L1 containing 12.5 units/ml MGL and incubated at 37°C for 30 min. MGL was heat-inactivated at 96°C for 3 min, and the denatured enzyme was removed by centrifugation. The supernatant (50 µl) was added to 50 µl of Reagent A2 containing 5 units/ml GPO, 5 units/ml peroxidase, and 300 µM Amplex Red. After 30 min of incubation at room temperature, 20 µl of Stop Reagent was added. The fluorescence intensity (excitation 544 nm, emission 590 nm) was measured using a fluorescence microplate reader. Each point represents the mean ± SD of triplicate measurements. Background fluorescence was 211 ± 2 (A and B), which was subtracted from each value. The lines were obtained by linear regression analysis (A) and quadratic regression analysis (B). The correlation coefficients were $r = 0.9979$ (A) and $r = 0.9997$ (B). The background fluorescence with (+) or without (−) heat inactivation and removal of the denatured enzyme was measured (C). Each bar represents the mean ± SD of triplicate measurements. The absence of an error bar signifies an SD value smaller than graphic symbol.

and LPA. In the case of samples containing both PA and LPA, the PA concentration can be calculated by subtracting the LPA concentration from the total concentration of PA and LPA.

PA content in HEK293 cells

Because Reagent A2 for PA measurement detects G3P, the measurement may be confounded by G3P. G3P is a metabolic intermediate, and cultured cells contain a large amount of G3P compared with PA. Therefore, lipid ex-
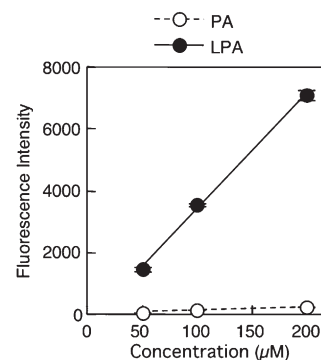

Fig. 6. Fluorescence changes in response to PA and LPA treated with MGL. The PA (open circles) or LPA (closed circles) standard solution (20 µl) was added to 80 µl of Reagent L1 containing 12.5 units/ml MGL and incubated at 37°C for 30 min. MGL was heat-inactivated at 96°C for 3 min, and the denatured enzyme was removed by centrifugation. The supernatant (50 µl) was added to 50 µl of Reagent A2. After 30 min of incubation at room temperature, 20 µl of Stop Reagent was added. The fluorescence intensity was measured using a fluorescence microplate reader. Each point represents the mean ± SD of triplicate measurements. Background fluorescence was 260 ± 14, which was subtracted from each value. The lines were obtained by linear regression analysis (PA, $r = 0.9946$; LPA, $r = 0.9991$).

traction from cells is recommended for the enzymatic measurement of PA. The lipid extract from HEK293 cells by the method of Bligh and Dyer (31, 40) did not contain detectable amounts of G3P or LPA, which was tested by the enzymatic assays.

Mitogenic stimulation of mammalian cells leads to DGK- or phospholipase D-dependent production of PA, which binds to and positively regulates mTOR, a master controller of cell growth (5, 6). mTOR plays a major role in regulating cell growth through the control of protein synthesis. The HEK293 cell line is widely used to dissect the mTOR pathway (5, 6). It has been also proposed that the generation of PA facilitates the recruitment of Raf-1 to the plasma membrane, where it can participate in the activation of the mitogen-activated protein kinase pathway, which is implicated in the mitogenic signaling (47).

To study the effect of cell density on the cellular PA content, we measured the PA content in the lipid extract from HEK293 cells at various cell densities. The PA/PC ratio was also determined as an index of the PA concentration in the cell membrane, because PC is the most abundant lipid in the membrane of mammalian cells. As shown in **Fig. 7A**, the cellular content of PA was negatively correlated with the cell density. In contrast, the PC content increased in a cell density-dependent manner (Fig. 7B). The PA content at low cell density (5.85 µg protein/cm^2) was 5.7-fold larger than that at high cell density (53.9 µg protein/cm^2), while the PC content at low cell density was 44.6% of that at high cell density. Consequently, the PA/PC ratio in the cells remarkably decreased with increasing cell density (Fig. 7C). The PA/PC ratio at low and high cell density (5.85 and 53.9 µg protein/cm^2, respectively) was 0.15 and 0.012, respectively. The PA/PC ratio at low cell density was unexpectedly high, because the concentration
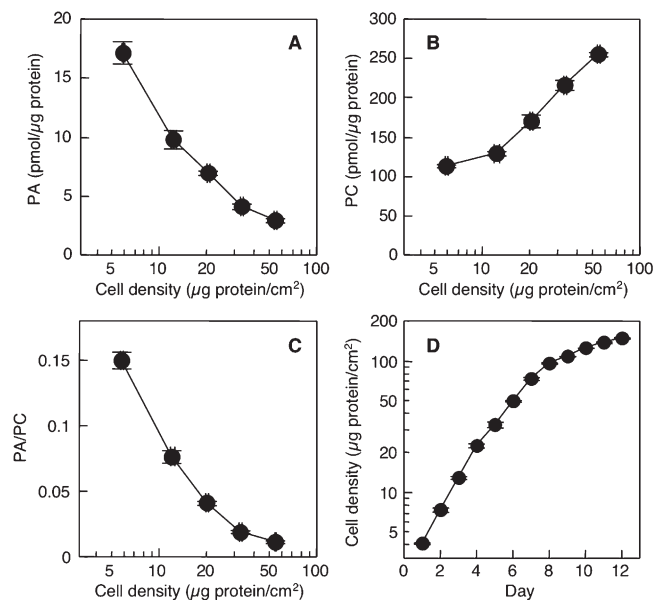
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Fig. 7. Effect of cell density on PA content in HEK293 cells. HEK293 cells were cultured on 10 cm dishes in DMEM containing 10% FBS at 37°C. PA content (A), PC content (B), PA/PC ratio (C), and proliferation (D) of HEK293 cells were determined by enzymatic measurements of PA and PC, and protein assay. Each point represents the mean ± SE of three measurements.

of PA in mammalian cell membranes has been thought to be normally <5 mol% of that of PC (48). We determined for the first time, to our knowledge, the PA/PC ratio in sparse cell cultures using the novel enzymatic assay. The relationship between the proliferation rate and the PA/PC ratio in HEK293 cells was also evaluated. Fig. 7D shows the exponential increase in the cell density. The proliferation rate of the cells was constant until day 4, and then the rate reduced gradually. The cell densities at days 1 and 4 were 4.13 and 23.1 µg protein/cm^2, respectively. The PA/PC ratio at high cell density (5.85 µg protein/cm^2) was 3.6-fold higher than the ratio at medium cell density (20.2 µg protein/cm^2) (Fig. 7C), although the proliferation rate was not altered between these cell densities. These results suggested the PA content and the PA/PC ratio in HEK293 cells were dependent on the cell density and were not correlated with the proliferation rate.

Effect of DGK inhibitor on PA content in HEK293 cells

DGK phosphorylates diacylglycerol to produce PA. DGKζ is highly expressed in HEK293 cells (6). To assess the involvement of DGK in the production of PA in HEK293 cells, the effect of DGK inhibitor R59949 on the cellular content of PA was examined. The addition of R59949 to HEK293 cells resulted in a dose-dependent decrease in the PA content (**Fig. 8A**). On the other hand, R59949, at concentrations below 30 µM, had no effect on the PC content in the cells, but the PC content was slightly reduced by R59949 at concentrations above 60 µM (Fig. 8B). Therefore, the PA/PC ratio in HEK293 cells was markedly decreased due to the inhibition of DGK by R59949 (Fig. 8C). The addition of 90 µM R59949 led to a 69.4% decrease in the PA/PC ratio in the cells. The cell

density was not increased by R59949 (Fig. 8D), indicating the decrease in the PA/PC ratio caused by R59949 was not attributed to the change of cell density. These findings demonstrated that the DGK activity accounted for a large part of the PA generation in HEK293 cells. The enzymatic measurement of PA was valid in the sense that this method clearly showed the effect of the DGK inhibitor on the PA content in HEK293 cells.

The relationships between cell density and metabolism of phospholipids, including PA and PC, have not yet been characterized. DGK activation has been reported to be required for hepatocyte growth factor- and v-Src-induced scattering and migration of epithelial cells (49). It is likely that signaling from cell-cell adhesion downregulates DGK activity and leads to a decline in PA concentration, which may impair cell scatter and migration. On the other hand, PC biosynthesis may show greater activation at a higher cell density, which is needed for the formation of various membrane structures. At present, the mechanisms influencing changes in PA and PC concentrations in relation to cell density remain unclear.

In conclusion, the enzyme-based, fluorometric method was developed for measuring PA in cultured cells. This new procedure can provide simple, rapid, sensitive, specific, and high-throughput PA quantification. The present method does not distinguish the species of the fatty acyl chain in PA. This assay requires only a 20 µl sample volume. The method is sensitive, with a detection limit of 50 pmol of PA. All enzymes and substrates are available commercially. In addition, the approach does not require radiolabeling. The usefulness of this procedure was demonstrated using the lipid extract from cultured cells.
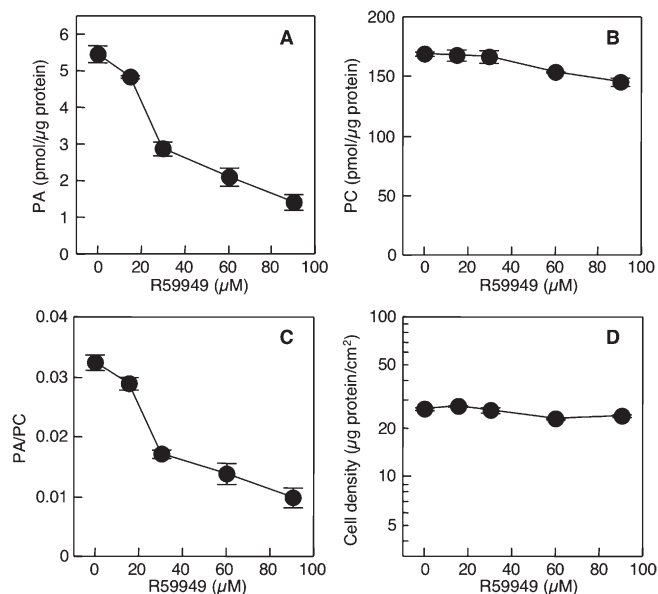

Fig. 8. Effect of DGK inhibitor R59949 on PA content in HEK293 cells. HEK293 cells on 10 cm dishes were incubated with the indicated concentrations of R59949 in DMEM containing 10% FBS for 24 h at 37°C. PA content (A), PC content (B), PA/PC ratio (C), and cell density (D) of HEK293 cells were determined by enzymatic measurements of PA and PC, and protein assay. Each point represents the mean ± SE of three measurements.

This method may also be extended to determine the PA content in a particular subcellular organelle. In principle, it appears applicable for measuring PA levels in lipid extracts under conditions where radiolabeling is extremely difficult, such as in animal tissues and fluids. This enzymatic measurement will be useful for further studies of the biological functions of PA and its related enzymes, including DGK, phospholipase D, LPA acyltransferase, and PA phosphatase.

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