Reconstitution of GTP-γ-S-dependent phospholipase D activity with ARF, RhoA, and a soluble 36-kDa protein

Kiyoshi Shimooku[a], Toshihiro Akisue[a], Hitoshi Jinnai[a], Tomohiro Hitomi[a], Chiaki Ogino**, Kimihisa Yoshida[a], Shun-ichi Nakamura[a,*], Yasutomi Nishizuka[a,b]

[a]*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan*
[b]*Biosignal Research Center, Kobe University, Kobe 657, Japan*

Received 1 April 1996; revised version received 30 April 1996

Abstract For activation of kidney membrane phospholipase D (PLD), cytosol is absolutely needed in addition to GTP-γ-S. The active component of cytosol consists of three protein factors: ADP-ribosylation factor, RhoA, and a soluble 36-kDa protein. Any combination of these two factors synergistically activates PLD to some extent, but the presence of the three factors causes full activation. The 36-kDa protein is stable at 60°C but inactivated at 80°C for 10 min. Tissue distribution of the 36-kDa protein roughly coincides with that of PLD, suggesting physiological relevance of the protein in the regulation of PLD.

Key words: Phospholipase D; ADP-ribosylation factor; RhoA; G-protein

1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PtdCho) in response to a wide variety of external signals (for review, see [1,2]). The mechanism of this enzyme activation has attracted considerable attention because the reaction may generate several lipid messengers or mediators for cell signalling (for review, see [3,4]). Extensive studies with cell-free systems have shown the existence of at least two types of PLD in mammalian tissues: one is dependent on oleic acid [5–7] and the other is activated by small GTP-binding regulatory proteins (G-proteins) such as ADP-ribosylation factor (ARF) [8,9], RhoA [10,11], or Ral [12]. More recently, an additional protein factor has been reported that is needed for guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S)-dependent PLD activity. PLD obtained from hematopoietic cell lines is shown to be activated by a 50-kDa soluble protein and either ARF [13,14] or RhoA [15]. Brain PLD is also activated by a cytosolic factor in a synergistic manner with ARF [16], and this factor has recently been shown to be indistinguishable from protein kinase C (PKC) [17].

An earlier report from this laboratory has described that under normal conditions membrane-bound PLD is in a latent form, but exhibits a high activity in the presence of ammonium sulfate and cytosol [18]. Using this enzyme assay, the kidney and spleen are shown to contain highest G-protein-sensitive PLD activity. It has been also shown that the partially purified kidney PLD utilizes PtdCho as a preferential substrate, and requires nearly absolutely phosphatidylethanolamine (PtdEtn) as a co-substrate [19]. The present study will show that the active component in the cytosol consists of ARF, RhoA, and an additional soluble 36-kDa protein, and that all of the three cytosolic proteins are needed to exhibit full PLD activation. Kinetics of the PLD activation by these factors and some properties of the 36-kDa protein will be described herein.

2. Materials and methods

2.1. Materials

L-α-Dipalmitoyl[palmitoyl-1-^{14}C]phosphatidylcholine ([^{14}C]PtdCho, 115 mCi/mmol) and [^{35}S]GTP-γ-S (1100 Ci/mmol) were purchased from DuPont-New England Nuclear. Phosphatidylethanol (PtdEtOH), a standard for TLC, was from Avanti Polar-Lipid, Inc. Plasmalogen-rich PtdEtn (60% plasmalogen) was from Serdary Research Laboratories (Englewood Cliffs, NJ). GTP-γ-S was from Boehringer-Mannheim. Glass-backed silica gel 60 was purchased from Merck. Monoclonal antibody against RhoA was purchased from Santa Cruz Biotechnology (California). Isoprenylated RhoA and non-isoprenylated RhoA fused to glutathione *S*-transferase expressed in and purified from Sf9 cell and *Escherichia coli*, respectively, were kindly donated from Dr. K. Kaibuchi (Nara Institute of Science and Technology). Polyclonal antibody, CK3 raised against a synthetic peptide (FARKGALRQKNVHEVKN) around the N-terminal portion of the α-subspecies of PKC was kindly donated from Dr. N. Saito (Biosignal Research Center, Kobe University). Purified conventional PKC was kindly donated from Dr. U. Kikkawa (Biosignal Research Center, Kobe University).

2.2. Preparation of crude cytosol

Proteins of the soluble fraction of rat kidney were precipitated with ammonium sulfate (30–70%), dissolved, dialyzed, and chromatographed on a Superdex 200 column as described earlier [18].

2.3. Preparation of PLD

PLD was solubilized from the bovine kidney particulate fraction, and precipitated with $MgCl_2$ as described previously [19]. The fraction (70 mg protein) that contained PLD was applied to a hydroxyapatite column (Pharmacia) (2.4×4 cm; 18 ml) which had been equilibrated with buffer B [19]. After washing the column with 100 ml of buffer B, PLD was eluted with a 200-ml linear concentration gradient (0–0.6 M) of potassium phosphate in buffer B at a flow rate of 1 ml/min. Fractions (5 ml each) were collected. The fractions containing PLD (potassium phosphate concentration at 0.25–0.4 M) were pooled and

* Corresponding author. Fax: (81) (78) 351-0082.

** On leave from the Graduate School of Science and Technology, Kobe University, Kobe 657, Japan.

The data are taken in part from the dissertation that will be submitted by K. Shimooku to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Sciences.

Abbreviations: PLD, phospholipase D; PtdCho, phosphatidylcholine; G-protein, GTP-binding regulatory protein; ARF, ADP-ribosylation factor; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PKC, protein kinase C

PII S0014-5793(96)00483-8

concentrated to a minimum volume with an Amicon ultrafiltration cell equipped with PM-30 membrane, and dialyzed overnight against 500 ml of buffer B. The dialyzed sample was clarified by centrifugation for 30 min at 100 000×*g*, and applied to a TSK heparin 5PW (Toyo Soda (Tokyo); 0.6×4 cm) equipped with buffer B. After washing with 10 ml of buffer B, the enzyme was eluted with a 20-ml linear concentration gradient (0–1.5 M) of NaCl in buffer B at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected. The fractions containing PLD (NaCl concentration at 0.8–1.2 M) were pooled and concentrated to a protein concentration of 0.5 mg/ml by ultrafiltration as above, and stored at −80°C. The PLD preparation at this stage was purified about 1000-fold from particulate fractions. This preparation was free of ARF, RhoA, and the 36-kDa protein (see below). The formation of PtdEtOH was linear with the enzyme protein up to 50 ng per tube, and the reaction proceeded linearly with incubation time up to 60 min. This purification made PLD more sensitive to the cytosol factors for its activation than that previously reported [18].

2.4. Purification of ARF

ARF was purified from the bovine brain as described [20]. The final preparation of ARF after gel filtration (Superdex 75, Pharmacia) was about 87% pure by silver stain. This preparation was free of RhoA and the 36-kDa protein factor described below, as judged by immunoblot analysis using a monoclonal antibody against RhoA and the factor activity (see below) to stimulate PLD with RhoA, respectively.

2.5. PLD assay

PLD activity was determined by measuring the formation of [^{14}C]PtdEtOH from [^{14}C]PtdCho in the presence of ethanol as described [18]. The reaction mixture (100 μl) contained 25 ng of PLD (see above), 6 nmol of [^{14}C]PtdCho (13 750 dpm/nmol), 7 nmol of plasmalogen-rich PtdEtn, 34.2 μmol of ethanol, 160 μmol of ammonium sulfate, 10 nmol of GTP-γ-S, and 2 μmol of HEPES-NaOH at pH 7.4. Where indicated, additional factors (see below) were added to the assay mixture.

2.6. Other procedures

Protein was determined by the method of Bradford [21].

3. Results

3.1. Reconstitution of PLD activity

Purified bovine kidney PLD per se was practically inactive, and required almost absolutely both crude cytosol and GTP-γ-S to exhibit enzymatic activity (Table 1). When the cytosol was replaced by ARF, the enzyme showed some activity, but did not exhibit full activation unless cytosol was added. Kinetic analysis indicated that this cytosolic factor consisted of at least two G-proteins and an additional 36-kDa protein (see below). Recombinant isoprenylated RhoA could substitute for one of the two G-proteins, although RhoA alone was inert to support the enzymatic activity both in the presence and in the absence of the 36-kDa protein (Table 2). Non-isoprenylated RhoA was without effect. With a saturated amount (5 μg) of the 36-kDa protein, which was inactive by itself, RhoA enhanced further the ARF-dependent activation of PLD (Fig. 1). The combined addition of the three components, ARF, RhoA, and the 36-kDa protein, fully restored the PLD activity that was observed in the presence of crude cytosol (Tables 1 and 2).

Table 1
Reconstitution of PLD activity with crude cytosol

Addition	PtdEtOH produced (dpm×10^{-2})	
	−GTP-γ-S	+GTP-γ-S
None	0.38±0.15	0.22±0.06
Crude cytosol	0.28±0.10	34.38±5.11

PLD activity was measured with 25 ng of enzyme in the presence or absence of 100 μg of crude cytosol from rat kidney and 100 μM GTP-γ-S as indicated. The reaction was carried out for 20 min at 37°C, and the radioactive PtdEtOH produced was determined. Results are the means±S.E.M. of duplicate determinations from three separate experiments.

Table 2
Reconstitution of PLD activity with the 36-kDa protein, ARF, and RhoA

Addition	PtdEtOH produced (dpm×10^{-2})	
	−GTP-γ-S	+GTP-γ-S
None	0.29±0.06	0.23±0.06
36-kDa protein	0.40±0.10	0.22±0.05
ARF	0.35±0.09	3.95±0.60
RhoA	0.15±0.05	0.76±0.20
36-kDa protein+ARF	0.36±0.11	18.00±2.30
36-kDa protein+RhoA	0.29±0.09	1.60±0.24
ARF+RhoA	0.23±0.15	9.01±0.98
36-kDa protein+ARF+RhoA	0.30±0.11	36.20±4.03

Crude cytosol from rat kidney was heated for 10 min at 60°C. Denatured proteins were removed by centrifugation for 10 min at 10 000×*g*, and the supernatant was used as a source of the 36-kDa protein. PLD was measured with 25 ng of enzyme in the presence or absence of 5 μg of the 36-kDa protein, 470 nM ARF, 60 nM recombinant RhoA, and 100 μM GTP-γ-S as indicated. Results are the means±S.E.M. of duplicate determinations from three separate experiments.

3.2. Properties of the 36-kDa protein

The 36-kDa protein retained its stimulatory activity after being heated for 10 min at 60°C, but was inactivated completely upon treatment for 10 min at 80°C. Small G-proteins were inactivated by the heat treatment for 10 min at 60°C.

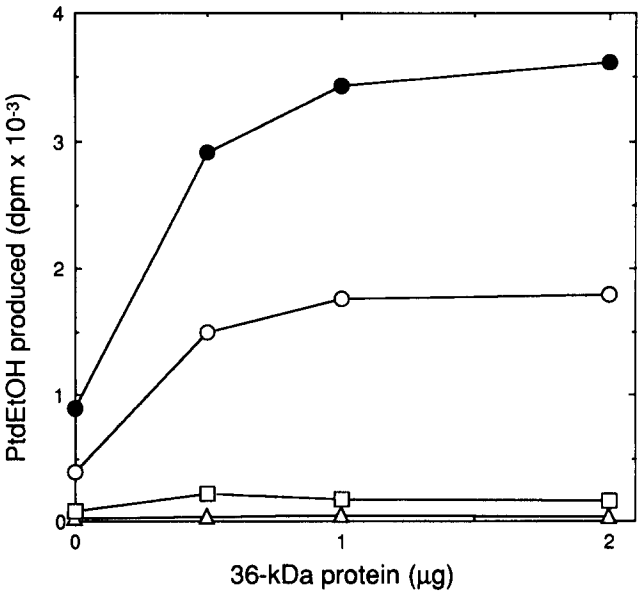

Fig. 1. Reconstitution of GTP-γ-S-dependent PLD activity with ARF, RhoA, and the 36-kDa protein. The 36-kDa protein was prepared as in the legend to Table 2. PLD activity was measured with 25 ng of enzyme in the presence or absence of 470 nM purified ARF, 60 nM recombinant RhoA, and various concentrations of the 36-kDa protein. ●-●, with ARF plus RhoA; ○-○, with ARF; □-□, with RhoA; △-△, without any G-protein.

This heated fraction was centrifuged for 10 min at 10000×*g* to remove denatured proteins, and the supernatant was subjected to a Superose 12 gel filtration column. The activity to support PLD activation was eluted as a single peak with an approximate molecular mass of 36 kDa (Fig. 2). Trypsin treatment completely inactivated the factor. The results indicate that the 36-kDa factor is a protein. The amount of the 36-kDa protein varied from tissue to tissue with the following order: kidney > liver > spleen > brain=lung (Fig. 3). The distribution pattern of the factor roughly coincided with that of PLD. The heat-stable factor did not affect [^{35}S]GTP-γ-S binding to ARF.

4 Discussion

Subsequent to the reports by Brown et al. [8] and Cockcroft et al. [9], several papers have appeared describing the involvement of small G-proteins in PLD activation. Brain membrane-bound PLD has been shown to be activated by the synergistic action of ARF and RhoA [16,22]. Liver membrane-bound PLD has also been shown to be activated by RhoA [11]. In HL-60 cells, cytosolic PLD is activated by ARF, whereas membrane-bound PLD is activated by ARF and RhoA synergistically [23]. The stimulatory mechanism of PLD by small G-proteins is at present unclear, and it remains uncertain whether the distinct sensitivity of the enzyme to several G-proteins reflects enzyme heterogeneity or impurity of the enzyme preparations employed. The present results suggest that one type of PLD in kidney is synergistically activated by ARF, RhoA, and the 36-kDa protein.

The 36-kDa protein may differ from the 50-kDa protein in hematopoietic cell lines [13–15] in molecular size. The 36-kDa protein showed no immunological cross-reactivity with an antibody raised against a synthetic peptide around the N-terminal portion of the α-subspecies of PKC, suggesting that the 36-kDa protein is not derived from PKC. Structural and kinetic analysis of the 36-kDa protein remains to be explored.

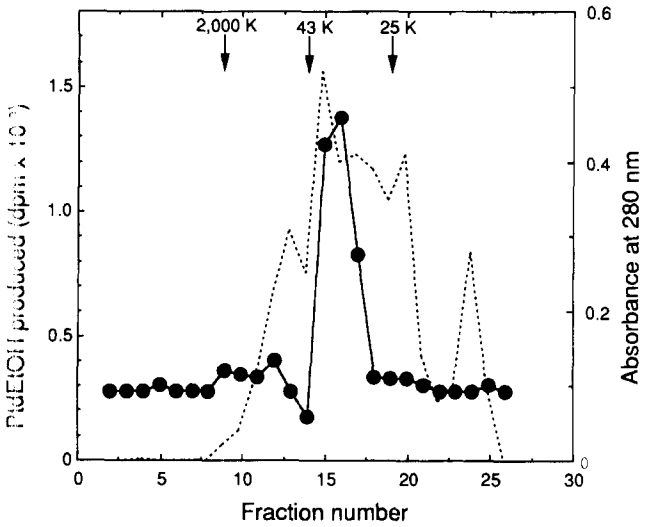

Fig. 2. Superose 12 column chromatography of the 36-kDa protein. The 36-kDa protein containing fraction (1 mg protein) prepared as in the legend to Table 2 was applied to a Superose 12 column which had been equilibrated with buffer A [18], and eluted with the same buffer at a flow rate 0.5 ml/min using an FPLC system (Pharmacia). Fractions (1 ml each) were collected. Each fraction (20 μl) was assayed for PLD activation with 25 ng of enzyme, 470 nM purified ARF, and 100 μM GTP-γ-S under the conditions specified. Arrows indicate the elution positions of blue dextran (2000 K), ovalbumin (43 K), and chymotrypsinogen A (25 K) which were run in parallel experiments.

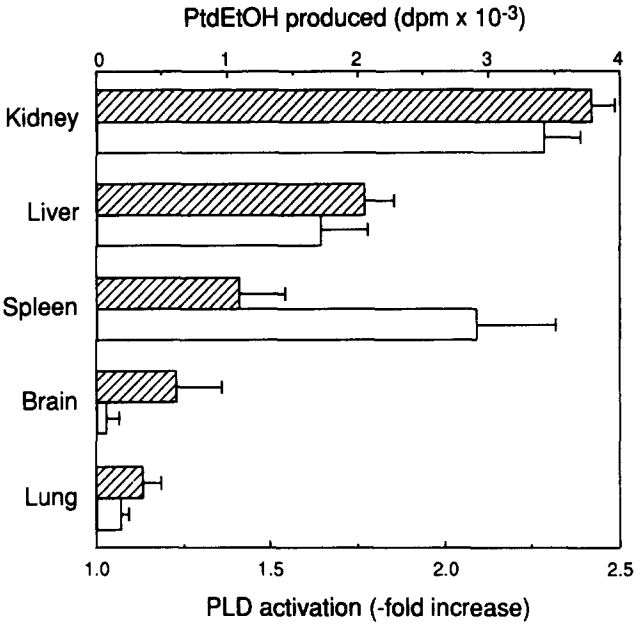

Fig. 3. Tissue distribution of the 36-kDa protein and PLD. Supernatant and particulate fractions were prepared from various rat tissues [18]. The soluble cytosols (10 mg/ml) were heated for 10 min at 60°C and centrifuged to remove the denatured proteins. PLD activation (hatched bar) by each cytosol (20 μl) was measured with 25 ng of partially purified enzyme, 470 nM purified ARF, and 100 μM GTP-γ-S under the conditions specified. Data are expressed as fold increase over control value obtained in the absence of the 36-kDa protein. PLD activity (open bar) of each particulate fraction (50 μg protein) was measured with 100 μg of crude cytosol from rat kidney and 100 μM GTP-γ-S as in [18]. Results are the means ± S.E.M. of duplicate determinations from three separate experiments.

The present studies with preparations of several protein components free of one another show that the kidney membrane PLD requires simultaneously at least three soluble proteins, ARF, RhoA, and the 36-kDa protein, to exhibit full enzymatic activity. ARF and RhoA have been shown to exist in kidney [24,25]. An earlier paper [19] has shown that PtdEtn is essential, perhaps as a co-substrate, for kidney PLD. In addition, it has been confirmed that tyrosine kinase and protein kinase C take part in the regulation of the enzyme activity [26]. Reconstitution analysis of these regulatory components described above with purified enzyme may be essential to clarify the mechanism of receptor-mediated activation of mammalian PLD.

Acknowledgements: The skilful secretarial assistance of Miss M. Maemori is acknowledged. This work was supported in part by research grants from the Special Research Fund of the Ministry of Education, Science, and Culture, Japan; the Yamanouchi Foundation for Research on Metabolic Disorders; the Sankyo Foundation of Life Science; and Merck Sharp and Dohme Research Laboratories.

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